Interactions between chitosans and bacteria:

flocculation and adhesion

by

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SUMMARY

Interactions between bacteria and biopolymers are essential in biofilm and floc formation. Chitosan represents an interesting model biopolymer whose properties may be manipulated both by its molecular weight and chemical composition, given by fraction of acetylated units (F_A), as well as by external conditions such as pH and ionic strength. Chitosan is also considered as feasible flocculant in many areas of water treatment and in downstream processing. The general scope of this work has been (1) to evaluate structure–function relationships in interactions of chitosans with bacteria and (2) to provide an empirical foundation for identification of important mechanisms of these interactions.

Prior to adhesion and flocculation studies, two methods were developed to quantify chitosan both in solution and bound to surfaces: a colorimetric method based on a reaction of chitosan with ninhydrin, and labeling of chitosan with the fluorophore 9-anthraldehyde. Furthermore, to be able to predict the charge density of different chitosans under different conditions, electrostatic properties of chitosans were examined by electrophoretic light scattering technique. It was shown that this method gave estimates of charge density based on the electrophoretic mobility of chitosan in solution. All chitosans had the same pK_a values of 6.5-6.6, independent of F_A.

Chitosans with different chemical composition were evaluated as flocculants by applying suspensions of *Escherichia coli* K12 as a model organism. The flocculation performance of chitosan was followed by residual turbidity measurements. It was found that flocculation efficiency increased by about a factor of 10 with increasing F_A, while pH and molecular weight were rather insignificant factors. Thus, the presence of acetylated residues appeared to be more important than charge density.

To examine the differences in flocculation closer, adsorption of labeled chitosan to *E. coli* cells during flocculation was quantified by fluorescence spectrometry, and the corresponding changes in zeta potential of bacterial cells were also recorded. The highly acetylated chitosan adsorbed most and were the most effective in neutralizing the cell surface charge. These data revealed that *E. coli* did not flocculate by a charge neutralization mechanism, but probably by bridging between cells.

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Other bacteria were also tested to obtain a more general picture of bacterial flocculation. Large differences were found in the flocculation efficiency of chitosans, both regarding the chitosan concentrations needed and the type of chitosan giving the best results. However, it seemed that Gram-negative bacteria flocculated generally better with highly acetylated chitosan, while Gram-positive flocculated better with low acetylated chitosan. No correlation was observed between the flocculation efficiency of different chitosans and general surface characteristics such as bacterial cell surface charge or hydrophobicity.

Adhesion of *E. coli* and other bacterial species to chitosan coated glass was studied as a function of the same variables as in flocculation. Again, bacteria differed widely both in the adhesion pattern as well as degree of adhesion. Although it was expected to find similar trends as in flocculation studies, this could not be clearly documented.

In conclusion, it has been shown that interactions between chitosans and bacteria depend strongly on the specific structure of the actual chitosan as well as that of the actual bacterium, resulting in large differences in flocculation efficiency as well as in adhesion pattern at different conditions. These differences are difficult to explain by conventional colloid theories. The detailed structure of bacterial cell surface as well as the physiological responsiveness of bacteria has to be taken into consideration.

LIST OF APPENDIX PAPERS

I   Prochazkova S., Vårum K.M., Østgaard K. (1999) Quantitative determination of chitosans by ninhydrin. *Carbohydr. Polym.* **38**, 115-122.

II   Tømmeraas K., Strand S.P., Tian W., Kenne L., Vårum K.M. (2001) Preparation and characterisation of fluorescent chitosans using 9-anthraldehyde as fluorophore. Accepted in *Carbohydr. Res.*

III   Strand S.P., Tømmeraas K., Vårum K.M., Østgaard K. (2001) Electrophoretic light scattering studies of chitosans with different degrees of *N*-acetylation. *Biomacromolecules*, in press.

IV   Strand S.P., Vandvik M.S., Vårum K.M., Østgaard K. (2001) Screening of chitosans and conditions for bacterial flocculation. *Biomacromolecules* **2** (1), 126-133.

V   Strand S.P., Vårum K.M., Østgaard K. (2001) Interactions between chitosans and bacterial suspensions: adsorption and flocculation. Submitted to *Colloids Surfaces B: Biointerfaces.*

VI   Strand S.P., Nordengen T., Østgaard K. (2001) Efficiency of chitosans applied for flocculation of different bacteria. Submitted to *Water Res.*

VII   Strand S.P. and Østgaard K. (2001) Bacterial adhesion to different types of chitosan coated surfaces. In prep.

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LIST OF ABBREVIATIONS AND SYMBOLS

AFM	atomic force microscopy
CAM	contact angle measurements
CNP	charge neutralization point
CSC	cell surface charge
CSH	cell surface hydrophobicity
$\overline{DP}_n$	number-average degree of polymerization
DS	degree of substitution
ELS	electrophoretic light scattering
EM	electrophoretic mobility
F_A	fraction of acetylated units
G+	Gram-positive
G-	Gram-negative
GlcN	2-amino-2-deoxy-β-D-glucopyranose
GlcNAc	2-acetamido-2-deoxy-β-D-glucopyranose
HIC	hydrophobic interaction chromatography
LPS	lipopolysaccharid
MAC	microsphere adhesion
MATH	microbial adhesion to hydrocarbons
$\overline{M}_n$	number-average molecular weight
PBS	phosphate-buffered saline
pK_a	dissociation constant
R_g	radius of gyration
SAT	salt aggregation test
VDW	van der Waals forces
V_A	van der Waals attraction
V_E	electric double-layer repulsion
V_T	total energy of interaction
α	degree of ionization
κ	Debye length
ψ	electrostatic potential
ζ	zeta potential

1. INTRODUCTION

1.1 General background

The interactions between bacteria and biopolymers are essential in microbial adhesion and aggregation. This is due to a simple fact that virtually all natural surfaces and interfaces in aqueous systems are covered by a layer of adsorbed macromolecules, referred to as a conditioning film (Marshall, 1985; Schneider, 1996). Such interactions play a central role in biological processes of biofilm and floc formation involved in certain diseases, dental plague formation, fouling of surfaces, interactions within microbial communities and survival of microorganisms in natural habitats (Costerton *et al.*, 1985).

Despite the widespread and crucial nature of these processes and extensive research, there is still much to be known about the nature of interactions and their relative importance. Since natural systems are extremely complex, it may be an advantage to use well-characterized model systems and compounds. Chitosan is an interesting biopolymer whose properties may be widely manipulated in laboratory studies. The cationic nature of chitosan is a rather unique feature, since natural polymers as well as almost all biological and natural surfaces tend to develop a negative charge. Systematic studies of interactions between bacteria and chitosans may thus reveal valuable information about the nature of actual forces and at the same time, they may show how important the structure of chitosan may be for its function in biological systems.

Moreover, chitosan is becoming an increasingly popular alternative to synthetic polymers due to its biocompatibility, biodegradability and non-toxicity. These properties as well as the cationic nature may be exploited in such diverse fields as drug and gene delivery (Felt *et al.*, 1998; Köping-Höggård *et al.*, 2001), water treatment (No and Meyers, 2000) or downstream processing (Agerkvist, 1992). However, it is relatively often neglected that the potential of chitosan for a particular application may be greatly dependent on the chitosan structure. To be able to fully exploit the potential of chitosans in different applications, more basic research is needed.

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1.2 Chitosan

1.2.1 Structure and properties

Chitosan is a biopolymer derived from chitin, widely distributed and abundant in the exoskeleton of crustaceans and insects. The estimated steady-state levels of chitin on earth are in the order of 10^{10}-10^{11} tons, with high turnover (Gooday, 1990). Chitosan can be produced from chitin by homogeneous or heterogeneous alkaline de-*N*-acetylation. The latter procedure is used for commercial production of chitosans, where seafood waste is utilized as the chitin source. The term chitosan refers to a whole family of linear heteropolysaccharides composed of varying amounts of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; **A**-unit) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN; **D**-unit). The structure of a partially de-*N*-acetylated chitosan is schematically shown in Figure 1.1.
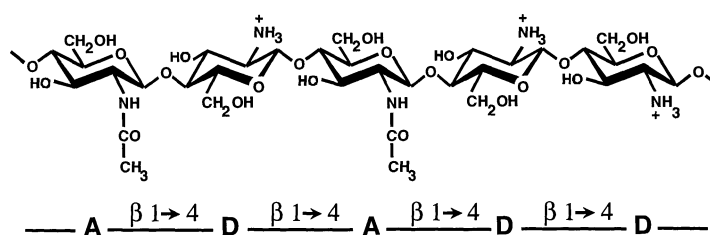
Figure 1.1 Chemical structure of a partially de-*N*-acetylated chitosan.

The β-(1→4) linkages connecting the monomers are responsible for the relatively high chain stiffness, resulting in extended conformation and high solution viscosity. The effect of the presence of *N*-acetylated residues on the chain stiffness is a matter of debate, some studies suggest that presence of *N*-acetylated residues further increases the stiffness of chitosan chain (Anthonsen *et al.*, 1993).

The chemical composition of chitosans is described by the molar fraction of GlcNAc units (F_A) which may be determined by ^{1}H-NMR or ^{13}C-NMR spectroscopy (Vårum *et al.*, 1991 a, b). The acetyl groups along the chain of water-soluble chitosans, prepared both homogeneously and heterogeneously, have been shown to be randomly distributed (Vårum *et al.*, 1991 a; Ottøy *et al.*, 1996). The fraction of acetylated units affects strongly chitosan properties such as solubility, charge density and conformation. It has been shown that

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chitosans with F_A 0-0.2 are only soluble in aqueous acidic solutions while chitosans with F_A 0.4-0.6 are also soluble at neutral pH (Vårum *et al.*, 1994).

The degree of protonization of amino groups of GlcN in solution, and thus the charge density of chitosan, is determined both by chitosan composition (F_A) and external variables such as pH and ionic strength. The reported values of the dissociation constant, pK_a, for chitosan range from 6.2 to 7, dependent on chitosan and conditions of measurement (Domard, 1987; Rinaudo and Domard, 1989; Anthonsen and Smidsrød, 1995). The literature describing the relationship between F_A and pK_a of chitosans is not consistent. Results of Domard (1987), recently extended by Sorlier *et al.* (2001) showed increase in apparent pK_a values with increasing F_A, whereas Anthonsen and Smidsrød (1995) found the same pK_a for all chitosans tested. Another discrepancy seems to be the reported changes in the apparent pK_a with the degree of ionization (α) and the values of the intrinsic pK_a, referred to as pK_0, for different chitosans, for details see Section 2.4.1.

1.2.2 Applications

The chitosan research has literally exploded during the past decade. This is due to its biocompatibility, biodegradability, non-toxicity, and other more unique properties such as film-forming ability, chelation and adsorption properties or antimicrobial activity as reviewed by Kumar (2000). Chitosans and chitosan derivatives are currently being studied for potential application in such diverse fields as drug delivery (Schipper *et al.*, 1996; Felt *et al.*, 1998; Paul and Sharma, 2000), gene transfection (Richardson *et al.*, 1999; Mao *et al.*, 2001; Köping-Höggård *et al.*, 2001), cholesterol lowering effect (Koide, 1998; Gallaher *et al.*, 2000), antimicrobial activity (Rhoades and Roller, 2000) or different aspects of water treatment (see below).

The ability of chitosan to interact and bind strongly to polymers, tissues and cells forms the basis for numerous biomedical applications. Interactions of chitosan with a number of polyanions such as alginate (Gåserød *et al.*, 1998), collagen (Taravel and Domard, 1996), chondroitin sulfat and hyaluronate (Denuziere *et al.*, 1996), and other materials such as liposomes (Henriksen *et al.*, 1994), undecylenic acid (Demarger and Domard, 1993 and 1994) and gastric mucin (Deacon *et al.*, 2000) have been studied thoroughly.

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The major commercial application of chitosan is currently in wastewater treatment (No and Meyers, 2000; Meyer *et al.*, 2000). Suspended solids, dyes, heavy metals, pesticides and other toxic compounds may all be efficiently removed by chitosan. In many cases, chitosan has been found to be superior to synthetic polymeric flocculants (Kawamura, 1991). Chelation of heavy metal ions by chitosan has been extensively studied (Dambies *et al.*, 2001; Wu *et al.* 2001). Chitosan has been used for recovery of feed-grade material from food processing wastes (Selmer-Olsen *et al.*, 1996; Savant and Torres, 2000). Removal of humic substances from drinking water (Eikebrokk, 1999) is also a growing field of application. Chitosan has been also tested for stabilization and destabilization of oil in water emulsions (Del Blanco *et al.*, 1999; Pinotti *et al.*, 2001).

1.3 Bacterial cell surfaces

The bacterial cell surface is in direct contact with its external environment and is involved in all kinds of interactions with surfaces and interfaces. The bacterial envelope is of prior importance for cell survival and it is estimated that approximately ¼ of the genetic potential is devoted to its synthesis, regulation and maintenance (Neidhardt *et al.*, 1990). Structural and functional properties of the cell surface reflect its heterogeneous composition and vary considerably in Gram-positive and Gram-negative bacteria. The combination of the different molecules and structures imparts the characteristic physicochemical properties such as cell surface charge and cell surface hydrophobicity to any particular bacterium.

The determination of the structure, chemical composition and physicochemical properties of the cell surface is not an easy task. The analytical techniques usually cannot be applied for surfaces in their native state, and different preparation methods are necessary. These were shown to cause severe modification or even damage of different structures (Pembrey *et al.*, 1999). Therefore, new nondestructive methods of cell surface analysis need to be developed. Recently, it has been shown that atomic force microscopy (AFM) has a great potential for characterizing the physicochemical properties of microbial cells (Boonaert *et al.*, 2000; van der Mei *et al.*, 2000).

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1.3.1 Chemical structure of bacterial envelopes

The important distinction between Gram-positive (G+) and Gram-negative (G-) bacteria is based on their differences in cell wall structures schematically shown in Figure 1.2, and described in detail by Neidhardt (1990), Hancock (1991) or Brock *et al.* (1996). Briefly, the G+ cell wall consists of a thick layer of peptidoglycan with smaller amounts of other polymers, especially teichoic acids and teichuronic acids. The glycan portion consists of two repeating sugars: *N*-acetylglucosamine (G) and *N*-acetylmuramic acid (M) always connected by strong β-(1$\rightarrow$ 4) linkage. The glycan chains are crosslinked by peptide cross-links through the M residues. The peptidoglycan is a rigid but highly elastic structure responsible for the shape of a bacterial cell. Teichoic acids are polyols of glycerol phosphate or ribitol phosphate connected by phosphate ester bonds often containing also amino acids. They are highly antigenic and can vary considerably in chemical composition. The teichoic acids are partially responsible for the negative charge of the cell due to the presence of dissociated phosphate groups. Recently, it has been reported that the charge of teichoic acids plays a pivotal role in the initial step of biofilm formation by pathogenic *S. aureus* (Gross *et al.*, 2001).
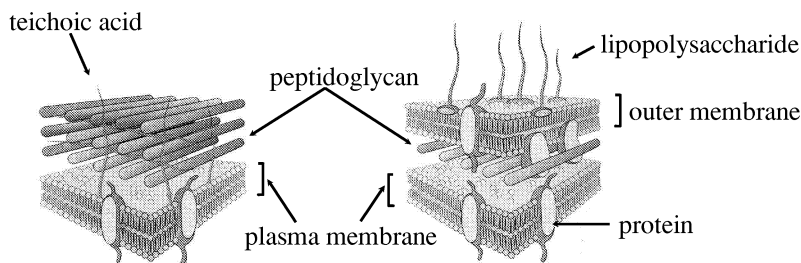
Figure 1.2 Schematic structure of Gram-positive (left) and Gram-negative (right) cell wall (Wallace *et al.*, 1996).

The G- cell wall is a multilayered complex structure (Fig. 1.2). The thin peptidoglycan layer forming only about 10 % of the cell wall is surrounded by the outer membrane, a highly organized structure with an inner part resembling the cytoplasmatic membrane and an outer part consisting of lipopolysaccharides (LPS). LPS are unique complex glycolipids, not found elsewhere in nature. They consist of three main parts: lipid A anchoring the LPS molecule in the membrane, a core-short sequence of characteristic sugars and a specific long hydrophilic carbohydrate chain (O-chain or O-antigen). The O-chains of different strains exhibit great heterogeneity in their length and composition. As LPS is the outermost structure on the cell

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surface, they have been shown to influence the adhesion behavior (Williams and Fletcher, 1996; Mackin and Beveridge, 1996).

Many bacteria possess surface appendages that can be classified into flagella, pili and fimbriae (Hancock, 1991). Flagella, responsible for bacterial motion, are very common and may extend 5-10 μm into the medium (Brock *et al.*, 1996). Usage of the terms pili and fimbriae is confused (Hancock, 1991). Fimbriae, filamentous protein structures that extend up to 1 μm from the bacterial surface, have often been regarded as important adhesins both in non-specific adhesion (Otto *et al.*, 1999) and in receptor-mediated adhesion to tissues (Klemm and Schebri, 2000). Pili are generally involved in conjugation, are longer than fimbriae and only one or a few are present on the surface. Several bacterial pathogens attach to the surface of eukaryotic cells through specific binding between the pili and the receptor on the host cell surface (Doyle, 2000).

1.3.2 Cell surface hydrophobicity (CSH)

There are many indications that CSH is an important factor involved in bacterial adhesion and aggregation both in different model systems (van Loosdrecht *et al.*, 1987 a; Rijnaarts *et al.*, 1995 b) and in natural systems of practical interest such as activated sludge (Zita and Hermansson, 1997 a, b; Olofsson *et al.*, 1998; Jorand *et al.*, 1998). There is also a growing evidence that especially adhesion of pathogens to tissues may be driven by hydrophobic interactions (Doyle, 2000).

The most common hydrophobic components on a cell surface are cell wall associated proteins, lipoteichoic acids, lipopolysaccharides, mycolic acids, fimbrial proteins, or even secreted polysaccharides containing hydrophobic residues. The list of so-called hydrophobins may be found in reviews by Rosenberg and Doyle (1990) and Doyle (2000).

Despite the importance of CSH, its measurement is a subject of continuing debate and criticism. Commonly employed methods are the microbial adhesion to hydrocarbons (MATH), hydrophobic interaction chromatography (HIC), contact angle measurements (CAM), microsphere adhesion (MAC) or salt aggregation test (SAT) described by Rosenberg and Doyle (1990). It has been observed that the outcomes of different tests often do not correlate (Pembrey *et al.*, 1999). This should not be surprising considering the different character of the assays. Adhesion based hydrophobicity assays such as MATH and HIC do

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not measure the overall hydrophobicity of the cell surface, but rather give an indication of presence of any structures with affinity for hexadecane or octyl-Sepharose (Rosenberg and Doyle, 1990). Especially the MATH test, commonly used due to its simplicity, has been very criticized lately. It has been argued that this test measures a complex interplay of interactions, rather than cell surface hydrophobicity (Busscher *et al.*, 1995 a; van der Mei *et al.*, 1995). It has also been observed that even slight changes in conditions such as cell concentration, pH, ionic strength, time or temperature can influence the results of these tests (Bunt *et al.*, 1993), and that MATH test may severely modify the constitution of microbial surfaces and reduce viability of some bacteria (Pembrey *et al.*, 1999).

CAM, involving the measurement of the contact angle of a liquid droplet placed on a smooth lawn of dried cells, is probably the most definitive way to determine cell surface hydrophobicity (Doyle, 2000). This method gives an average value of the overall hydrophobicity of the surface. A reference guide to microbial cell surface hydrophobicity based on contact angles for 142 different isolates has been published (van der Mei *et al.*, 1998), emphasizing the need to accept this method as the standard for CSH. However, air drying during the sample preparation has also been shown to affect the cell viability and cell surface composition (Pembrey *et al.*, 1999).

MAC is a relatively new method especially suitable for determination of hydrophobicity in environmental samples without prior cultivation (Zita and Hermansson, 1997 b). It also gives detailed information about CSH of a population, showing differences between single cells (Zita and Hermansson, 1997 a, b; Olofsson *et al.*, 1998).

1.3.3 Cell surface charge (CSC)

Almost all bacteria have a negatively charged surface at physiological pH values (James, 1991). The charge originates from dissociation of surface groups and from the adsorption of charged ions. The most common ionizable groups on the bacterial surface are phosphate, carboxylate or amino groups. The net charge depends on the relative amounts of positively and negatively charged groups, and also on the extent of their mutual interactions and thereby localization on the surface (Hancock, 1991).

The CSC of bacteria may be estimated by measurement of electrophoretic mobility (U) (James, 1991; Wilson *et al.*, 2001). A derived quantity, the so-called electrokinetic or zeta

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potential (ζ) (see Section 1.4.2), may be calculated from the electrophoretic mobility. The relationship between the mobility of a rigid particle and the zeta potential is rather complicated and depends on radius of the particle (a), the thickness of the double layer (κ^{-1}) defined in Section 1.4.2 and the properties of the suspending medium. In a limiting case, when the particle is large and the double layer thin, giving $\kappa a \gg 1$, the zeta potential may be calculated from the simple Smoluchowski expression (Mørk, 1994):

$$U = \varepsilon\zeta/\eta$$

where ε is the permitivity and η the viscosity of the suspending medium. This relationship is strictly valid only for rigid non-permeable and non-conducting particles. Bacterial cells covered with polymer layers may show an electrokinetic behavior rather different from rigid particles, and a more appropriate theory allowing for non-zero mobility at high electrolyte concentration was developed by Oshima and co-workers (Oshima and Kondo, 1991; Oshima, 1995). In agreement with this theory, the polymer layers on the bacterial surfaces have been shown to reduce the surface potential significantly, especially at high ionic strengths (Morisaki *et al.*, 1999), showing a limitation of the Smoluchowski equation. However, due to its simplicity, the Smoluchowski formula still clearly dominates when the zeta potentials of bacteria are reported (Ong *et al.*, 1999; Châtellier *et al.*, 2001; Wilson *et al.*, 2001).

1.4 Interactions in colloidal systems

Colloid science concerns systems where particles or large molecules, having at least one dimension within the range of 1 nm to 1 μm, are dispersed in a medium (Hiemenz and Rajagopalan, 1997). Since bacterial suspensions and chitosan solutions belong to such colloidal systems, their behavior is governed by principles of colloid chemistry. The characteristic properties of colloidal systems, such as their stability, are determined by the dimensions of colloids as well as mutual interactions between dispersed components and their interaction with the dispersing medium. These colloidal forces originate in a myriad of electromagnetic intermolecular interactions in the corresponding colloids and in the dispersing medium and manifest themselves in such diverse phenomena as microbial adhesion and aggregation, polymer adsorption or self-assembly of biological structures.

The fundamental forces governing the behavior of colloidal systems are briefly outlined below. Some of them are reasonably well understood on molecular level, whereas others are

still in the inceptive state. The magnitude of these forces depends both on the size of colloids and on the distance between them. Thus, the interactions between macroscopic bodies will be of much longer range than those between molecules even though the same basic type of force may be operating in each case (Israelachvili, 1998). Furthermore, the different power laws of distance dependencies of individual forces imply that the total energy of interaction may be both attractive and repulsive, depending on the distance. A more thorough treatment of colloidal interactions can be found elsewhere (Evans and Wennerström, 1994; Mørk, 1994; van Oss, 1994; Hiemenz and Rajagopalan, 1997; Israelachvili, 1998).

1.4.1 van der Waals forces

Van der Waals forces (VDW) are universal attractive forces between molecules originating in the dipole or induced dipole interactions at the atomic level. They are essential in determining properties of materials and behavior of colloidal systems. There are three major types of VDW forces:

I. Keesom forces are forces between molecules with permanent dipoles, that is molecules possessing an asymmetrical distribution of electrons. The particularly strong example of dipole-dipole interaction is hydrogen bonding. Keesom interactions are important in highly polar molecules such as water, HF or NH_3, both in the liquid and solid state.

II. Debye interactions, also referred to as permanent dipole-induced dipole forces, usually make the smallest contribution to the total VDW.

III. London dispersion forces, or induced dipole-induced dipole interactions leading to an attractive force between any pair of atoms and molecules (Evans and Wennerström, 1994), are perhaps the most important contribution to the total VDW forces. They play a role in adhesion, surface tension, adsorption, cohesive properties of liquids and solids or stability of colloidal dispersions (Israelachvili, 1998).

The strength of VDW forces increases in the case of interaction between macroscopic objects such as colloidal particles since typically each particle consists of a large number of atoms or molecules. Approaches used to calculate the VDW interactions between macroscopic bodies would be far beyond the scope of this text and may be found elsewhere (Evans and Wenerström, 1994; Hiemenz and Rajagopalan 1997; Israelachvili, 1998). Briefly, the first

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approach, developed by Hamaker, is based on summation of all interactions between all molecules in the bodies, leading to rather simple expressions. However, this approach is undoubtedly oversimplified and a macroscopic approach according to Lifshitz is considered as more correct. The Lifshitz approach is based on bulk properties of the interacting media rather than molecular parameters, however it is rather complicated and difficult to apply.

The VDW forces are always attractive when any two bodies interact in vacuum or when they are of the same material. However, VDW repulsion may occasionally arise between bodies of significantly different properties such as between bacteria and Teflon in water (van Oss, 1994). The VDW forces are long-ranged and strongly dependent on separation distance (r). At short separation, r < 10 nm, the VDW forces decrease proportionally with $1/r^6$. With increasing r to 100 nm, the forces become retarded and decay faster, approximately with $1/r^7$ (Hiemenz and Rajagopalan, 1997; Israelachvili, 1998).

1.4.2 Electrostatic interactions

Electrostatic interactions play an essential role in the conformation and structure of macromolecules, stability of colloidal dispersions or transport functions associated with biological membranes.

Surfaces immersed in a liquid acquire charges mainly by the dissociation of a surface group or by the adsorption of an ion or polyelectrolyte from a solution. In an electrolyte solution, the charge on the surface is balanced by the presence of counterions, whose distribution around the surface is not uniform and gives rise to an electric double-layer (Hunter, 1981; Evans and Wenerström, 1994). The double-layer forms spontaneously by two opposing forces: electrostatic attraction tending to localize the counterions close to the particles (Coulombic forces) and the tendency of ions to diffuse randomly through the solution (entropic effect).

The most widely accepted model of the electric double-layer is the Stern model shown in Figure 1.3. In this model, a part of the counterions is bound to the particle surface and forms so-called Stern layer, and the rest is distributed in the diffuse layer. The interaction between charged objects is mainly governed by the overlap of diffuse layers. Consequently, it is the electric potential at the boundary between the Stern and diffuse layer (Ψ_δ), rather than that on the surface (Ψ_0), that is the most relevant for interactions. However, there are no experimental

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methods to measure it, and as a substitute, the electrokinetic or zeta potential (ζ) on the shear plane is used. The magnitude of the effective potential (assumed to be ζ) and the extent of the diffuse layer are two major factors influencing electrostatic interactions between two objects.
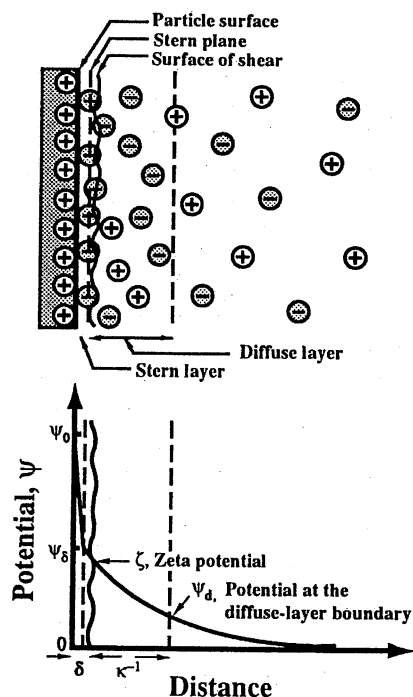
Figure 1.3 Schematic illustration of the Stern model of the electric double-layer and variation of the electric potential with distance (Hiemenz and Rajagopalan, 1997).

Both the surface potential and the extent of the double-layer are strongly dependent on the ionic strength. Increasing the concentration or the charge of the electrolyte in the solution decreases the "surface" potential, compresses the double-layer and shortens its extent as illustrated in Figure 1.4. At a distance x from a surface, the potential ψ decays according to Poisson-Boltzmann approximation as:

$$\Psi = \Psi_\delta \exp(-\kappa x) \qquad\qquad \kappa = 2.3 \times 10^9 \left(\sum c_i z_i^2 \right)^{1/2}$$

where κ (m^{-1}) is the Debye-Hückel parameter or Debye length, given here as a function of the molar concentration (c_i) and the valence (z_i) of ion i in water at 25°C. The inverse Debye length, $1/\kappa$, is a measure of the thickness of the diffuse double-layer. It ranges from about 1000 nm in pure water to about 1 nm in 0.1 M NaCl solution. However, it is important to note

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that the electric potential at the distance $1/\kappa$ is not negligible and the actual distribution of counterions in the vicinity of a charged surface approaches the bulk value only at large distances from the surface.
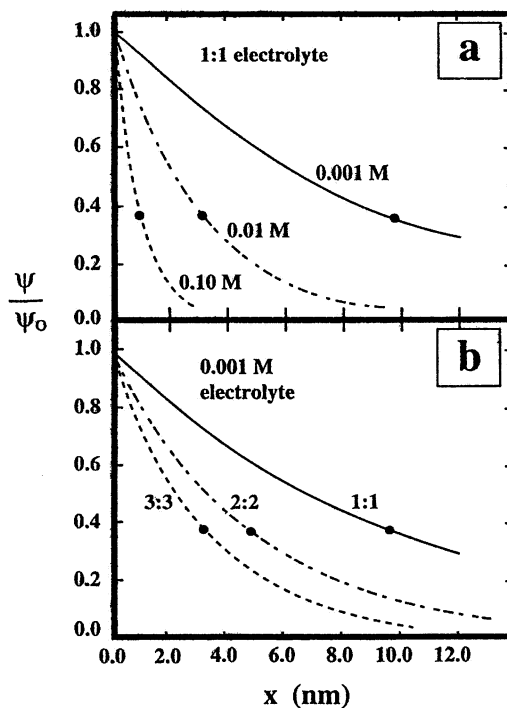
Figure 1.4 Fraction of electric potential versus distance from a surface for (a) various salt concentrations of 1:1 electrolyte and (b) electrolytes of three different valence types at a concentration of 0.001 M (Hiemenz and Rajagopalan, 1997).

When two charged objects approach each other, they start to influence each other as soon as their double-layers overlap. If particles are of the same sign they experience a repulsive interaction. When the double-layer is more diffuse, the repulsion occurs at longer separation distance (see Figure 1.4). If the electrolyte concentration is increased, particles may approach closer and the attractive van der Waals forces may then overtake the repulsion and cause aggregation. This is a general principle of the so-called DVLO-theory of colloid stability, developed independently by Deryagin and Landau and Verwey and Overbeek in 1940s (Hiemenz and Rajagopalan, 1997). In this quantitative theory, the van der Waals attraction (V_A) and electric double-layer repulsion (V_E) are assumed to be additive and combined to give the total energy of interaction (V_T) between particles as a function of separation distance (d) as shown in Figure 1.5. V_T first passes through a shallow secondary minimum, then

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through a maximum forming an energy barrier against aggregation, before reaches a deep primary minimum. DVLO-theory provides a basic framework for thinking in colloid chemistry, but since it ignores other types of interactions, such as those discussed below, it cannot fully explain the behavior of more complex colloidal systems.
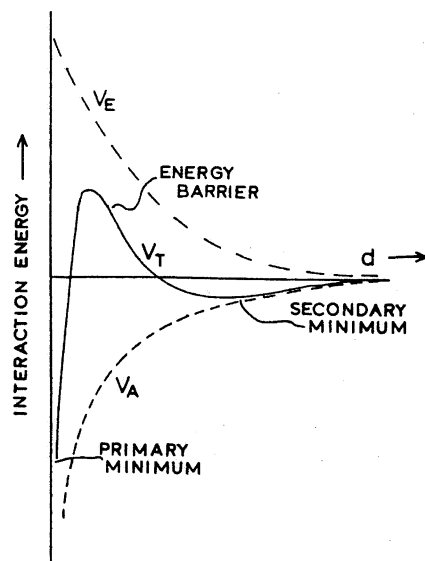
Figure 1.5 Potential energy diagram for the interaction of colloidal particles according to DVLO theory of colloid stability. The van der Waals attraction (V_A), electrical repulsion (V_E) and the total interaction energy (V_T) are shown as a function of particle separation (d) (Gregory, 1989).

1.4.3 Solvent structure-based interactions

Hydration and hydrophobic interactions are sometimes referred to as solvent structure-based interactions (Cohen Stuart *et al.*, 1991) to emphasize their relationship to the nature and properties of water.

I. Hydrophobic interactions denote unusually strong attraction between hydrophobic molecules and surfaces in water (Israelachvili, 1998). However, the term "hydrophobicity" is confusing itself (Rosenberg and Doyle, 1990) and still have no clear and satisfying definition (Tanford, 1997). Traditionally, the reluctance of apolar compounds to dissolve in water has been attributed to their hydrophobicity, or phobia of water. However this is rather misleading since the London dispersion interactions between water and apolar compounds are favorable (Israelachvili, 1998; Doyle, 2000). The hydrophobicity may be better explained when looking at the special properties of water with its high cohesive energy and strong inclination of water

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molecules to form hydrogen bonds (O···H) with each other. These are reluctant to sacrifice their hydrogen bonds leading to reorientation and disruption of existing water structure. Consequently, the strong attraction of water molecules for one another dominates, or as expressed by Israelachvili (1998), water simply loves itself too much to let some substances to get in its way. The *hydrophobic effect* can be looked upon as a tendency of certain molecules, or their parts, to associate with similar structures rather than disrupt the structure of water.

The recognition of the role of the hydrophobic interactions in biology is increasing as illustrated by Tanford (1997): "Though diverse factors are involved in determining the precise specificity of molecular interactions in biology, the hydrophobic force is the energetically dominant force for containment, adhesion, *etc.*, in all life processes". It has been recognized that hydrophobic interactions are an important factor in protein structures and biological recognition (Rose, 1993; Tanford, 1997; Karpluss, 1997) or in stability of nucleic acids. Hydrophobic interactions also play a role in colonization of surfaces by microorganisms. There is growing evidence that especially adhesion of pathogens to tissues may be driven by hydrophobic interactions (Doyle, 2000). It has been reported that the range of hydrophobic interactions seems to be longer than believed, and extends up to 80 nm (Christenson *et al.*, 1987).

II. Hydration interactions are responsible for strong binding of water to ions, charged species, polar and hydrophilic molecules, making water a good solvent or suspending medium for colloidal and biological interactions. The fact that even uncharged molecules and particles may be dissolved or suspended in water implies that additional repulsive force between them must operate and exceed the attractive van der Waals forces. This repulsion force was traditionally believed to arise from the strongly bound and oriented layer of water molecules that induce the orientation of water molecules in subsequent layers. When two approaching bodies are about to make contact, this water must be removed and work must be done.

Israelachvili and Wennerström (1996) have suggested an alternative theory of the origin of hydration forces. They postulate that the repulsion often observed between colloidal and biological objects in water are not due to a layer of structured water, but rather due to the entropic repulsion arising from the interacting surface groups and ions.

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Whatever the molecular origin of the hydration forces, they are typically short-ranged with extent only about 10 Å.

III. Acid-base interactions (AB-interactions) concept is a more quantitative approach to hydrophobic and hydration interactions, developed by van Oss (1994). He treats these interactions as polar forces resulting from electron-donor/ electron-acceptor (Lewis acid-base) interactions between polar compounds in a polar medium such as water. The AB-interactions are considered to be long-range forces, which may be much stronger than VDW and electrostatic interactions.

1.4.4 Polymer-induced interactions

The presence of polymers may lead to macroscopic interactions, also called osmotic and entropic interactions related to macromolecular adsorption (Cohen Stuart *et al.*, 1991). It has been known and exploited for centuries that small amounts of added polymer may destabilize colloids, whereas larger amounts can have stabilizing effect. According to van Oss (1994), polymer interactions are secondary colloidal forces, which are essentially just a special kind of manifestation of the primary driving forces, discussed in Sections 1.4.1-1.4.3. These interactions are extensively described in a number of books (Tadros, 1982; Napper, 1983; Evans and Wennerström, 1994; Hiemenz and Rajagopalan 1997), and only a brief summary is given below.

Provided there is any affinity between a polymer and a surface, polymers in solution adsorb strongly and irreversibly to a surface due to a high number of contacts that may be established between a polymer molecule and a surface and thereby high total energy of adsorption (Robb, 1984; Evans and Wennerström, 1994). This is also the reason why there is no such a thing as a clean surface in a natural environment- any surface will be immediately covered by a layer of adsorbed organic molecules, especially proteins and other polymers, modifying the properties of that surface (Marshall, 1985; Busscher *et al.*, 1995 b; Schneider, 1996). Since adsorption of polymers to surfaces and interfaces is utilized in many applications, considerable scientific interest is devoted to this field. The main trends of the adsorption behavior of neutral polymers and polyelectrolytes have been summarized in a review by Cohen Stuart *et al.* (1991) and in an excellent monograph by Fleer *et al.* (1993).

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The adsorption of polymers is governed by interplay between molecular weight, solvent quality and interaction energy between the monomers and surface. For charged polymers, the polyelectrolyte charge, the surface charge and ionic strength are the essential variables. The main conclusions of the studies are that the adsorbed amount of polyelectrolyte depends strongly on charge density, which is controlled by ionic strength, and in the case of weak polyelectrolytes, also pH (Böhmer *et al.*, 1990). If the polyelectrolyte is fully charged, the adsorption layer is thin and electrostatic repulsion opposes further adsorption. Increasing salt concentration reduces this repulsion, more polymer adsorbs and the layer becomes more extended. In this case, the adsorption also increases with increasing molecular weight of the polymer. The theory was shown to agree well with experimental results of Blaakmeer *et al.* (1990), Bauer *et al.* (1999), Bremmel *et al.* (1998). Figure 1.6 schematically illustrates the possible configurations of a polymer chain adsorbed to a surface and the terminology used to describe it.
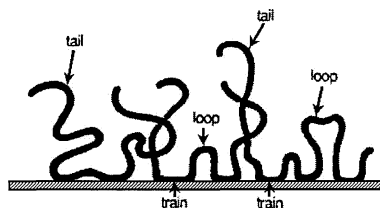
Figure 1.6 Possible configurations of adsorbed polymer chain (Fleer *et al.*, 1993).

Surfaces covered with polymers will "see" one another through the outer part of the polymer layer. These layers will start to overlap at surface separation distances in the range of approximately 10-100 nm, depending on the dimensions of the adsorbed layer which corresponds to the radius of gyration (R_g) (Robb, 1984; Evans and Wennerström, 1994). At these distances, the magnitude of van der Waals and double-layer forces between the bare surfaces is usually negligible and polymer interactions dominate (Robb, 1984). An adsorbed polymer in a good solvent expands away from the surface to gain configurational entropy. When it comes in contact with another chain, its allowed conformations are restricted and the free energy increases. This is a principle of a repulsive force between surfaces covered with adsorbed polymers, referred to as steric stabilization and illustrated in Figure 1.7. The range of this interaction is determined by the dimensions of the polymer chain and its configuration. The more detailed description of these repulsive forces may be obtained from advanced textbooks by Napper (1983) or Israelachvili (1998).

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The attractive type of polymer interaction is called bridging and occurs only if there is sufficient unoccupied particle surface for contact, that is at low added amount of polymer (Pelssers *et al.*, 1989; Gregory, 1993). If a single polymer molecule becomes attached to more than one particle, the particles become " bridged" by the adsorbed polymer as schematically shown in Figure 1.7. Polymer bridging is often observed with high molecular weight polymers adsorbing in "loops and tails" conformation, that is with a significant fraction of polymer segments extruding from the surface (Eriksson *et al.*, 1993; Baran and Gregory, 1996).

(a)   (b)
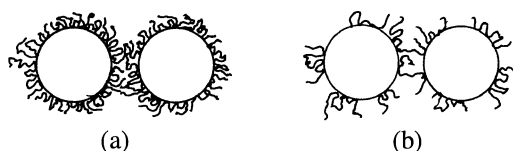
Figure 1.7 Schematic representation of steric stabilization (a) and bridging (b) (Luckham, 1987).

1.5 Bacterial adhesion and flocculation

The principles of bacterial adhesion and flocculation are closely related since both processes involve the interaction between microorganisms and some type of surface. These surfaces may be inert materials, the exterior of human or animal, or other microorganisms of the same or different species (Costerton *et al.*, 1985). As outlined above, both bacterial cell surfaces and most surfaces in natural environment are covered by polymeric material and consequently, the polymer-induced interactions will play a dominant role in these processes.

1.5.1 Flocculation

The terms flocculation and coagulation are used to describe a general situation when colloidal particles form aggregates. These two terms are used in different ways according to the area of application and mechanisms of aggregation. However, as there is a lack of agreement on the distinction, flocculation is often used as a generic term (Gregory, 1989). Flocculation may proceed either naturally, such as in formation of activated sludge flocs, aggregation of myxobacteria or brewing yeasts, or artificially, after addition of flocculating agents, as an important part of solid-liquid separation processes widely used in wastewater treatment and downstream processing. Flocculation agents may be inorganic salts, polymers or their

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combination (Gregory, 1989; Kawamura, 1991). The majority of flocculating agents used in practice are polymers and polyelectrolytes, mostly of synthetic origin, with derivatives of polyacrylamide as a typical example (Gregory, 1989; Hocking *et al.*, 1999).

Flocculation may be caused by any of the following mechanisms or their combination: double-layer compression, charge neutralization, charged patch flocculation, bridging and colloid entrapment. Figure 1.8 summarizes the possible mechanisms for floc formation involving polyelectrolytes. Charged patch flocculation results from an attraction between oppositely charged domains on a particle (Gregory, 1973). Charge neutralization is achieved by adsorption of a oppositely charged flocculant on the surface of a colloid, resulting in zero net charge (Durand-Piana *et al.*, 1987; Ashmore and Hearn, 2000). Bridging occurs when a polymer molecule attaches to several colloids, binding them together, as also shown in Fig. 1.7 (Eriksson *et al.*, 1993; Chaplain *et al.*, 1995). The colloid entrapment involves polyelectrolyte network formation as shown in Fig. 1.8 C or addition of large doses of metal salts such as aluminum or iron (Gregory, 1989). These will precipitate as hydrous metal oxides and sweep the colloids out of the suspension within the precipitating mass.
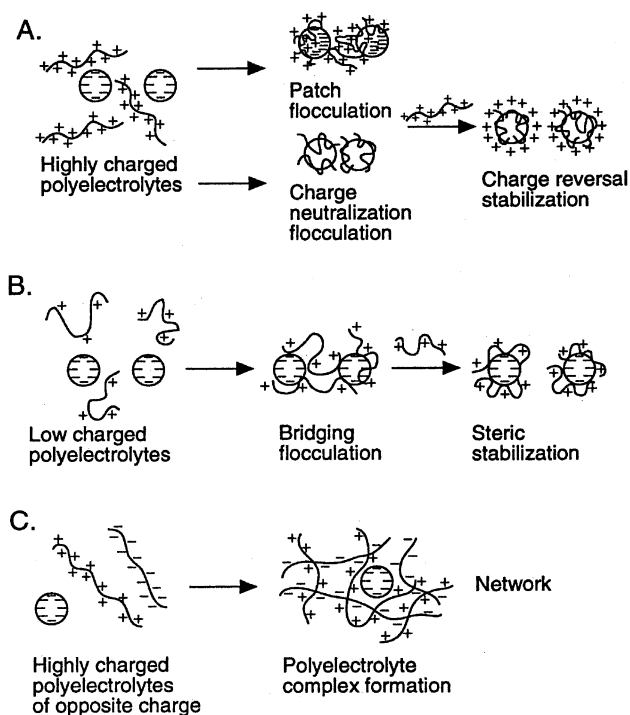

Figure 1.8 Mechanisms of flocculation involving charged polyelectrolytes (Agerkvist, 1992).

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The colloidal interactions, described in Section 1.4, determine the structure and the strength of the formed aggregates as well as the collision efficiency. However, since the range of these interactions is usually below 100 nm, particles must be first transported into sufficient distance to encounter these forces. Hence, the flocculation process significantly depends on mixing conditions (Gregory and Guibai, 1991) as well as the concentration of particles (Gregory and Sheiham, 1974; Chaplain *et al.*, 1995). Therefore, the actual flocculation mechanism will also depend on the relative rates of polymer adsorption, polymer chain rearrangements and particle collision rate (Pelssers *et al.*, 1989; Chaplain *et al.*, 1995).

As schematically shown in Fig. 1.8, flocculation occurs only in a certain range of polymer concentration and larger doses of polymers may lead to restabilization of colloidal dispersion. This restabilization may occur due to an entropic effect known as steric stabilization discussed in Section 1.4.4 or due to the reversal of charge (Fig. 1.8). Stabilization of colloidal dispersion by polymers is very important in many applications, such as in paints or emulsions.

Despite extensive research and tremendous amounts of literature on the subject of flocculation by polymers, a weak point remains the missing rational basis for the choice of optimal flocculants (Leu and Ghosh, 1988; Gregory, 1989; Hocking *et al.*, 1999). According to Gregory (1989), the subject of flocculation by polymers, although understood reasonably well in broad outline, needs a good deal of systematic study before a more rational application of polymeric flocculants can be achieved.

1.5.2 Adhesion

Bacterial adhesion is the first step of biofilm formation and may be defined as a process of transfer of a cell from an unbound state in the bulk phase to a more or less firm attached state at an interface (Hermansson, 1999). Bacteria associated with interfaces are found in most natural and engineered environments, where they often cause serious problems (Costerton *et al.*, 1995; Flemming, 1995). On the other hand, adhesion of bacteria is crucial in a number of biotechnological application or for a function of many natural ecosystems. As the process of bacterial adhesion is manifested in so many forms in a wide variety of disciplines, different theories and models to describe this phenomenon have been developed, however, there is still no general agreement on which of them is best suited (Marshall, 1985; Busscher and Weerkamp, 1987; van Loosdrecht *et al.*, 1989; Hermansson, 1999).

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It is generally accepted that both specific surface components and the macroscopic surface properties such as cell surface charge, hydrophobicity or surface free energy are important in adhesion. The specific surface components are involved in so-called specific adhesion requiring a contact between stereochemically complementary structures (Busscher and Weerkamp, 1987). The specific adhesion is usually mediated by protein molecules located in fimbriae, pili or as a single protein species on bacterial surface (Klemm and Schembri, 2000). These so-called adhesins allow targeting of a given bacterium to a specific surface and are thereby essential for virulence of most pathogens (Bertin *et al.*, 1996; Doyle, 2000).

The non-specific interactions in adhesion are defined as interactions due to overall macroscopic surface properties of a cell (Busscher and Weerkamp, 1987). These can extend over large distances where any recognition necessary for the intermolecular interactions is not possible. The same authors postulated a hypothesis for mechanism of bacterial adhesion to solid surfaces: If a cell experience attractive non-specific interactions at larger distances, where macroscopic cell surface properties play the dominant role, it may approach the surface close enough to enable short-range interactions to occur, either mediated by specific adhesins, or by hydrophobic effect and polymer bridging.

The adhesion of a negatively charged bacterium to a negatively charged surface has traditionally been regarded as a two-step event, where the first step is a reversible adhesion due to long-range forces, possibly followed by an irreversible attachment (Norde and Lyklema, 1989; van Loosdrecht *et al.*, 1989). The reversible adhesion is an attraction by long-range forces holding bacteria near a surface, so that bacteria continue to exhibit Brownian motion and can be readily removed from the surface (Marshall, 1985; Meinders *et al.*, 1995). Both steps of the bacterial adhesion may be explained by the DVLO theory mentioned in Section 1.4.2, and recently reviewed by Hermansson (1999). The "classical" DVLO theory describes the net interaction between a cell and a surface as a balance between van der Waals and the electrostatic interactions as a function of separation distance. Other interactions such as hydrophobic and polymer interactions are neglected. Generally, at low and intermediate ionic strength, the adhesion will occur in the so-called secondary minimum located typically 10-20 nm from the surface (Busscher and Weerkamp, 1987; Rijnaarts *et al.*, 1995 b). At high ionic strength, usually over 0.1 M, electrostatic interactions are strongly reduced (see Section 1.4.2) and cells may approach close to the surface and adhere irreversibly. There are

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numerous reports where the DVLO theory has been shown to describe well the bacterial adhesion at low ionic strength, while poorly at high ionic strength (van Loosdrecht *et al.*, 1989; Norde and Lyklema, 1989; Zita and Hermansson, 1994; Rijnaarts *et al.*, 1995 b).

A more adequate model to describe bacterial adhesion seems to be the extended DVLO-AB theory including the attractive hydrophobic interactions and repulsive hydration effects (van Oss, 1994; Meinders *et al.*, 1995; Jucker *et al.*, 1996). However, even the DVLO-AB model often fails to describe bacterial adhesion since it still does not take into account the presence of surface polymers which may cause adhesion even when the cells do not experience DVLO-AB attraction (Jucker *et al.*, 1998 a). Unfortunately, the quantification of polymer interactions is complicated by poorly accessible physical and chemical properties of bacterial cell surfaces (Jucker *et al.*, 1998 a). As there is an increasing evidence that polymer interactions may dominate the interaction of bacteria with surfaces (Jucker *et al.*, 1997, 1998 b; Ong *et al.*, 1999; Camesano and Logan, 2000), especially at higher ionic strengths, a more detailed and precise information about the cell surface architecture is required. As pointed out in Section 1.3, AFM may be used both for characterization of cell surfaces (Boonart *et al.*, 2000; van der Mei *et al.*, 2000) and for direct measurement of forces between bacteria and surfaces, thereby assessing the role of molecular interactions in cell adhesion and cell aggregation (Razatos *et al.*, 1998; Ong *et al.*, 1999; Camesano and Logan, 2000).

1.5.3 Limitations of physicochemical theories

The physicochemical approaches to bacterial adhesion and flocculation are based on an assumption that bacteria behave as colloidal particles. However, it should be clear from Section 1.3 that bacteria cannot be regarded as smooth and rigid colloidal particles. Bacteria may neither be regarded as inert particles, but living organism capable of metabolism, growth and, in some instances, independent motion. Consequently, cell surface characteristics may rapidly change as a response to external conditions. These limitations may be illustrated by a few considerations.

As already mentioned in Section 1.4, the different forces vary strongly in their magnitude and distance dependence. The total interaction energy for two particles may be attractive at contact, but repulsive at longer separation and particles will thus remain separated (Fig. 1.5). Furthermore, the net interaction energy is proportional to the radius of particles. As even the

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long-range interaction forces discussed in Section 1.4 have range of action below 100 nm (Israelachvili, 1998), it is necessary to "to look" at the cells at the same scale. At this scale, the definition of a distance from the cell to the surface looses its meaning due to the heterogeneity and presence of various cell appendages as described in Section 1.3. Consequently, the different parts of a cell surface may be exposed to forces of different strength and maybe also of different kind. For instance, a fimbriated bacterium in a distance of 300 nm from the surface may easily establish contact with the surface through fimbriae without itself being affected by any forces. Similarly, a negatively charged G- bacterium with LPS protruding 20 nm into the medium may likewise be attached to a negatively charged surface through its LPS chains due to their smaller radius and thereby, lower electrostatic repulsion, or any kind of specific-interaction.

Furthermore, some biological factors of possible importance should be also mentioned. Bacteria are metabolically active and may synthesize and excrete different metabolites. It is generally recognized that adhered bacteria start to produce extracellular polymers shortly after adhesion. These polymers effectively bind the cells to the surface and later provide a protective matrix surrounding the attached cells. There is even some evidence that bacteria are able to sense the surfaces and alter the composition of outer membrane proteins (Otto *et al.*, 2001).

Binding of highly charged polymers to cell surface may also affect the ionic balance of the cell membrane, thereby inducing a physiological stress response aiming to maintain proper ionic and osmotic intracellular conditions.

Despite these limitations, it may be concluded according to Hermansson (1999): A correct translation of colloidal theories to bacterial adhesion is never the less very useful in order to form a framework in which biological factors may be added, eventually forming a unified adhesion theory.

1.6 Scope

The general scope of this work has been (1) to evaluate structure –function relationships in interactions of chitosans with bacteria and (2) to provide an empirical foundation for identification of important mechanisms of these interactions.

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As this study was the first of this kind, method development and screening studies have formed a considerable part of this work. Especially methods developed to quantify chitosan both in solution and bound to surfaces, and to estimate its electrostatic properties were essential, and are thus presented as an independent chapter (Chapter 2). Most experiments were performed with *E. coli* K12 wild type strain (DSM 498), arbitrarily chosen as a model organism. Later, other bacterial species were included and these were also chosen randomly due to their relatively prevalent occurrence and simplicity of cultivation.

The rest of this work consists of two major topics, reflecting the two different experimental systems where these interactions were studied:

1) Interaction of bacteria with chitosan added to bacterial suspension – flocculation (Chapter 3)
2) Interaction of bacteria with chitosan bound to glass - adhesion (Chapter 4)

Despite increasing interest in application of chitosan for flocculation and removal of different materials, relatively little is known about how the chitosan structure affects the flocculation performance. According to my knowledge, many studies on the flocculation by chitosan generalize the results based on the use of one particular commercial chitosan type (usually F_A 0-0.2), without considering the large differences in chitosan composition and properties. Since it is traditionally expected that the flocculation efficiency of chitosan is directly proportional to its charge, the involvement of other non-electrostatic interaction is usually neglected.

Chitosan represents an interesting model biopolymer whose properties may be manipulated both by its chemical composition and molecular weight as well as by environmental conditions such as pH and ionic strength. The systematic variation in chitosan-related variables (F_A, molecular weight), environmental conditions (pH, ionic strength) may establish relationship between the structure of chitosan and flocculation performance and reveal the relative importance of different forces and mechanisms.

It is generally recognized that mechanisms of adhesion and flocculation are closely related. It seemed therefore logical to compare the trends observed in flocculation studies with those obtained in adhesion experiments summarized in Chapter 4. The same variables mentioned above were also studied with chitosan bound to a planar glass surface. Since the same kind of interactions is to be expected between the bacterial cells and chitosan, similar trends as found

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in flocculation may be valid also in adhesion. The aim of our adhesion studies was merely to compare the process of adhesion and flocculation and possibly show any common patterns, not to investigate the mechanisms of adhesion.

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2. QUANTIFICATION AND CHARACTERIZATION

2.1 Introduction

This chapter summarizes our studies concerned with the development of methods to quantify chitosan, the characterization of electrostatic properties of chitosan and the characterization of cell surface properties of bacteria.

During the investigation of adhesion and flocculation, it was necessary to quantify chitosan bound to a solid surface such as glass as well as chitosan adsorbed to bacterial cells. Since no convenient methods were available, a new method had to be developed for quantification of bound chitosan. The amino group of GlcN residue is rather reactive, and chitosan may be quantified by an analytic method based on its determination. The reaction of chitosan with ninhydrin is one example, see Appendix Paper 1.

Another possibility is to label chitosan with a chromophore or a radioactive label. The amount of incorporated marker, that is degree of substitution (DS), determines the detection limit. However, a high degree of modification may influence the properties of chitosan. Radioactive labeling of chitosans by ^{3}H and ^{125}I isotopes was described by Gåserød *et al.* (1998). However, a direct measurement of ^{3}H-labelled chitosan bound to alginate capsules (Gåserød *et al.*, 1998) or to glass (unpublished results) seemed difficult. It was also found inconvenient to break the glass prior to scintillation counting, and this method was therefore excluded. A fluorophore, 9-anthraldehyde, was instead used to label the chitosan, see Appendix Paper 2.

The methods for determination of the chemical composition (F_A) and molecular weight of chitosans are well established (Vårum *et al.*, 1991 a; Anthonsen *et al.*, 1993) and were routinely performed for all chitosans samples used. However, uncertainty still exists concerning the estimation of charge density of different chitosans since the value of the dissociation constant pK_a of chitosan, and especially then the relationship between F_A and pK_a, are a subject of debate. The electrostatic properties of chitosans are essential in interactions with charged bacteria or surfaces. It is important to be able to predict the charge density of different chitosans and how changes in pH and ionic strength will affect it.

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Therefore, we have examined the chitosans by electrophoretic light scattering technique (ELS) and ^{1}H-NMR as described in Appendix Paper 3.

Finally, bacterial strains applied through our study were also characterized with respect to their macroscopic surface properties such as cell surface charge or hydrophobicity, see Appendix Paper 6.

2.2 Quantification of chitosan by ninhydrin method

A primary amino group reacts with ninhydrin to form a colored reaction product, diketohydrindylidene-diketohydrindamine, also called Ruhemann's purple (Moore and Stein, 1948; Friedman and Williams, 1974). This reaction has been known and studied for years (Moore and Stein, 1948) and is extensively used for amino acids analysis. Application to other compounds is often complicated by the fact that the amount of color formed from a given compound may not correspond with the expected theoretical yield (Yanari, 1955; Friedman and Williams, 1973 and 1974). The possible reasons for the apparent non-ideal stoichiometry of the ninhydrin reaction may include slow formation of Ruhemann's purple, side reactions, color instability as well as interfering color (Friedman and Williams, 1974).

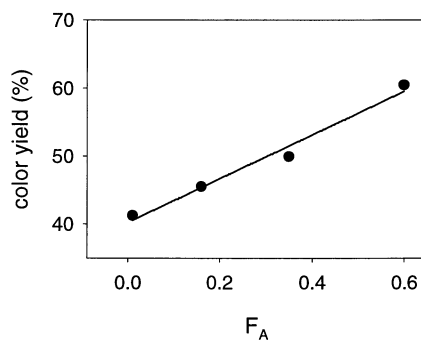**Figure 2.1** Effect of fraction of acetylated units (F_A) of chitosan on the reactivity in ninhydrin reaction (Appendix Paper 1).

Curotto and Aros (1993) used the ninhydrin reaction for quantitative determination of chitosan as well as the percentage of free amino groups. However, our preliminary experiments showed unexpected anomalies and the reaction of chitosans with ninhydrin was thoroughly examined, see Appendix Paper 1.

It has been found that the color yield per mole of amino groups depended on the composition of chitosan (F_A): the higher the amount of GlcN units, the lower was the molar color yield. Moreover, chitosans with F_A values of 0.01-0.6 reached only 41% -60% of the possible theoretical yield, assuming that the monosaccharide GlcN gave the maximum obtainable color yield. As shown in Figure 2.1, a linear relationship existed between F_A of the chitosan and the degree of reactivity in the ninhydrin reaction.

Figure 2.2 shows the reactivity of short β-(1$\rightarrow$4)- linked glucosamine oligomers compared to the monomer. Considerable difference in the reactivity was observed for short GlcN oligomers (DP 2-4), while the molar color yields of longer oligomers with $\overline{DP}_n$ between 10 and 1000 remained constant.
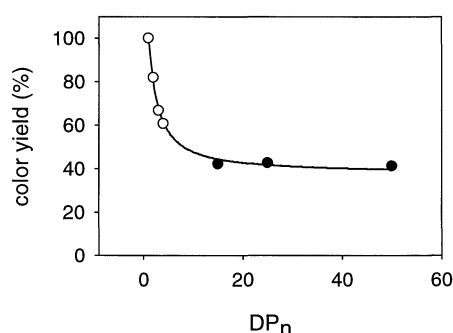
Figure 2.2 Effect of degree of polymerization ($\overline{DP}_n$) of a fully deacetylated chitosan on the reactivity in ninhydrin reaction (Appendix Paper 1). Symbol key: (o) monodisperse samples, (●) polydisperse samples.

The comparison of the reaction rates of chitosans and oligomers showed that incomplete color formation within 30 min standard reaction time may be of importance for some chitosans. While the monomer, dimer, tetramer and chitosan with F_A 0.6 fully completed the reaction, the chitosans with F_A 0.0, 0.09 and 0.16 did not, and a slow increase in color yield was observed, although most of the color was formed during the first 10 minutes. However, the differences in the reaction kinetics of chitosans could only partly explain their different color yields. The extension of the reaction time to 120 min did still not result in the same molar yield for all chitosans (Appendix Paper 1).

The most probable reason for the non-quantitative reaction yield is an unknown side reaction between amino groups and ninhydrin or any of the intermediates. The presence of several

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GlcN units at adjacent sites in the chain had a profound effect on the yield (Fig. 2.2). Possibly, some cyclic products between ninhydrin and another amino group located close to the reacting amino group may be formed as suggested by Friedman and Williams (1974). If such side reaction is reversible, a slow increase in color yield could be observed long after most of the color was formed, depending on the stability of the cyclized product. This could explain both the F_A dependence of the yield of chitosans, the low yields of GlcN oligomers and differences in the reaction rates (Appendix Paper 1).

Although several features of the ninhydrin reaction seemed to be anomalous, this has only limited implications for the practical use of the method for chitosan quantification. Since chitosan samples usually range from 10^3 to 10^6 g/mol, the color yield would be independent on the molecular weight of the sample. The only parameter of importance remains F_A, however, even here the knowledge of an accurate value is not required provided a calibration curve of the unknown sample is available.

Application of the ninhydrin method for the studies of adsorption of chitosan is limited by several facts. First, the quantitative determination of chitosans by this method is only possible in absence of proteins and other amino-group consisting compounds. This is clearly not a case in presence of bacteria and this method cannot be directly used to measure chitosan adsorbed to bacterial cells. Regarding chitosan adsorption to glass surfaces, amounts seems so low that detection limits in the order of 10 mg/L require rather large area to detect amounts adsorbed. It is therefore not sensitive enough for analysis of planar glass surfaces such as microscopic slides. The adsorbed chitosan might only be detected when larger surface area for adsorption was available as in case of glass beads.

2.3 Fluorescence labeling of chitosan

Labeling of chitosan with a fluorophore allows both direct observation of the polymer by fluorescent microscopy and quantification by fluorescence spectroscopy. Appendix Paper 2 describes a method for preparation and characterization of fluorescent chitosans by condensation of chitosan with 9-anthraldehyde (Schiff base formation) followed by reduction using sodium cyanoborohydride. Such fluorescent chitosans emit at Em_{max} 413 nm when excited at Ex_{max} 250 nm. The degree of substitution (DS) of the obtained fluorescent chitosan depends on the molar ratio of the two starting materials, and can be quantitatively

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determined by UV or ^{1}H-NMR spectroscopy. The derivatization procedure lead only to a negligible decrease in intrinsic viscosity of the chitosans (Appendix Paper 2). The conformation of fluorescent chitosans with low degrees of substitution was not altered (Cölfen *et al.*, 1996). Solubility also remained unchanged, fluorescent chitosans were water soluble at acidic pH values, and insoluble in methanol and ethanol.

Fluorescent chitosans were used to quantify the chitosan adsorbed to *Escherichia coli* cells, see Appendix Paper 5. Incorporation of less than 1% of 9-anthraldehyde into the chitosan molecule did not show any effect on the flocculation performance as concluded by comparing the same polymer with and without the label. Our choice of DS of approximately 1% should both allow sufficiently low detection limits and assure that each chitosan molecule will be labeled. The detection limits for a chitosan with 0.8% DS under conditions of our experiments were approximately 0.1 mg/L at pH 5 and 1 mg/L at pH 6.5.

2.4 Characterization of chitosans

2.4.1 Electrostatic properties of chitosans

As mentioned in Section 2.1, the relationship between pK_a and F_A of chitosan is a subject of debate. Anthonsen and Smidsrød (1995) reported in their ^{1}H-NMR study that chitosan oligomers with F_A 0.5 and F_A 0 had the same pK_a of 6.6. Potenciometric titration carried out by Domard (1987) and later Sorlier *et al.* (2001) showed an increase of pK_a with increasing F_A from 6.3 to 7.2. Another discrepancy seems to be the reported changes in the apparent pK_a with the degree of ionization (α) and the values of the intrinsic pK_a, referred to as pK_0, for different chitosans. While Anthonsen and Smidsrød (1995) reported a linear decrease in the apparent pK_a values with increasing α for all chitosans, Domard (1987) and Sorlier *et al.* (2001) have shown that the relationship between apparent pK_a values and α for different chitosans was strongly dependent on F_A. The extrapolation to zero charge density ($\alpha = 0$) gave a pK_0 value of about 9 for both chitosan with F_A 0.5 and F_A 0 (Anthonsen and Smidsrød, 1995). In the contrary, Domard (1987) and Sorlier *et al.* (2001) reported the intrinsic pK_0 values to increase from 6.4 for F_A 0.05 to 7.1 for F_A 0.89.

The mean electrophoretic mobilities (EM) of 3 chitosans with F_A 0.01, 0.13 and 0.49 in buffers of an ionic strength of 0.1 M are shown in Figure 2.3 as a function of pH. It is interesting to note that the ELS measurements did not seem to be affected by precipitation of

low F_A chitosans occurring at higher pH (Vårum *et al.*, 1994), as can be seen from the continuity of the curves presented in Figure 2.3. To determine pK_a, logistic sigmoid curves of the type $y = a/[1+(x/x_0)^b]$ were fitted to the experimental data. At any pH, the EM of chitosans was linear function of F_A, showing that EM was indeed proportional to the charge density of chitosan (see Appendix Paper 3). The pK_a values calculated from the inflection points of the regression curves (x_0) showed that all three chitosans, irrespective of F_A, had nearly identical pK_a values, ranging from 6.52 to 6.57. This is consistent with previous results reported by Anthonsen and Smidsrød (1995) on depolymerized chitosans, where no F_A dependence of pK_a was observed.
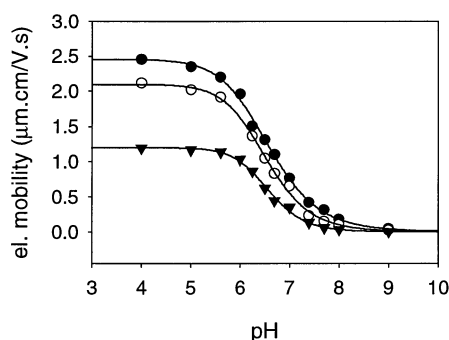
Figure 2.3 Electrophoretic mobility of chitosans as a function of pH (Appendix Paper 3). Symbol key: F_A 0.01 (●), F_A 0.13 (o) and F_A 0.49 (▼).

The effect of NaCl concentration on the EM of chitosans F_A 0.01 and 0.49 is shown in Figure 2.4. As expected, the increase in ionic strength resulted in a decrease of EM, clearly demonstrating the screening effect of counterions. The straight lines obtained show again that in the NaCl concentration range tested, chitosan did not undergo conformational changes causing abrupt changes in EM. Similar relationship between EM and ionic strength has been reported for polystyrenesulfate and DNA (Hoagland *et al.*, 1999).

The constructed Katchalsky plot (Katchalsky, 1954), described in Appendix Paper 3, showed that for all chitosan tested, the apparent pK_a values decreased proportionally with increasing charge density in the range $0.1 < \alpha < 0.8$. Extrapolation of data to zero charge density ($\alpha = 0$) gave pK_0 of about 8.8. This is also in agreement with the previous results of Anthonsen and Smidsrød (1995) and also with a parallel ^{1}H-NMR study of the same chitosans (see Appendix Paper 3).

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An important conclusion is that ELS technique was shown to be very useful method for estimation of electrostatic properties of chitosans as EM was directly proportional to the charge density of chitosan and seemed not to be perturbed by phase or conformational transitions. The data given in Figures 2.3 and 2.4 are important for interpretation of results from flocculation and adhesion studies.
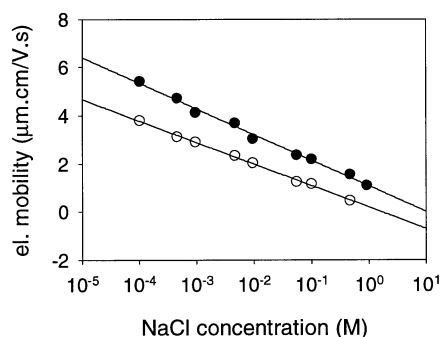
Figure 2.4 Electrophoretic mobility of chitosans as a function of NaCl concentration (Appendix Paper 3). Symbol key: F_A 0.01 (●), F_A 0.49 (o).

2.4.2 Hydrophobicity of chitosan

Chitosan is often described as an amphipilic polymer (Smidsrød, 2000; Domard, 2000). The methyl substituent of the acetyl group of GlcNAc increases the hydrophobic character of a polysaccharide (Neu, 1995). Hence, chitosans with high F_A should be more hydrophobic and may exhibit higher degree of hydrophobic interactions. Such interactions may lead to aggregation in chitosan solution. Indeed, aggregates in chitosan solution have been reported (Anthonsen *et al.*, 1994; Ottøy *et al.*, 1997) and recently, Philippova *et al.* (2001) have shown their hydrophobic nature.

We have attempted to quantify the differences in hydrophobicity of chitosans by measuring the contact angles of water droplets on glass slides with adsorbed chitosan. Unfortunately, no significant differences were observed between the chitosans with F_A 0.01, 0.13 and 0.49. The contact angles on glasses coated by all chitosans were $41 \pm 2°$. Such values of contact angles are typical for relatively hydrophilic surface, such as stainless steel (Egington *et al.*, 1995). However, since the original glass surface was very hydrophilic as shown by immediate wetting by water, a clear modification of the surface following chitosan adsorption was

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recorded. Harkes *et al.* (1991) have reported that the sessile drop technique for measuring of contact angles did not show any differences between glass slides coated with different polymethacrylates. By applying the Wilhelmy plate technique, significant differences were found in hydrophobicity of these surfaces (Harkes *et al.*, 1991). It seems therefore, that the sessile drop technique is not suitable to detect the minor changes in hydrophobicity of polymer coated solid surfaces.

2.5 Characterization of bacteria

2.5.1 Electrostatic properties

The cell surface charge (CSC) of a bacterium depends not only on biological factors affecting the cellular composition (growth phase, growth conditions), but also on external variables such as pH, ionic strength and type of the salt in medium. Most bacteria are negatively charged in the range of pH 4-10, with isoelectric point around pH 2-4 (James, 1991; Rijnaarts *et al.*, 1995 a). CSC is usually expressed as electrophoretic mobility U (μm.cm/V.s) or derived quantity, zeta potential ζ (mV), which is under certain assumptions considered as proportional with EM (see Section 1.3.3). The reported zeta potentials of bacteria under physiological conditions usually range from -6 to -25 mV, however values outside this range may also be found (Jucker *et al.*, 1996).

The zeta potentials of bacteria used in our study were determined by Doppler electrophoretic light scattering (ELS) as described in Appendix paper 6, and are given in Table 2.1. All data were measured in PBS buffer with pH 6.5 and ionic strength of 0.1 M, as these were typical conditions used in flocculation and adhesion experiments. All bacteria were harvested in the stationary growth phase and corresponding culture conditions are given in Appendix Paper 6. Great diversity of zeta potentials of different bacteria was recorded, from slightly negative potential in case of *P. putida* to highly negative potential of *M. luteus*.

It should be clear that despite negative zeta potentials, positively charged groups also exist on bacterial surfaces. The relative amounts of negatively and positively charged groups may be estimated by electrostatic interaction chromatography (ESIC) as described by Pedersen (1980). As the zeta potential values are indicative of total net charge, but not of the distribution of charges, significant electrostatic attraction between sites with positive charge and *e.g.* negatively charged surface may occur.

Table 2.1 Characteristics of bacteria used: zeta potentials and contact angles of water for bacteria resuspended in PBS of pH 6.5 and ionic strength of 0.1 M (Appendix Paper 6).

Bacterial species	Zeta potential (mV) mean ± s.d.	Contact angle of water (°) mean ± s.d.
Gram-negative:		
Escherichia coli	-16.0 ± 0.5	16 ± 1
Serratia marcescens	-14.8 ± 0.8	27 ± 1
Enterobacter cloacae	-7.2 ± 0.1	23 ± 3
Pseudomonas putida	-0.6 ± 0.5	31 ± 3
Pseudomonas sp. 1650	-10.8 ± 0.3	38 ± 4
Gram-positive:		
Micrococcus luteus	-26.6 ± 1.2	29 ± 2
Bacillus megaterium	-19.1 ± 1.9	17 ± 1
Rhodococcus sp. 094	-8.0 ± 0.2	53 ± 4
Reference:		
Polystyrene latex	-43.6 ± 1.6	n.d.[a]

[a] not detected

2.5.2 Cell surface hydrophobicity

Despite the problems with definition and measurements of CSH discussed in Chapter 1, it was shown to be an important factor in interaction of bacteria with various surfaces (van Loosdrecht *et al.*, 1987 a; Zita and Hermansson, 1997 a, b; Olofsson *et al.*, 1998). Similarly as CSC, hydrophobicity is also dependent on biological factors such as the growth phase and growth conditions. It is recognized that hydrophobicity of many cells is higher in exponential growth phase (Bredholt, 2000) or under conditions of nutrient depletion (van Loosdrecht *et al.*, 1989).

Several methods to assess CSH of bacteria were initially attempted in our studies: microbial adhesion to hydrocarbons (MATH), hydrophobic interaction chromatography (HIC) and contact angle measurements (CAM). The MATH assay, performed with hexadecane was strongly dependent on experimental conditions. It was shown that even small differences in the volume ratio of both phases or the duration of vortex mixing had large effect on results.

The HIC assay was complicated by high adhesion of bacteria to glass wool used in the bottom of columns. Only CAM gave satisfying results.

Relative hydrophobicity of the bacterial cells was estimated by measuring the contact angles of water droplets placed on bacterial lawns as described in Appendix paper 6. As shown in Table 2.1, most bacteria exhibited relatively hydrophilic surface with contact angles within 16-30°. *Pseudomonas* sp. and *Rhodococcus* sp. had more hydrophobic surfaces with 38° and 53°, respectively. According to literature (van der Mei *et al.*, 1998), bacteria were shown to range from very hydrophilic with low contact angles resembling glass (< 20°) to very hydrophobic with contact angles comparable to those of Teflon (around 100°). It is also remarkable that the contact angles may vary considerably within taxonomically related species or even between different strains (van Loosdrecht *et al.*, 1987 a; van der Mei *et al.*, 1998).

2.5.3 Other characteristics

Unfortunately, characterization of more microscopic surface properties, such as presence of surface appendages, distribution of hydrophobic/hydrophilic sites, surface microtopography, *etc*. was not carried out. Since all these factors may be of importance in interactions with chitosans, more detailed cell surface analysis would be definitely beneficial.

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3. FLOCCULATION

3.1 Chitosan as a flocculant

Chitosan represents a promising alternative to synthetic polymeric flocculants both in downstream processing (Agerkvist, 1992; Weir *et al.*, 1994) and water treatment (No and Meyers, 2000; Meyer *et al.*, 2000). As mentioned in Section 1.2, especially water treatment offers many possibilities ranging from humic acid removal from drinking water (Eikebrokk, 1999) to treatment of diverse wastewaters (Ganjidoust *et al.*, 1997; Savant and Torres, 2000; Pinnoti *et al.*, 2001) or sludge dewatering (Kawamura, 1991).

Despite increasing interest, the structure-function relationship has been rarely addressed in flocculation studies. Generally, one particular type of chitosan, typically a commercial chitosan with F_A 0-0.2, is used in most studies. As discussed in Chapter 2, by changing the chitosan composition and environmental conditions, the properties of chitosans may be altered considerably. Especially the changes in polymer properties such as charge density, solubility and conformation are generally recognized to influence its flocculation performance. By studying systematically the effects of these properties on flocculation, the relative importance of different interactions may be identified.

Studies of chitosan flocculation considering the effects of chitosan structure and external conditions on flocculation efficiency include an investigation of flocculation of *Escherichia coli* disintegrates (Agerkvist, 1992), flocculation of polystyrene latex particles (Ashmore and Hearn, 2000; Ashmore *et al.*, 2001), flocculation of kaolin suspensions (Domard *et al.*, 1989) and flocculation of undecylenic acid dispersions (Demarger and Domard, 1993 and 1994). In all of these studies, flocculation efficiency of chitosan has been shown to increase with decreasing F_A and pH, and thereby increasing charge density. Consequently, electrostatic interactions have been implicated to play a dominant role in interactions of chitosans with negatively charged materials. However, preliminary experiments showed that especially chitosans of low charge density, *i.e.* high F_A, were excellent flocculants of *E. coli* suspensions. Therefore, we have carried out following studies on flocculation:

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1) Flocculation of polystyrene latex particles applied as a reference (Appendix Paper 6, Section 3.2).

2) A comparative study of flocculation of *E. coli* suspensions by different chitosans and at different conditions (Appendix Paper 4, Section 3.3 and 3.4).

3) Chitosan adsorption to *E. coli* suspension and coupling between adsorption and flocculation: effect of chitosan composition, pH, ionic strength (Appendix Paper 5, Section 3.5 and 3.6).

4) Efficiency of chitosans for flocculation of different bacterial species (Appendix Paper 6, Section 3.7).
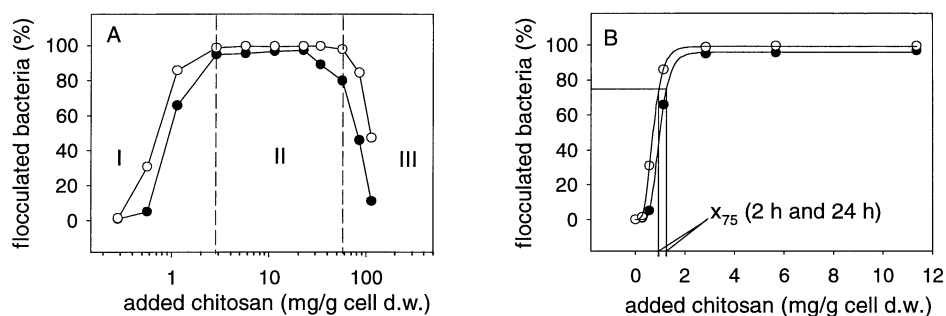
Figure 3.1 Illustrative example of a flocculation curve (A) and determination of x_{75} concentration (B): *E. coli* was flocculated by chitosan F_A 0.13 at pH 6.5 and 0.1 M and flocculation was measured after 2 h of sedimentation (●) and 24 h of sedimentation (o). Three distinct regions of flocculation referred to as initial increase (I), optimal flocculation (II) and restabilization (III) are schematically shown on the flocculation curve after 24 h of sedimentation (o).

The main intention with the series of flocculation studies listed above was to carry out an empirical screening to map the range of variations in flocculation efficiency. Indirectly, these data would also reflect the fundamental interactions between bacteria and chitosan in the flocculation process. A better understanding of the flocculation mechanisms and the nature of interactions will be beneficial for the application of chitosan as a flocculant. The possibility to modify chitosan properties by both internal and external variables may give an opportunity to develop an optimal flocculant for a given application. Regarding such option, the basic question is then simply whether any correlation between the structure of chitosan and surface properties of bacteria exists.

Appendix Papers 4-6 were based on a simple experimental assay, where the flocculation process was monitored by residual turbidity measurements, see procedure in Appendix Paper

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4. Figure 3.1 illustrates a typical flocculation curve, obtained by plotting the relative fraction of flocculated bacteria as a function of chitosan concentration, expressed relative to the bacterial concentration (mg/g cell dry weight). The three distinct regions of flocculation referred to as initial increase, optimal flocculation and restabilization are clearly distinguished on the semilogarithmic abscissa scale. To simplify the comparison of different chitosans at different conditions, a parameter referred to as critical concentration, x_{75}, was determined from the non-linear regression of the experimental data, expressing the chitosan concentration needed to obtain 75% flocculation as shown in Fig. 3.1. See Appendix Paper 4 for details.

3.2 Flocculation of polystyrene latex particles

Polystyrene latex (PS latex) particles represent typical model suspensions used in flocculation studies (Eriksson *et al.*, 1993; Ashmore and Hearn, 2000; Ashmore *et al.*, 2001). They may be well characterized, surface charge density may be quantified and different surface functional groups are available. The flocculation of PS latex by chitosan has been thoroughly studied by Ashmore and Hearn (2000) and Ashmore *et al.* (2001). They concluded that flocculation of PS latex proceeded by a charge neutralization mechanism enhanced by a "charge patch" mechanism, schematically shown in Fig. 1.8. Although flocculation occurred in a wide range of F_A and pH, highly charged chitosans with low F_A and at low pH were the most efficient (Ashmore and Hearn, 2000).
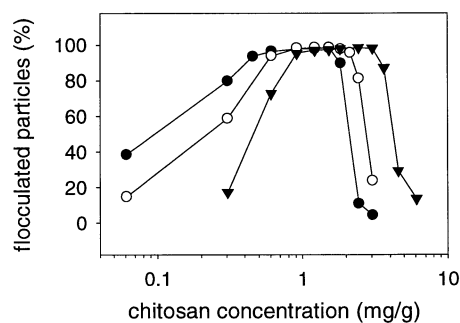

Figure 3.2 Flocculation of polystyrene latex particles by different chitosans at standard conditions of pH 6.5 and ionic strength of 0.1 M (Appendix Paper 6). Symbol key: F_A 0.01 (●), F_A 0.13 (o), F_A 0.49 (▼).

We have used negatively charged PS latex with surface sulfate groups and diameter of 1 μm as a reference material (Appendix Paper 6). As shown in Figure 3.2, the particles were flocculated at very low chitosan concentrations and in a narrow range of concentrations. The efficiency of chitosans slightly decreased with increasing F_A. The observed flocculation pattern, as well as the effect of F_A agreed with the reported results of Ashmore and Hearn (2000) and Ashmore *et al.* (2001). See Appendix Paper 6 for details.

3.3 Flocculation of *E. coli*

In the vast amount of literature on flocculation, a relatively small part deals with bacteria. There are reports comparing the efficiency of different polymers including chitosan (Baran, 1988), showing the effect of charge and hydrodynamic dimensions of the flocculant (Tarasova *et al.*, 1985). Some investigations of the flocculation mechanisms and the effects of polymer structure on flocculation involve biological systems of practical interest, such as activated sludge (Eriksson and Alm, 1991 and 1993) or cell disintegrates (Agerkvist, 1992). Chitosan flocculated *E. coli* disintegrates mainly by non-equilibrium bridging and exhibited a hydrogen bonding capacity towards cell debris (Agerkvist, 1992). Synthetic cationic polymers of low charge density flocculated activated sludge by a bridging mechanism, producing flexible shear resistant flocs, while highly charged polymers gave rigid open flocs with good filtration properties (Eriksson and Alm, 1993). The formation of activated sludge flocs was shown to involve electrostatic as well as other interactions (Eriksson and Alm, 1991).

A more fundamental approach to flocculation was used by Busch and Stumm (1968) and recently by Châtellier *et al.* (2001 a, b). The latter group has studied the flocculation of *E. coli* by a highly charged quaternized polyvinylpyridine. They concluded that flocculation followed the charged patch model due to the inhomogeneities of charge on the bacterial surfaces and the flat configuration of adsorbed polymer layer (Châtellier *et al.*, 2001 b).

In our study, *E. coli* K12 strain DSM 498 was arbitrarily chosen as a model organism. The suspensions of *E. coli* cells are under normal environmental conditions stabilized by the virtue of electrostatic or steric repulsion (see Section 1.4). The objective of Appendix Paper 4 was to compare the performance of a wide range of different chitosans for flocculation of *E. coli* suspensions in order to investigate the importance of variables such as the chemical composition, molecular weight, pH and ionic strength.

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3.3.1 Effect of F_A

The fraction of acetylated units (F_A) of the chitosan determines chitosans solubility (Vårum *et al.*, 1994), charge density (Appendix Paper 3) and conformation (Anthonsen *et al.*, 1993). Generally, charge density has been found to be one of the most important factors for performance of chitosans in flocculation (Domard *et al.*, 1989; Agerkvist, 1992; Ashmore and Hearn, 2000; Ashmore *et al.*, 2001). However, chitosans with high F_A were clearly better flocculants in our study, as shown in Figure 3.3. The increase in F_A resulted in a rather dramatic decrease of x_{75} concentrations, in some cases by a factor of 10 or more. Similar relationships between F_A and x_{75} were obtained at pH 5, where all chitosans are completely soluble and fully charged, and at pH 6.5, where low acetylated chitosans precipitate and the charge density is lowered to 50% (Fig. 2.3).
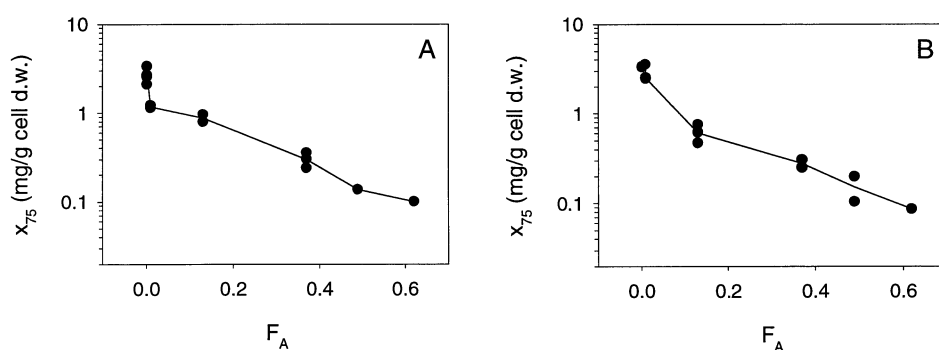
Figure 3.3 Critical chitosan concentration at 75% flocculation (x_{75}) as a function of F_A at pH 5 (A) and pH 6.8 (B) (Appendix Paper 4).

3.3.2 Effect of pH

In addition to F_A, pH is another important factor influencing both solubility and charge density of chitosan. Therefore, it is expected, and often found, that flocculation efficiency largely depends on pH (Agerkvist, 1992; Ashmore and Hearn, 2000; Ashmore *et al.*, 2001). However, as shown in Figure 3.4, no such dependence was observed in our study. The low acetylated chitosans had similar x_{75} concentrations in the range of pH 4.0-7.5. A further increase in pH resulted in dramatic increase in critical concentration, probably caused by so-called sweep flocculation (Bache *et al.*, 1997) with excess insoluble chitosans. Highly

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acetylated chitosan flocculated at similar x_{75} concentrations in the range of pH 4-7.4, but they did not flocculate at higher pH.

As both F_A and pH affect the charge density, it is interesting to note that these variables exhibited very different effects on flocculation. In the case of F_A, the flocculation efficiency increased with F_A and thereby with decreasing charge density, whereas in the case of pH the flocculation remained largely unaffected by pH and consequently, charge density was of minor importance.

3.3.3 Effect of molecular weight

As shown in Figure 3.4 (B), the chitosans with higher molecular weights flocculated most efficiently, but no dramatic effect was observed in the range of molecular weights of 50 000-290 000 g/mol, corresponding to $\overline{DP_n}$ of 250-1300. Similar results have been also reported by Agerkvist (1992). Such observation may point to bridging as a flocculation mechanism, since longer polymer may span over longer distances (Gregory and Sheiham, 1974; Baran and Gregory, 1996). However, also chitosans with molecular weight of approximately 10 000 g/mol, corresponding to $\overline{DP_n}$ 50, flocculated.

Figure 3.4 Critical chitosan concentration at 75% flocculation (x_{75}) as a function of pH (A) and $\overline{DP_n}$ (B) (Appendix Paper 4). Symbol key: F_A 0.01 (●), F_A 0.13 (o), F_A 0.37 (▼) and F_A 0.49 (▽).

3.3.4 Effect of ionic strength

As shown in Appendix Paper 4, ionic strength strongly influenced stability of *E. coli* suspension both with and without chitosan. At concentrations of 0.5 M NaCl and higher, *E.*

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coli cells flocculated without any chitosan present, showing clearly the significance of double layer repulsion for stability of colloidal dispersions. Upon addition of chitosan, this flocculation was reversed and bacteria were restabilized in suspension. As illustrated in Figure 3.5, ionic strength was shown to affect the x_{75} concentrations of chitosan: the higher ionic strength, the lower x_{75}. Such a behavior is rather typical for polymeric flocculants, showing the aid from double-layer compression. The observed broadening of flocculation intervals with increasing ionic strength has also been reported previously (Ashmore *et al.*, 2001).
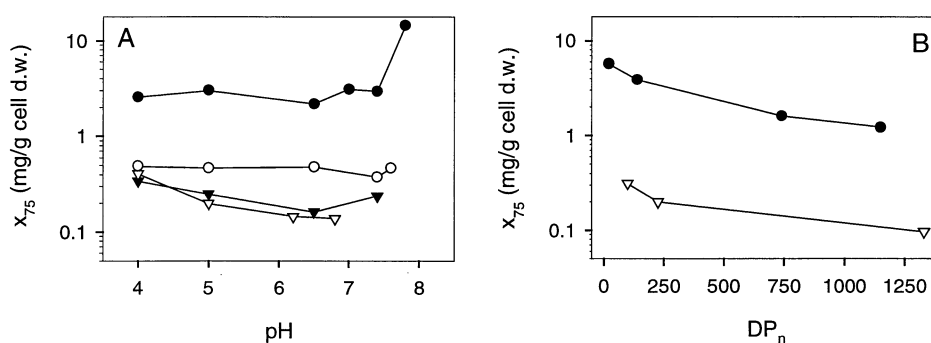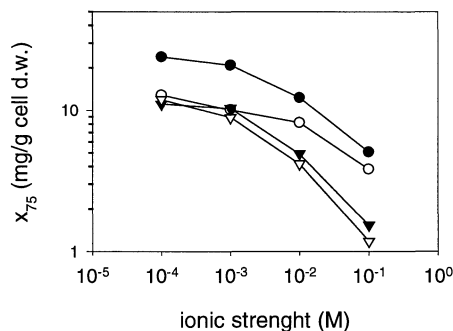

Figure 3.5 Critical chitosan concentration at 75% flocculation (x_{75}) as a function of ionic strength (Appendix Paper 4). Symbol key: F_A 0.01 (●), F_A 0.13 (o), F_A 0.37 (▼) and F_A 0.49 (▽).

3.3.5 Other factors

In addition to the variables discussed above, the effects of bacterial concentration and other bacteria related factors were also examined. As shown in Appendix Paper 4, the effect of bacterial concentration under the conditions of our assay was only minor. More than 10-fold decrease was necessary to obtain significant deviation from the typical flocculation pattern.

It is recognized that the growth medium and growth phase may influence the properties of bacteria. However, the flocculation of *E. coli* cells harvested in exponential and stationary growth phase was similar (unpublished data). Similarly, cells grown on mineral medium with glucose and rich LB medium also flocculated in the same way (unpublished data).

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3.4 Restabilization

As pointed out in Section 1.5.1, addition of excess amounts of a polymer may increase the stability of colloidal dispersions. Such a phenomenon is clearly shown in Fig. 3.1, where high chitosan concentration restabilized the suspension of *E. coli*. Similarly as flocculation, the chitosan concentration at the onset of restabilization depended on chitosan composition and molecular weight, pH and ionic strength (Appendix Paper 4). Compared to polystyrene latex particles, bacteria flocculated over much wider range of chitosan concentrations. It has been reported that natural polyelectrolytes, such as chitosan and alginate, often flocculate in wide range of concentrations, while synthetic polymers give narrow intervals of flocculation (Kawamura, 1991). This is clearly an advantage, making dosing of natural polymers easier. Since stabilization of colloidal dispersions is at least as much practically important as flocculation, chitosans may also be exploited as stabilizers.

The analysis of the restabilization data may reveal information about the interactions between adsorbed chitosan layers, but such a discussion would be beyond the scope of this work.

3.5 Adsorption of chitosan to *E. coli* cells

The general aspects of adsorption behavior of polyelectrolytes were summarized in Section 1.4. Adsorption of chitosan to the surface of *E. coli* is a necessary prerequisite for flocculation to occur. The flocculation mechanism largely depends on the conditions of the adsorption process and the configuration of the adsorbed polymer layer (Pelssers *et al.*, 1989; Chaplain *et al.*, 1995). Appendix Paper 5 presents a study of adsorption of chitosans to *E. coli* cells.

Due to strong coupling between adsorption and flocculation, one objective of our study was to test a hypothesis that F_A dependency of flocculation reflects different adsorption behavior of chitosans. If the highly acetylated chitosans have significantly higher affinity for the cell surface, flocculation may occur at lower added amounts whereas in case of low F_A chitosans, lower portion is adsorbed and more added chitosan is therefore needed to obtain the same effect. Moreover, the effect of ionic strength on adsorption may reveal the role of electrostatic interactions in interactions between bacteria and chitosans. Adsorption is mainly driven by electrostatic interactions when increase in ionic strength results in higher adsorption. When

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only a small effect of ionic strength is observed, contribution of non-electrostatic interactions is expected.

Alternatively, F_A dependency may be caused by different flocculation mechanisms. If high F_A chitosans flocculate predominantly by bridging, but low F_A by charge neutralization, large differences in critical concentrations may be observed. Bridging generally occurs at lower concentration and charge density is less important. This hypothesis was tested by zeta potential measurements of *E. coli* cells in the presence of chitosan as summarized below, see also Appendix Paper 5.

3.5.1 Effect of pH and F_A

As shown in Figure 3.6, the adsorption of all tested chitosans to *E. coli* depended strongly on pH, the adsorbed amounts increased approximately 40% if pH increased from pH 5 to pH 6.5. This is not surprising, since the charge density of chitosan is greatly influenced by pH (Fig. 2.3, see also Appendix Paper 3). Such a pH dependence is in good agreement both with theory and experimental studies, showing that the adsorption of highly charged polyelectrolytes onto oppositely charged surfaces is limited by intra- and intermolecular electrostatic repulsion (Blaakmeer *et al.*, 1990; Rustemeier and Killmann, 1997; Bauer *et al.*, 1999). At pH 6.5, when charge density of chitosan is lower, the electrostatic repulsion is reduced and higher amounts of chitosan may be accumulated. Furthermore, an increase in the non-electrostatic contribution to the adsorption energy may also be expected due to the decrease in the solvent quality and increasing attraction between the segments (Parazak *et al.*, 1988; Claesson and Ninham, 1992). There was a high attraction between the chitosan and the cell surface at pH 5 and 6.5, whereas the adsorption of chitosan F_A 0.49, soluble also at neutral pH values, at pH 7.8 followed a different low-affinity curve (Fig. 3.6).

As shown in Fig. 3.6, the shape of the adsorption isotherms changed as pH increased; a plateau was generally reached at pH 5 but never at pH 6.5, within the concentration range tested. At pH 6.5, the adsorbed amounts continued to increase proportionally to the bulk chitosan concentration. Similar results have been reported earlier both for chitosan on kaolin (Domard *et al.*, 1989), and other cationic polymers on latex (Eriksson *et al.*, 1993). This has often been explained by molecular weight polydispersity and preferential adsorption of larger molecules exchanging initially adsorbed smaller ones (Cohen Stuart, 1991), or rearrangement

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of chains on the surface (Rustemeier and Killmann, 1997). The plateaus reached at pH 5 may reflect that the electrostatic barrier, which builds up due to the adsorption of highly charged polymer, became too high to allow such exchange. Alternatively, assuming adsorption in "train" conformation at pH 5 (Claesson and Ninham, 1992), there may be too many contacts between initially adsorbed chains and the cell surface components to be broken in order to replace or rearrange the initially adsorbed molecules.
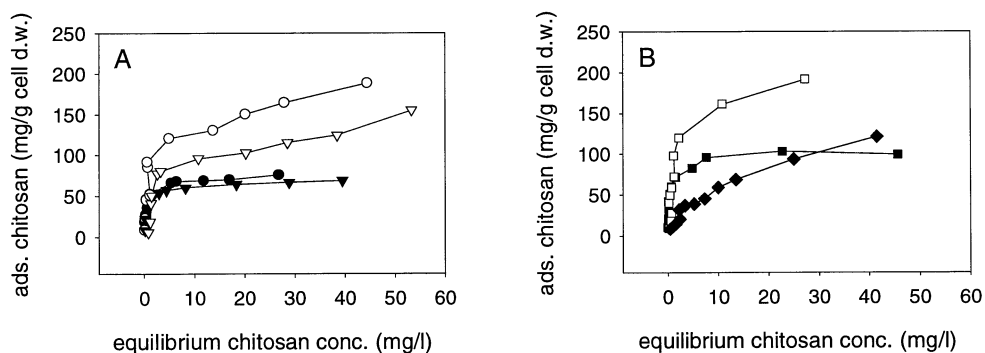
Figure 3.6 Effect of pH on adsorption of chitosans with F_A 0.01, F_A 0.13 (A) and F_A 0.49 (B) to *E. coli* (Appendix Paper 5). Symbol key: F_A 0.01 at pH 5 (●) and pH 6.5 (o), F_A 0.13 at pH 5 (▼) and pH 6.5 (▽), F_A 0.49 at pH 5 (■), pH 6.5 (□) and pH 7.8 (♦).

Since the chemical composition of chitosan (F_A) determines the charge density at given pH (Fig. 2.3), the adsorbed amounts of chitosans should increase with F_A, assuming the principles as discussed above. However, the relationship between F_A and adsorbed amount is less obvious (Fig. 3.6). The chitosan F_A 0.49 adsorbed most, both at pH 5 and pH 6.5, followed by F_A 0.01 and F_A 0.13. See also Appendix Paper 5.

3.5.2 Effect of molecular weight

In the study presented in Appendix Paper 5, it was found that the low molecular weight chitosans adsorbed to *E. coli* cells in higher amounts than the respective high molecular weight chitosans. This seems to be inconsistent with results for other polyelectrolytes described elsewhere (Domard *et al.*, 1989; Blaakmeer *et al.*, 1990; Rustemeier and Killmann, 1997). The effect of M_n on the adsorption of polyelectrolytes depends on ionic strength as well as pH for weak polyelectrolytes, and reflects the changes in configuration of the adsorbed layer (Cohen Stuart *et al.*, 1991; Fleer *at al.*, 1993). At low ionic strength and high

polymer charge density, the adsorption occurs in flat layers, so-called "trains" (Fig. 1.6), and no effect of M_n is observed. At higher ionic strength or lower polymer charge density, increased screening of charges will occur and polymers adsorb in loops and tails conformation (Eriksson *et al.*, 1993). As polymers with higher M_n may form longer loops and tails, higher amount of polymer per surface area can be adsorbed. However, all experimental data are derived from model suspensions of latex or mineral particles that are hard spheres with relatively flat surface. In our case, the completely different nature of a bacterial surface with projecting polymers has to be taken into consideration. See Appendix Paper 5 for details.

3.5.3 Effect of ionic strength

The increase in ionic strength from 0.001 to 0.1 M affected only the adsorption of chitosan F_A 0.01, while no effect was observed on the adsorption of F_A 0.49, see Appendix Paper 5. The major consequence of an increase in the ionic strength is increased screening of charges and thereby reduction in electrostatic interactions. If accumulation of a polyelectrolyte on a surface is limited by inter- and intramolecular repulsions between segments or chains, the increased screening of charges will result in increased adsorption (Rustemeier and Killmann, 1997) as in case of F_A 0.01. On the other hand, the adsorption of chitosan with lower charge density (F_A 0.49) was not influenced by ionic strength (Appendix Paper 5). The difference in the adsorption patterns of the two chitosans may reflect different chemical contributions to the total adsorption energy, with chitosan F_A 0.49 possessing significant non-electrostatic contribution to the adsorption.

3.5.4 Zeta potential of E. coli cells

The adsorption of chitosan to negatively charged *E. coli* cells was also monitored by recording changes in zeta potential of cells as described in Appendix Paper 5. The zeta potentials of *E. coli* cells in the absence of chitosan were found to be rather stable in the pH range 5-7.8, with a mean value of -13.4 ± 0.4. Figure 3.7 shows changes in zeta potential of *E. coli* cells caused by adsorption of chitosans as a function of the added chitosan concentration at pH 5, 6.5 and 7.8. It is striking that adsorption of chitosan with low charge density (F_A 0.49) resulted in the steepest increase of zeta potentials, and that the charge neutralization points (CNP) were reached at much lower concentrations than for the F_A 0.01 and F_A 0.13.

Apparently, there is a discrepancy between quantitative adsorption data (Fig. 3.6) and adsorption estimated by changes in zeta potential of *E. coli* cells (Fig. 3.7). In the latter case, the steepest increase of zeta potentials in presence of the chitosan with F_A 0.49 indicated higher initial adsorption. However, no significant differences in the amounts of adsorbed chitosans of different F_A were observed at low chitosan concentrations added where charge reversal occurred. Since the equilibrium bulk concentrations in the initial stages of adsorption were practically under the detection limits, a firm conclusion cannot be made. The consequences of the differences in cell concentration in both experiments are discussed in Appendix Paper 5, however these cannot explain the relative differences between chitosans.
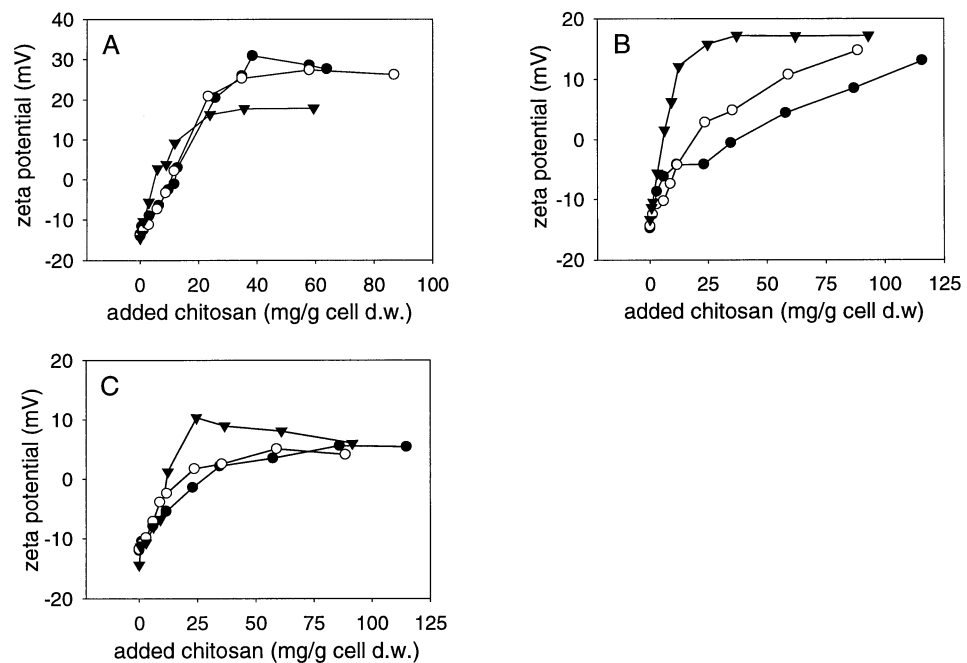
Figure 3.7 Changes in zeta potential of *E. coli* cells due to adsorption of chitosans at pH 5 (A), pH 6.5 (B) and pH 7.8 (C) (Appendix Paper 5). Symbol key: F_A 0.01 (●), F_A 0.13 (o), F_A 0.49 (▼).

3.6 Conclusions: mechanisms of *E. coli* flocculation by chitosans

Summarizing the results of Appendix Papers 4 and 5, some hypothesis about flocculation mechanisms may be postulated. Some conclusions derived from the combination of flocculation, adsorption and zeta potential data as illustrated in Figure 3.8 are listed below:

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1) Charge neutralization was not the main flocculation mechanism. As shown in Appendix
   Paper 5 and exemplified in Figure 3.8, the onset of flocculation was always located at
   concentrations lower than CNP. Furthermore, flocculation proceeded also after charge
   reversal, despite a high positive charge attained (Fig. 3.8).
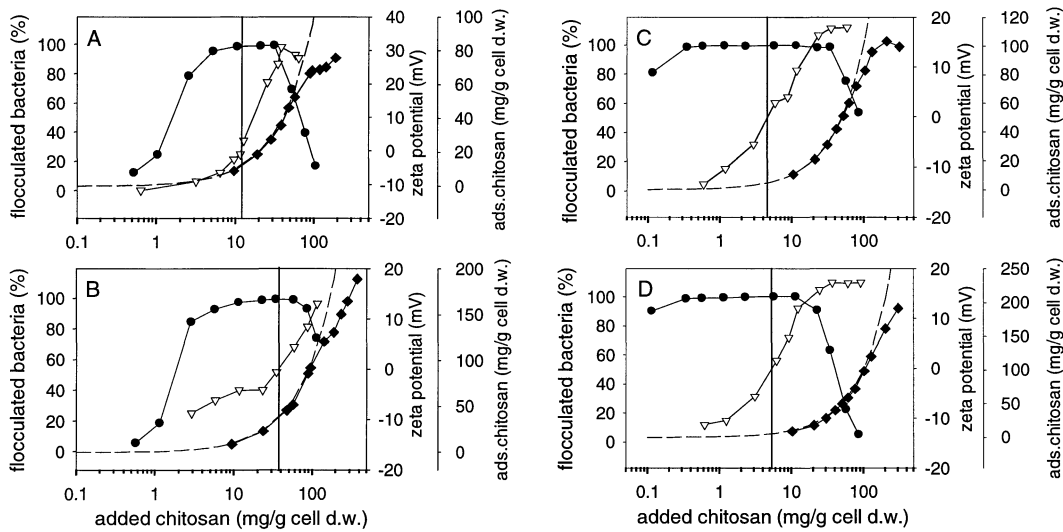
Figure 3.8 Adsorption to and flocculation of *E. coli* by chitosan F_A 0.01 at pH 5 (A), pH 6.5 (B) and
F_A 0.49 at pH 5 (C) and pH 6.5 (D) (Appendix Paper 5). Symbol key: % of flocculated bacteria (●),
adsorbed amount of chitosan (♦), zeta potential of bacterial cells (▽), theoretical 100% adsorption (---)
Vertical lines indicate the position of charge neutralization point.

2) A probably dominant mechanism of flocculation was bridging between cells. As shown in
   Fig. 3.8, flocculation occurred at very low chitosan concentrations when hardly any
   increase of zeta potentials was recorded and only low amounts of chitosans were
   adsorbed. The observed M_n dependency of flocculation (Fig. 3.4 B), although not
   dramatic, also supports the bridging mechanism. Since only a few charged monomers may
   be involved in a bridge formation, the net charge density of a bridging polymer will be of
   minor importance and this may explain the minor effect of pH upon chitosan flocculation
   performance (Fig. 3.4 A). The large increase in the chitosan concentration needed for
   flocculation at low ionic strength (Fig. 3.5) may also be explained. As the range of double
   layer repulsion increases at low ionic strengths, cells cannot come close enough to
   establish sufficient contact through bridging, and higher chitosan amounts may be
   necessary.

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3) The reasons for relative differences in flocculation efficiency of different chitosans remain unknown. Clearly, the presence of GlcNAc units in the chitosan molecule had beneficial effects on adsorption and flocculation (Fig. 3.2). This may reflect increasing contribution from hydrophobic interactions or any kind of specific interactions between GlcNAc and any molecule on the cell surface.

4) *E. coli* cells behave completely different from latex particles. It may reflect fundamentally different structure of their surfaces, the former covered with polymer layers. It is also possible that adsorption of chitosan to the bacterial surface triggers a biological response to changes in their local environment. It is generally recognized that polycations such as polyethylenimine disorganize and increase permeability of outer membrane of Gram-negative bacteria without being directly biocidal (Vaara, 1992; Helander *et al.*, 1997 and 1998). Lately, Châtellier *et al.* (2001 b) have shown that adsorption of highly charged cationic polymer to *E. coli* resulted in the release of organic matter of biological origin from the cells. It is possible that adsorption of highly charged chitosan also induces similar process, thereby reducing the charge accumulated in the adsorbed layer. This might explain the relatively low efficiency of low acetylated chitosan to neutralize the surface charge (Fig. 3.7), despite their high charge density.

3.7 Flocculation of different bacteria

As discussed in Section 1.3, bacteria show enormous diversities in chemical composition and structure of their surfaces. Thus, it may be expected that the adsorption of chitosan to bacterial surfaces, as well as the resulting flocculation patterns, may vary considerably for different bacterial strains. The objective of our investigation presented in Appendix Paper 6 was to show if there is any correlation between chitosan structure and bacterial cell surface properties in flocculation. As discussed in Section 2.4.1, cell surface hydrophobicity (CSH) is regarded as an important factor in adhesion. Correlation between CSH and adhesion has been often found (van Loosdrecht 1987 a; Zita and Hermansson, 1997 a; Olofsson *et al.*, 1998), but it has been rarely implicated in flocculation. On the other hand, the role of cell surface charge (CSC) in adhesion is questionable, but it is of prior importance in flocculation. The flocculation of bacteria varying in CSH and CSC with chitosan of different F_A and thus, charge densities and hydrophobicity, may reveal the relative importance of electrostatic and hydrophobic interactions.

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Fig. 3.9 summarizes the results of Appendix Paper 6, where different bacterial species with known zeta potentials and hydrophobicity were flocculated with three different chitosans with F_A 0.01, 0.13 and 0.49. The concentration of chitosans applied to achieve effective flocculation varied more than a factor of 100 in some cases. Generally, flocculation of *E. coli*, *S. marcescens* and *M. luteus* required lowest concentrations, whereas that of *B. megaterium* and *Rhodococcus sp.* 094 required the highest. The effect of chitosan composition, *i.e.* F_A, was also rather profound, especially for some of bacteria. Interestingly, the three Gram-positive species were more efficiently flocculated with high charge density chitosan with F_A 0.01, while the Gram-negative species were generally flocculated better with chitosans with higher F_A.
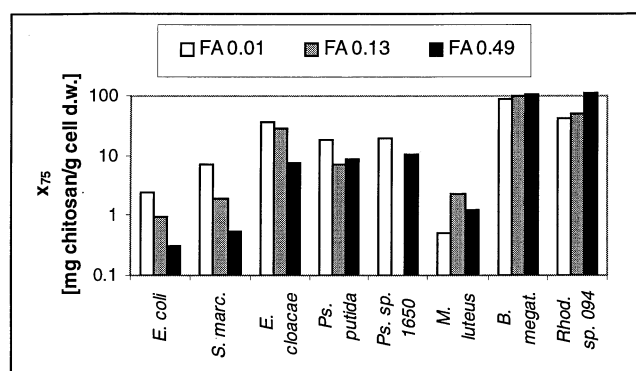
Figure 3.9 Critical chitosan concentration at 75% flocculation (x_{75}) for different bacteria and chitosans (Appendix Paper 6).

No clear correlation was found between the zeta potentials given in Table 2.1 and flocculation behavior, neither concerning the chitosan concentrations, nor the chemical composition of chitosan. Similarly, contact angles (Table 2.1) did not show any correlation with flocculation patterns observed. These results were not unexpected, considering that both contact angles and zeta potential measurements give rather macroscopic characteristics of a cell surface. The structural complexity of bacterial surfaces implies many different possibilities for interactions on the molecular level, such as involvement of different surface appendages projecting from the surface. The structural and molecular heterogeneity of a bacterial surface may affect the configuration of adsorbed chitosan layer resulting in different mechanisms and thereby different efficiency.

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The observed wide differences between bacteria clearly illustrate the limitation of traditional colloidal description of bacterial adhesion and flocculation, as in DVLO theory (Section 1.4). Regarding bacteria simply as colloidal particles with smooth surface of average properties, the bacteria of similar zeta potentials and hydrophobicity, as for instance *B. megaterium* and *E. coli* in our study, should behave similarly. Apparently, individual differences in composition and structure of cell surfaces may result in a vast array of possible polymer-polymer interactions causing attachment through different short-range interactions. Since most natural surfaces are conditioned with polymeric material of natural origin, a similar situation may be expected also in such cases. Therefore, a more microscopic approach yielding information about surface architecture is necessary to explain these findings.

4. ADHESION

4.1. Introduction

This chapter summarizes our investigation of bacterial adhesion to chitosan coated glass slides presented in detail in Appendix Paper 7. This work was based on preliminary pilot studies not included in this thesis (Prochazkova *et al.*, 1997). As mentioned in Section 1.5, mechanisms of adhesion and flocculation are believed to be closely related. Flocculation may be looked upon as adhesion of cells to each other, often mediated through adsorbed polymers such as chitosan.

Bacterial adhesion to solid surfaces is an extensively studied phenomenon and its principles were summarized in Chapter 1. The research has been focused on following areas: the role of cell surface properties on adhesion (van Loosdrecht *et al.*, 1987 a, b; Makin and Beveridge, 1996; Otto *et al.*, 1999), the role of substratum properties (Egington *et al.*, 1995; Wiencek and Fletcher, 1997; Cunliffe *et al.*, 1999) and the effects of hydrodynamic and environmental conditions on adhesion (Rijnaarts *et al.*, 1993; Meinders *et al.*, 1995). The aim of all of these studies has been to elucidate the mechanisms and identify the most important factors to allow control and reduce negative consequences of the adhesion process, commonly denoted biofouling. Most adhesion studies describe adhesion of negatively charged bacteria to negatively charged surfaces as it usually occurs in nature. In such cases, electrostatic repulsion between bacteria and a substrate has to be overcome and it is believed that adhesion initially takes place at the so-called secondary minimum, see section 1.5.2. Adhesion to positively charged surfaces is rarely addressed since it has been of relatively little practical interest (Harkes *et al.*, 1991).

4.2 Chitosan coating of glass

4.2.1. Coating

Glass is a negatively charged and hydrophilic surface. The negative charge develops through dissociation of surface silanol groups having isoelectric point around pH 2 (Parks *et al.*, 1965). The zeta potential of glass in 0.1 M PBS has been reported to be around –21 mV

(Rijnaarts *et al.*, 1995 b). The reported contact angles usually range from 0 to 30° (Rijnaarts *et al.*, 1995 b). No contact angles could be measured in this work due to immediate wetting by water, see Section 2.4.2.

Two methods of preparation of chitosan coated glass surfaces were tested. Initially, chitosan was covalently bound to glass through an epoxyfunctional silane-coupling agent as described by Prochazkova *et al.* (1997). However, this procedure was later found to have problematic reproducibility and coating was sometimes irregular and unstable. These drawbacks were largely avoided when a simple adsorption of chitosan from solution was used as described in Appendix Paper 7. Adhesion tests showed that both methods of chitosan coating gave the same amount of adhered bacteria, and much higher than bare glass. Therefore, the simpler adsorption procedure was preferred in the current studies (Appendix Paper 7).

4.2.2 Quantification

Unfortunately, all attempts to quantify the amount of bound chitosan on glass slides by ninhydrin method (Appendix Paper 1) or fluorescence spectrometry (Appendix Paper 2) failed due to insufficiently low detection limits. Similar problems with monitoring of the extent of glass coating by fluorescence spectroscopy were also reported by others (Cunliffe *et al.*, 1999). The ninhydrin method was successfully applied to measure amounts of chitosan bound to glass beads, indicating values below 20 μg/g at pH 6-6.5, corresponding to 17 mg/m^2. The amounts of chitosan bound by both methods were similar and the amounts increased with pH, see Appendix paper 7. However, since the physical and chemical properties of different glass materials may grossly vary (Eaton, 1980), the chitosan binding to glass beads and glass slides does not have to be similar.

The fact that the exact amount of bound chitosan could not be quantified is clearly a limit in our study. It is difficult to compare adhesion to different chitosans without knowing their relative amounts bound. Another factor of importance is the actual charge of chitosan coated glass at actual conditions. Since methods to measure streaming potential of surfaces (Hunter, 1981) were not available, the zeta potential of the modified glass surfaces could not be measured. Depending on the charge density of the surface, type of chitosan and conditions, the sign and size of charge of the chitosan modified glass surface may vary. It may become positive, if chitosan can reverse the originally negative glass charge, neutral, when glass and

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chitosan charge will compensate each other, or negative, when glass surface charge dominates. Claesson and Ninham (1992) have shown that chitosan with F_A 0.05 strongly adsorbed on mica reversing the surface charge at low pH while at pH 6.2 merely neutralized it.

4.2.3 Stability

The stability of chitosan coating under storage as well as during adhesion assay is also of interest. It has been reported that adsorption of chitosan to mica was irreversible and no desorption occurred as pH was raised (Claesson and Ninham, 1992). Our experiments showed that the adsorption was practically irreversible, provided pH was kept constant as during adsorption. The release of fluorescent chitosan bound to glass beads showed that desorption of chitosan occurred when pH was lowered to 4.5, showing a loss of approximately 50% (Appendix Paper 7).

4.3 Adhesion assay

The adhesion was performed as a short-term test where bacteria resuspended in PBS or acetate-NaCl buffer were allowed to adhere to different types of glass slides for 2 h, stained by Hucker crystal violet and quantified by absorbance measurements as described in Appendix Paper 7. In the case of *E. coli*, the absorbance values were calibrated against numbers of adhering cells per surface area determined by direct microscopic counting.

Generally, reproducibility in adhesion assays is often a problem. It may be largely affected by even slight differences in cell surface characteristics, changes in properties of substrata, hydrodynamic or external conditions. Although the difference between replicates of glass slides was generally low (± 10%), repeated experiments occasionally showed significant variation in numbers of adhered cells or even adhesion patterns observed. The reasons for this remain unknown. Therefore, the adhesion studies are presented here merely to evaluate possible similarities between adhesion and flocculation regarding the structure and properties of chitosans.

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4.4 Adhesion of polystyrene latex particles

Adhesion of polystyrene latex particles was included as a reference (Appendix Paper 7). As shown in Figure 4.1, good reproducibility between independent experiments was observed, showing that problems with bacteria are either connected to the dynamic nature of bacterial properties or to the desorption of adhered bacteria during staining and rinsing procedure applied only for bacteria. Coating of glass slides with all chitosans significantly increased the adhesion of particles compared to the original glass. The relative differences between different chitosans were only small, with adhesion increasing with decreasing F_A.
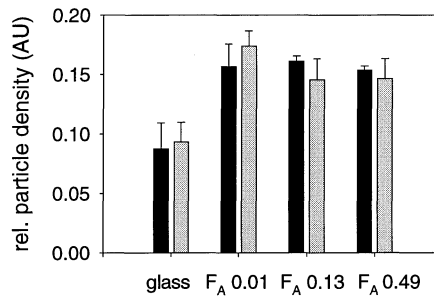
Figure 4.1 Adhesion of polystyrene latex particles to glass and glass coated with different chitosans (Appendix Paper 7). Two sets of bars represent two independent experiments.

4.5 Adhesion of *E. coli*

As in the flocculation studies (Chapter 3), *E. coli* K12 strain (DSM 498) was applied as model organism also here. Appendix Paper 7 presents data where a multitude of factors possibly influencing the adhesion of *E. coli* to glass and chitosan coated glass was examined. Generally, adhesion of *E. coli* to chitosan coated glass was much higher than adhesion to the original glass surface, although a strong variation in the degree of adhesion occurred. Microscopic observation revealed very dense and homogeneous cell distribution on the chitosan coated glass slides at pH 6.5 and ionic strength of 0.1 M, irrespective of the type of chitosan (Appendix Paper 7).

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4.5.1 Effect of pH and F_A

As shown in Figure 4.2, adhesion clearly decreased with increasing pH. While relatively small differences were observed in the range of pH 5-6.5, the adhesion at pH 7.8 dramatically decreased. Although the net charge of chitosan modified glass is not known, some conclusions can be drawn considering the charge density of chitosan at these pH (see Section 2.4.1). The chitosan coated glass slides will be probably positively charged at pH 5 whereas the low charge density of chitosan at pH 7.8 could hardly compensate the negative glass charge at this pH. Therefore, an electrostatic repulsion between bacteria and glass slides may be expected at pH 7.8. However, the range of this repulsion will be very short due to the high ionic strength, approximately a few nm (see Section 1.4.2), and any attractive force of longer range could easily overcome such repulsion. Therefore, the low adhesion at pH 7.8 suggests that the positive charge of chitosan is important for the interactions. Similar results were obtained in flocculation studies, showing that flocculation either ceased or considerably altered character at pH 7.4 and higher, see Fig. 3.4 A.

Figure 4.2 Adhesion of *E. coli* to glass and chitosan coated glass as a function of pH (Appendix Paper 7). Symbol explanation: Symbol key: glass (●), F_A 0.01 (o), F_A 0.13 (▼), F_A 0.49 (▽).

Figure 4.2 also illustrates the effect of chitosan composition on adhesion. Chitosan with F_A 0.49 seemed to attract *E. coli* more strongly than those with lower F_A. This is also in agreement with flocculation studies, where chitosans with high F_A were the most effective flocculants (see Fig. 3.3). The difference between F_A 0.01 and 0.13 is rather small. These results show that the degree of adhesion is not simply proportional to the charge density of chitosan and other non-electrostatic interactions may therefore play an important role. However, since we do not know the exact amounts of chitosan bound, no firm conclusions can be made.

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4.5.2 Effect of ionic strength

The effect of ionic strength shown in Figure 4.3 clearly demonstrates the most significant difference between the chitosans tested. While the adhesion to glass coated with chitosan F_A 0.49 appeared to be unaffected by ionic strength, the adhesion to low acetylated chitosans dramatically increased with increasing salt concentration. Since the pH of the bacterial suspension was approximately 6.5, *i.e.* close to the pK_a value of chitosan (Section 2.4.1), it is difficult to assess the actual net charge of the glass surface with adsorbed chitosan. Since no increase in adhesion to glass coated with chitosan F_A 0.01 and 0.13 was observed in 1mM solution, it seems that both bacteria and glass were negatively charged, giving rise to relatively long-range electrostatic repulsion preventing adhesion at this ionic strength. When increasing ionic strength to 10 mM and further to 100 mM, the range of this repulsion decreased and adhesion increased. This is typical for adhesion of bacteria to negatively charged surfaces (Zita and Hermansson, 1994; Rijnaarts *et al.*, 1995 b). It is important to note that low adhesion was not caused by desorption of chitosan, as shown by repeating adhesion test at 0.1 M with the same glass slides (unpublished data).
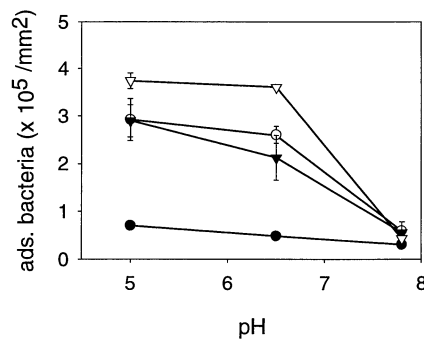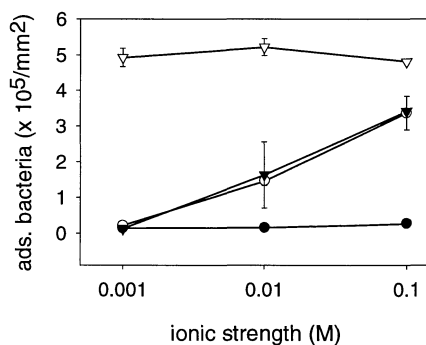

Figure 4.3 Adhesion of *E. coli* to glass and chitosan coated glass as a function of ionic strength (Appendix Paper 7). Symbol key: glass (●), F_A 0.01 (o), F_A 0.13 (▼), F_A 0.49 (▽).

The adhesion of *E. coli* to chitosan F_A 0.49 was much higher and unaffected by ionic strength. It seems that in this case no electrostatic repulsion between cells and chitosan F_A 0.49 coated glass existed. Furthermore, the fact that variation in ionic strength did not affect adhesion suggests that non-electrostatic interactions dominated adhesion. Again, some information about the relative amounts of bound chitosan would be very appreciable. When comparing

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with flocculation studies, a similar pattern was observed in the adsorption of chitosans. The adsorption of chitosan F_A 0.49 to *E. coli* was not affected by ionic strength, while it increased with ionic strength for F_A 0.01 (see Section 3.5.3).

4.5.3 Other factors

The adhesion of *E. coli* was also found to depend on growth medium and growth phase as documented in Appendix Paper 7. Furthermore, it was also affected by the type of buffer used and type of mixing during adhesion assay (unpublished results).

4.6 Adhesion of other bacteria

As in the flocculation studies (Section 3.7), adhesion of six other bacterial species was also investigated in order to obtain a more general picture of bacterial adhesion to glass with and without chitosan coating, see details given in Appendix Paper 7. Adhesion assay was carried out at pH 6.5 and the net charge of the glass surfaces remains unknown. As shown in Figure 4.4, large differences in adhesion behavior of different bacteria were observed. Since direct microscopic counting was unfortunately not carried out, an exact quantitative comparison of data for different bacteria could not be accomplished. Although absorbance values may reflect differences in biomass, differences in stain affinity cannot be excluded. Therefore, only relative differences between chitosans and glass without chitosan are discussed below.

Surprisingly, the effect of chitosan coating on adhesion shown in Fig. 4.4 seems rather inconclusive. In some cases, for instance for *S. marcescens*, only a small increase in amounts of adhered bacteria to chitosan modified glass compared to the original glass was shown. Adhesion of *E. cloacae* was significantly improved only by coating of glass by chitosan F_A 0.49, whereas the low acetylated chitosans did not show any difference from the original glass. *P. putida* adhered even less to glass coated with chitosan than to clean glass surface. *Pseudomonas* sp. adhered better to glass coated with chitosan F_A 0.01 and F_A 0.49. *Rhodococcus* adhered most to low acetylated chitosan with F_A 0.01, while for *M. luteus* only 15-30% increase of adhesion by chitosan coating was obtained. Comparing to flocculation results presented in Section 3.7, the adhesion showed only a few similarities. The clear distinction between G- and G+ bacteria in flocculation, showing preferential adhesion to chitosan F_A 0.49 and F_A 0.01, respectively, was not apparent in adhesion.
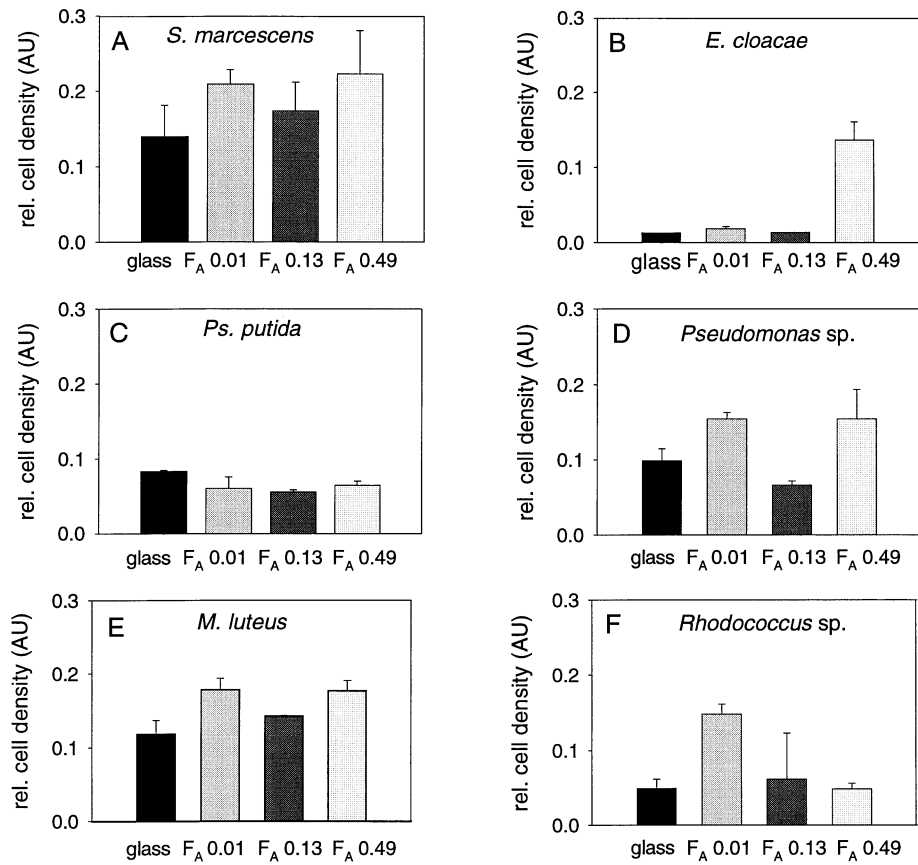
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Figure 4.4 Adhesion of *Serratia marcescens* (A), *Enterobacter cloacae* (B), *Pseudomonas putida* (C), *Pseudomonas* sp. 1650 (D), *Micrococcus luteus* (E) and *Rhodococcus* sp.094 (F) to glass and glass coated with different chitosans at pH 6.5 and ionic strength of 0.1 M.

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5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 Main findings

In the first part of this work, two methods for quantification of chitosan were developed (Appendix Papers 1, 2). A colorimetric ninhydrin method permitted quantification of chitosan in solution or bound to glass, but the detection limits were too high to quantify chitosan bound to glass slides used in the adhesion studies. Labeling with fluorescent 9-anthraldehyde allowed both quantification and visualization of chitosan and was successfully used for quantification of chitosan adsorbed to bacterial cells.

The electrophoretic light scattering studies of chitosans with different chemical composition showed that all chitosans had the same pK_a value of 6.5, irrespective of F_A (Appendix Paper 3). Similarly, the intrinsic pK_a of 8.8 was also independent of F_A. Consequently, the charge density of chitosan at given conditions is proportional to F_A.

The main part of research presented herein has dealt with flocculation and adhesion. It has been shown that chitosan flocculated all bacterial suspensions tested, however, there were large differences in the flocculation patterns and doses needed. The studies performed with *E. coli* as a model organism revealed that there is a strong relationship between the chemical composition of chitosan, given by F_A, and its efficiency in flocculation. This efficiency was found to increase proportionally with F_A: the choice of highly acetylated chitosans with the range of F_A 0.5-0.6 caused a reduction of necessary doses by a factor of 10 or more compared to low acetylated commercial chitosans (Appendix Paper 4). This finding shows that the conventional view of highly charged chitosans, that is samples with low F_A and at low pH, as the best flocculants, should not be generalized.

The primary aim of this work was an empirical screening of the range of variations at widely different conditions. However, the systematic investigation of chitosan adsorption during flocculation together with accompanying changes in the electrokinetic potential of bacterial cells contributes to a better understanding of the actual mechanisms involved. Thus, it was shown that a simple neutralization of bacterial charges was not the main mechanism of flocculation (Appendix Paper 5). Chitosan seemed to bridge the cells, producing large and

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open flocs. Interestingly, highly acetylated chitosan with the lowest charge density was the most effective in neutralizing and reversing the bacterial charge. This finding, however confusing, may point to a biological response following adsorption of strongly cationic polymers, possibly disturbing the cell wall components. It still remains a puzzle why the presence of GlcNAc residues (A-units) in the chitosan chain had such a profound effect on flocculation of *E. coli* and other Gram-negative bacteria used in this study. However, besides being a natural component of peptidoglycan in the bacterial cell wall (Hancock, 1991), GlcNAc is also a well-known receptor of different lectins and fimbriae (Bertin *et al.*, 1996) and also a specific target of lysozyme and wheat germ agglutinin (Kristiansen, 1998). Therefore specific interactions of similar nature cannot be excluded.

Studies of flocculation of different bacteria showed large differences in their flocculation characteristics (Appendix Paper 6), and thereby documented that it is impossible to generalize the results from one bacterium to another. The chitosan concentration needed to achieve effective flocculation varied by a factor of 100, depending on the bacterial species. Nevertheless, distinct difference between flocculation of Gram-positive and Gram-negative bacteria emerged from our studies: the former appeared to flocculate better with chitosans with low F_A, while the latter flocculated better with chitosans with high F_A. The general validity of this finding is still to be confirmed. It seemed impossible to correlate the observed flocculation characteristics to general properties of the bacterial surface such as cell surface charge or hydrophobicity. Consequently, the application of physicochemical theories describing bacteria as colloid particles with average surface properties seems oversimplified. The individual structure and topography of bacterial surfaces has to be considered.

The studies of bacterial adhesion to chitosan coated glass surfaces were expected to give similar trends and patterns as observed in flocculation. Unfortunately, the combination of experimental difficulties and small amount of reliable data makes it impossible to formulate any general conclusions yet. In the case of *E. coli*, the effect of pH and F_A on adhesion agreed with that observed in flocculation. Concerning other bacterial species, no clear correlation was obtained.

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5.2 Consequences for applications

The idea of using chitosan as a flocculant is definitely not new, and chitosan has been successfully applied in numerous areas of water treatment (No and Meyers, 2000). It has also been tested for harvesting cell debris in downstream processing (Agerkvist, 1992). Clearly, chitosan may be considered as a possible alternative to synthetic polymeric flocculants. Although there are vast amounts of literature describing particular applications of chitosan in water treatment, the more fundamental literature on this topic seems to be missing.

Despite the basic character of the presented study, dealing only with bacterial suspensions of pure cultures, some of the conclusions presented above may also be of importance for application of chitosan in wastewater treatment or downstream processing. Since our studies show that generalization of results from one bacterial suspension to another is impossible, generalizations to more complex system such as mixed cultures or sludge would be even more meaningless. The optimal concentration of chitosan needed for flocculation has to be experimentally determined in each particular case. Any application of chitosan should also consider the relationship between the chitosan structure and its flocculation efficiency. The fact that the flocculation doses may vary by a factor of 10 or more, depending on F_A, is clearly of considerable economic interest when evaluating any large-scale applications. Since the majority of bacteria naturally occurring in wastewater treatment systems belong to Gram-negative species (Henze, 1992), attention should be paid to highly acetylated chitosans. Unfortunately, the currently available commercial chitosans have F_A ranging only from 0 to 0.2 and the availability of highly acetylated chitosans is rather limited, probably due to low demand for these chitosans, so far.

The most apparent hinder for larger application of chitosan seems to be its cost. Compared to synthetic polyacrylamide-based flocculants with costs of approximately 30 NOK/kg (Paus & Paus pers. comm.), the price of 150-180 NOK/ kg of industrial grade chitosan (Primex Ingredients, pers. comm.) is generally too high to compete. However, in applications where special consideration about the risk to human health has to be taken, such as in drinking water treatment, the advantages of a FDA accepted and natural polymer seems to counteract the cost, and the interest in chitosan seems therefore to increase (Eikebrokk, 1999). In wastewater treatment, however, the environmental aspects are traditionally beaten by low costs of

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synthetic polymers. Hopefully, the increasing focus on waste recycling, biodegradability and environmental impacts may increase future prospects of chitosan also in this area. Increased demand for chitosans may also reduce the cost considerably.

5.3 Future studies

Due to the basic character of this study, the investigation should be extended to more applied aspects. Some relevant examples are flocculation of problematic activated sludge such as pint-point flocs, or flocculation of excess biomass from biofilm reactors. Another interesting topic, although a more distant from this study, would be sludge-dewatering. It would also be of interest to test the possibility of chitosan coating for quick biofilm establishment.

Furthermore, there are many questions of more academic interest that remained unanswered and would deserve further attention. Firstly, regarding the confusing relationship between the charge density of adsorbed chitosan and its efficiency to neutralize the bacterial charge (Section 3.5.4), the biological activity of bacterial cells following adsorption of different chitosans should be examined. It would be very interesting to study eventual response to chitosan accumulation on the cell surface, especially concerning the permeability of the outer membrane, the activity of ionic pumps and possible release of ions or molecules. Another logical extension of our studies would be to include better characterization of bacterial surfaces, preferably also including a series of mutants with subtle differences in the composition of surface structures. The nature of interactions between G- bacterial surfaces and highly acetylated chitosans also requires further attention.

To be able to draw any conclusions from the comparison of flocculation and adhesion studies, the latter should be performed in a better-characterized and more controlled model system. This includes the characterization of the chitosan modified glass slides with respect to the actual charge and the amounts of bound chitosan, replacement of the current bacterial staining procedure and absorbance measurements by fluorescent staining combined with microscopic image analysis (Rasmussen, 2001). Also, the incubation systems should have controlled hydrodynamic conditions, such as in the rotating annual reactor system applied by Rasmussen (2001).

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Paper I