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The Study of Environmental Biopolymers by Mathematical Modeling and Single Molecule Detection Techniques

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Abstract: Three examples of ***CABE's*** Polymer Science projects are presented: (i) the modeling of polyelectrolyte adsorption on charged particles; (ii) scaling properties of biopolymers studied by fluorescence correlation spectroscopy and (iii) the determination of biopolymer conformations by atomic force microscopy. Up-to date information on the group including current projects and literature citations can be found at: *http://www.unige.ch/cabe/*

Keywords: Atomic force microscopy · Biopolymers · Fluorescence correlation spectroscopy · Monte Carlo simulations · Polysaccharides

Introduction

CABE (Biophysical and Analytical Environmental Chemistry) belongs to both the Department of Inorganic, Analytical and Applied Chemistry of the University of Geneva and *CESNE* (Centre of Natural Environmental Sciences), the multidisciplinary structure of the Faculty of Sciences, which is designed to promote cooperative teaching and research in Environmental Sciences. ***CABE*** profits from long-standing expertise in analytical and environmental chemistry which has been developed over the past 20 years. Competence has been acquired in the development and application of a large number of instrumental techniques used for the analysis and physicochemical studies of complex environmental systems.

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Research Activities

CABE's interests lie in the study of environmental microstructures and microprocesses (nanometer to centimeter range), so as to better understand the macroscopic structures and behavior of environmental systems, in particular surface waters, sediments and soils. In order to achieve these objectives, research on dynamic processes related to physical and colloid chemistry, soft condensed matter, biochemistry and biophysics is performed both in laboratory and *in situ*. Projects concentrate mainly on fundamental research (environmental microprocesses and analytical principles) but links to applications exist, in particular, for instrument development and for environmental monitoring and water treatment.

CABE is presently examining some of the processes controlling the circulation of vital and toxic trace elements in aquatic systems. Because most trace compounds are associated with natural colloidal material, our objectives include the characterization of inorganic particles and biopolymers and investigations of their mechanisms of interaction (bridging flocculation, heterocoagulation). These processes are expected to strongly influence the sedimentation rate of detrimental and vital trace compounds and ultimately have an impact on biological productivity and biodiversity at the macroscopic level.

1. Modeling Polyelectrolyte Adsorption on Charged Particles

In polymer sciences, the complexation of polyelectrolytes (charged polymer chains) with oppositely charged particles has received a great deal of attention, because of potential industrial applications in food technology [1][2]. In biology, DNA binding to positively charged liposomes or proteins [3] is likely to find applications in gene therapy and genetic regulation [4]. In environmental chemistry the adsorption of charged polymers on particles is of great interest since this process is expected to control colloidal aggregation and thus the fate of numerous trace pollutants [5] that are associated with colloids in surface and waste waters. In all cases, the behavior of the aquatic system depends largely on the ability of the polyelectrolytes to adsorb and adhere to particle surfaces, and on the structure of the adsorbed layer [6]. However, due to the complexity of adsorption-coagulation processes, applications of polyelectrolytes to real systems are often based on empirical or semi-empirical observations [7][8] rather than on predictions based on

theoretical [9] or computational models [10]. Understanding of the thermodynamic and kinetic factors controlling the stability of colloidal dispersions of solid particles requires knowledge of the influence of a large number of parameters, including: polyelectrolyte molecular weight, concentration, molecular geometry; particle size and concentration, surface chemistry; solvent quality, pH, temperature, ionic strength; size and nature of the counterions; and the polyelectrolyte/particle relative concentration ratio [11–13]. Although a significant literature exists with respect to the interaction of polymers with flat surfaces [14][15] and the interaction between solid surfaces in the presence of polymers [16][17], far fewer papers have examined either the adsorption of supersized charged linear polymers on comparatively small oppositely charged objects or the adsorption of flexible polyelectrolytes onto strongly curved surfaces [18].

Computer simulations are used in the *CABE* to investigate the formation of a complex between a single polyelectrolyte chain and an oppositely charged particle. Using a Monte Carlo approach we have focused on the influence of the chain length and curvature effects by adjusting both the particle/polyelectrolyte relative sizes and the intrinsic flexibility of the chain. The influence of the electrolyte concentration, *Ci*, has also been examined since it is expected to play a key role in controlling both chain conformations and polyelectrolyte/particle interaction energies *via* screening effects. Attention has been given, in particular, to a) conditions of adsorption/desorption b) the interfacial structure of the adsorbed layer (monomer fraction in loops, trains and tails) and c) charge inversion and the overcharging problem.

MC simulations demonstrate that the extent of polyelectrolyte adsorption is controlled by two competing effects: i) electrostatic repulsion between the chain monomers, which forces the polyelectrolyte to adopt extended conformations in solution and limit the number of monomers which may be attached to the particle (Table 1) and ii) electrostatic attractive interactions between the particle and the monomers which force the polymer chain to undergo a structural transition and collapse at the particle surface.

By adjusting the polymer size, a maximum chain deformation is found when the chain radius of gyration is similar to the size of the colloidal particle. The adsorbed amount of the polymer is found to increase with chain length or a decreasing ionic strength. Trains are favored in low ionic strengths while loops become more favorable at higher ionic strengths. It is worth noting that above a critical chain length, electrostatic repulsion between the adsorbed monomers force the polyelectrolyte to protrude from the surface in a tail-like structure. For the short chains, a stronger electrostatic attraction is required at low ionic strength to overcome the loss of entropy per monomer due to adsorption (Fig. 1).

Simulation results also demonstrate that, depending on the chain length, the number of monomers close to the particle surface may be higher than necessary for charge neutralization. As a result, the particle associated to the polyelectrolyte undergoes a charge reversal. In presence of salt, simulations have pointed out the importance of two competing effects: on one hand, more monomers are adsorbed because of the higher capacitance of the polyion, and on the other hand, the electrostatic attraction between the particle and the monomer becomes less important, increasing the probability of a loss of monomer from the particle surface.

Surface curvature effects are expected to limit the adsorption of monomers. Comparison of the results for a relatively small surface with those obtained for a polyelectrolyte spread on an increasing large particle has shown that adsorption is favored by increasing particle size. Below a critical particle size (Table 2), electrostatic repulsion between the adsorbed monomers causes protruding tails of polyelectrolyte in solution. The low salt regime is dominated by monomer–monomer repulsion; the polymer partially wraps around the sphere and the two tails extend in opposite directions. Charge reversal increases with the salt concentration and exhibits a maximum value when the polyelectrolyte is able to completely wrap itself around the particle.

The results demonstrate that both the intrinsic rigidity and chain stiffening due to an electrostatic monomer-monomer repulsion play a key role. To achieve adsorption, the bending energy should be balanced by the gain of energy of the polyelectrolyte/particle association. Therefore, rigid chains are more difficult to adsorb on curved surfaces than are flexible ones. Chain rigidity clearly affects the adsorbed monomer layer structure by inducing the formation of 'solenoid' structures. Flexible chains are adsorbed in a more disordered way.

The simple model studied here, which

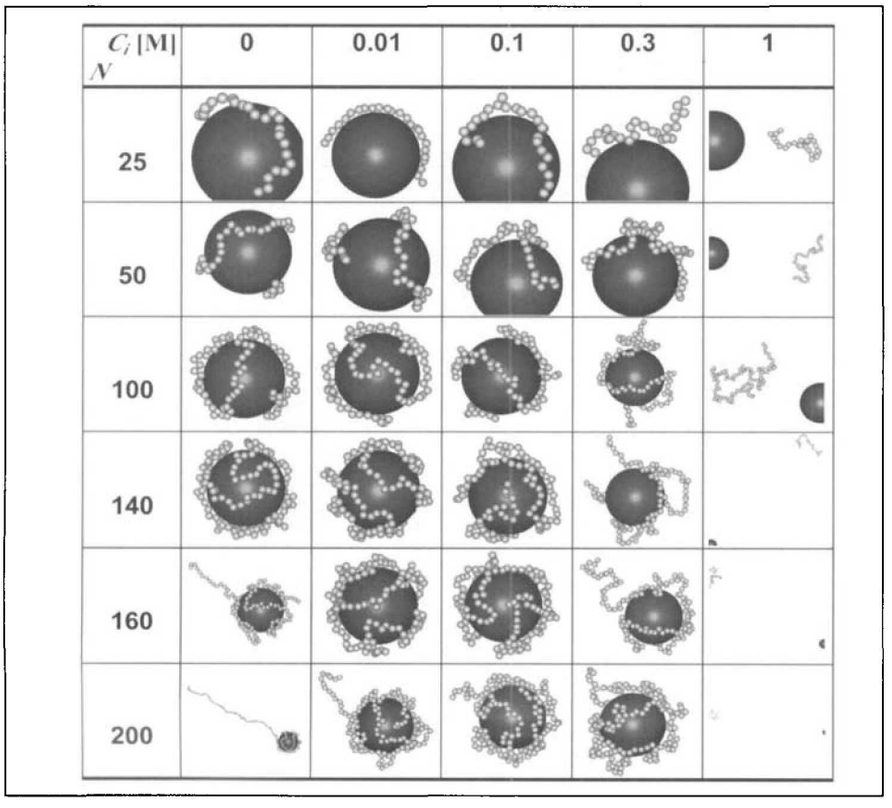


Table 1. Equilibrated conformations of polyelectrolyte/particle complexes as a function of *N* (polyelectrolyte monomer number) and C_i (electrolyte concentration).

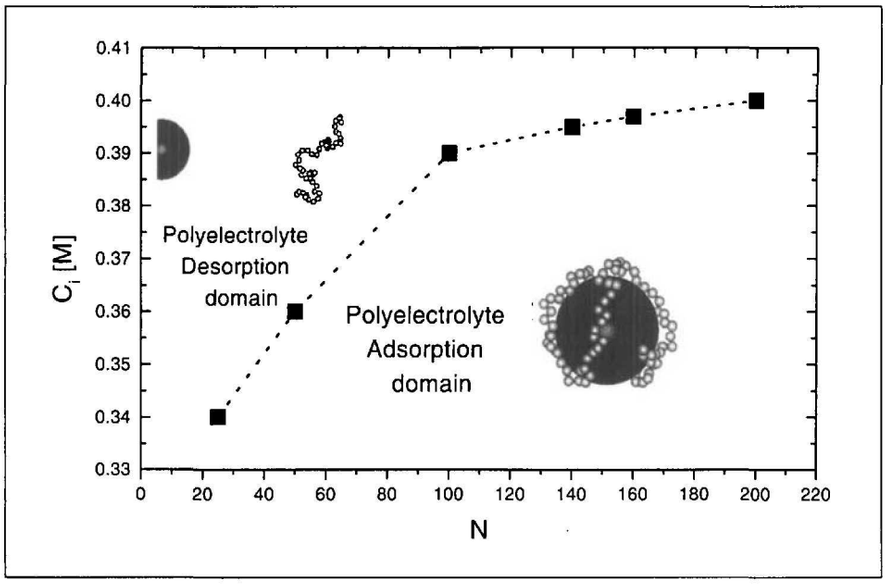


Fig. 1. Polyelectrolyte adsorption/desorption limits as a function of the electrolyte concentration C_i and polymer length, N.

involves one chain interacting with one particle, can be extended to more complicated systems involving several chains and/or colloidal particles and polydisperse systems that are more representative of natural systems. Computer simulations are in good agreement with analytical theory and enable a better understanding of the molecular factors that control the interfacial behavior of polyelectrolytes, which in turn, can facilitate a better understanding of environmental colloid-polymer mixtures.

2. Scaling Properties of Biopolymers Studied by Fluorescence Correlation Spectroscopy.

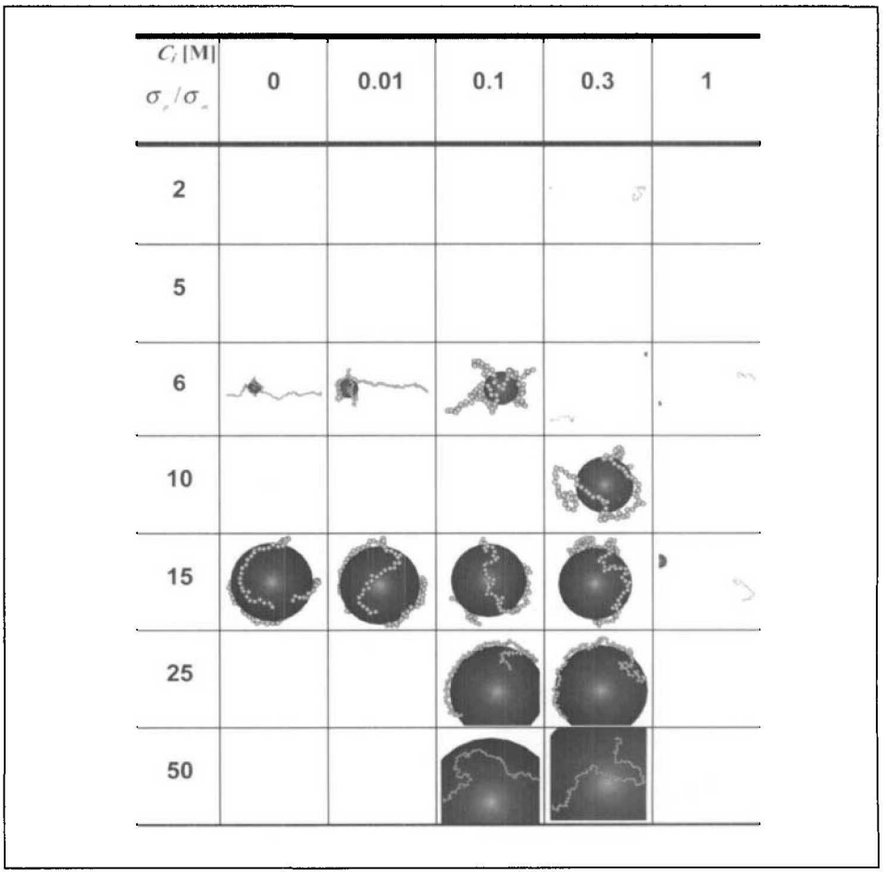
The scaling relationship between the size and mass of polymers has been of considerable interest over the past few years [19]. Fluorescence Correlation Spectroscopy (FCS) [20] provides new insight in this area due to the high sensitivity and selectivity of the method. In FCS, a small open volume (usually 2.5 10^{-16} l) called the 'sample volume' (SV) is strongly illuminated by a focused laser beam. Fluorescent molecules which diffuse into the volume produce fluctuations of emitted fluorescent light, which are captured by a detection system. The autocorrelation function of the fluorescence fluctuations are calculated and the characteristic time obtained from the function is related to the diffusion coefficient (D) and thus the hydrodynamic sizes of the diffusing molecules by a simple relationship:

$$\tau_1 = \frac{\omega_1^2}{4D} \quad (1)$$

where ω_1 is the transverse radius of the SV which is known from calibration using molecules with a known diffusion coefficient.

As already mentioned, the main advantage of the FCS is its high sensitivity, which is due to the difference between the fluorescence excitation and emission wavelengths. This difference allows a filtering of all non-fluorescent light (for example, that due to scattering or reflection from the cell borders and the surface) which strongly decreases the background and increases the signal-to-noise ratio [21]. Thus a single fluorescent molecule can be detected by FCS. Nonetheless, even the higher concentrations of ~10^{-8}M which are practically used in FCS are much lower than those used in light scattering. For this reason, the polymers can be studied in dilute concentrations under conditions which are similar to those found in natural waters. Another advantage of FCS is its specificity which is related to the fact that only molecules which fluoresce at a specific wavelength are observed. Therefore, the diffusion of specifically labeled molecules inside gels or in concentrated systems of non labeled molecules can be studied [22]. In recent applications, the diffusion of molecules in the cell cytoplasm [23][24] or cell

Table 2. Equilibrated conformations of the polyelectrolyte/particle complex as a function of the particle σ_p and monomer σ_m diameters.



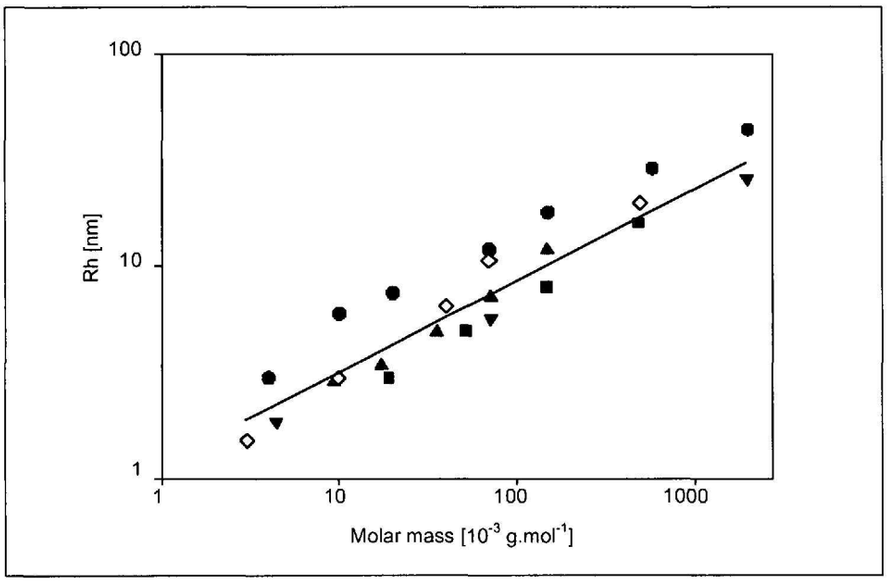


Fig. 2. Hydrodynamic radii of dextran measured by FCS (open diamonds) compared with literature data obtained by FRAP (closed symbols: squares [26], circles [27], triangles [28], inverted triangles [29]). The line is derived from the semi-empirical power law dependence found in the literature (Eqn (2)).

membrane [25] was studied *in vivo*. Because biological cells usually have a broad auto-fluorescence, labels such as Cy-5 which emit light in the red wavelengths, far from the cells' auto-fluorescence can be used. The physical principles of the FCS method are well described in the literature and will not be discussed further here. Below are examples of two applications of FCS for studies of the relationship between the molar mass and size of dextran and schizophyllan polymers.

Dextran is a branched (1-6) linked α-D-glucan polysaccharide. Dextran hydrogels are currently used as a column packing in chromatographic applications and recently in the field of controlled drug delivery [26]. It is valuable molecule for studying permeability and microcirculation *in vivo* [27–29]. Because dextran molecules are branched, they tend to collapse, suggesting that a scaling relationship between the radius of gyration and the molar mass, M, will have an exponent of less than 0.5. This result has indeed been observed in the literature [30]:

$$R_g \propto M^{0.43} \qquad (2)$$

Dextran samples labeled by tetramethylrhodamine (TMR) with molar masses of 3, 10, 40, 70, 500 kD were measured by FCS in our laboratory. The hydrodynamic radius R_h of the Dextran was calculated using the Stokes-Einstein equation. Data are presented in Fig. 2 along with literature data based on measurements made with the fluorescence recovery after photobleaching (FRAP) method. The FCS data are in agreement with the results of the literature within experimental error and with Eqn (2) demonstrating the capability of FCS for studying the diffusion dynamics of dextran. Even though the FCS and FRAP methods are similar, FCS has some advantages. For example, FCS requires lower concentrations than does FRAP. In addition, FRAP requires more powerful excitation energies which may perturb the sample.

Schizophyllan is a polysaccharide that consists of a main chain of (1→3)-β-linked D-glucose residues with one (1→6)-β-linked-glucosyl side chain for every three D-glucose residues [31]. In aqueous medium (pH<13), this polysaccharide adopts a triple-helical conformation and behaves as a rigid chain while above pH 13, the molecule is denatured and adopts a random coil conformation [32].

Different sizes of native and denatured schizophyllan labeled with rhodamine 6G in borate buffer have been studied by FCS [33] (Fig. 3). The length of the native schizophyllan can be estimated from diffusion coefficients using Broersma's equation. The obtained length of 189 ± 26 nm is close to that determined by AFM measurements: 185 ± 71 nm.

The relationship between R_h and the molar mass, M, of denatured Schizophyllan studied by FCS showed a scaling relationship $R_h \sim M^{0.59}$, in agreement with the random coil model with an excluded volume effect. The persistence length q_{denat} of the denatured Schizophyllan was determined by the Hearst's relationship, to be equal to 5.16 ± 0.75 nm. This work demonstrates the utility of the FCS method for dynamic investigations of biopolymers, especially in very dilute regimes where other techniques cannot be used because of lack of sensibility.

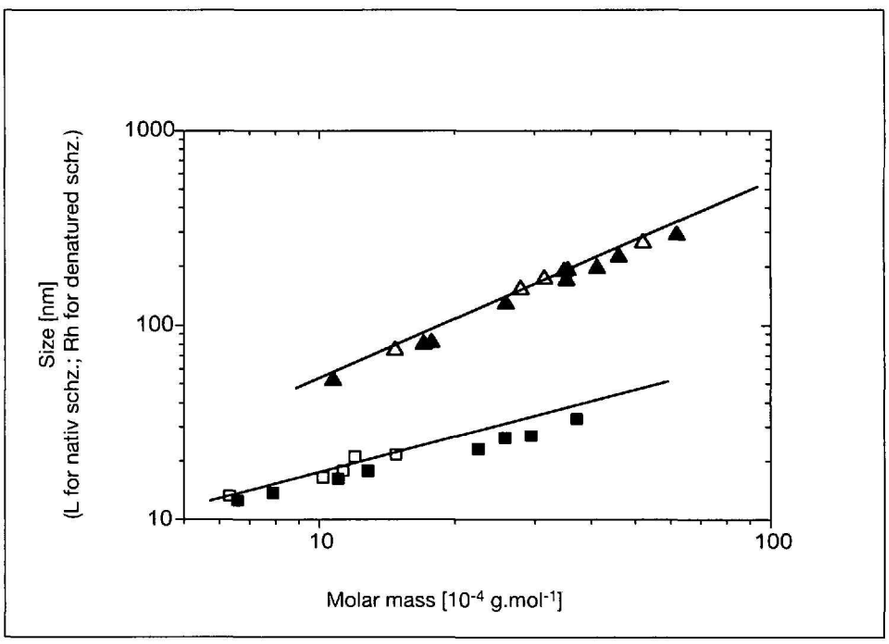


Fig. 3. Comparison of size data for native and denatured schizophyllan. Δ - lengths of non denatured schizophyllan determined in [33]; □ - radius of denatured schizophyllan determined in [33]; ▲ - lengths of non denatured schizophyllan extracted from the work Kashiwagi *et al.* [31]; ■ - radius of denatured scleroglucan from the work of Yanaki and Norisuye [32].

3. The Determination of Biopolymer Conformations by Atomic Force Microscopy

As mentioned above, the physicochemical structure of biopolymers influences their role(s) in the natural environment. Previous examinations of the role of natural organic biopolymers on the stabilization/destabilization of colloidal suspensions has demonstrated that biopolymers such as the humic substances that are small relative to the colloidal surface will, if adsorbed, modify colloidal stability due mainly to changing surface properties of the colloid [34–36]. On the other hand, relatively large biopolymers may destabilize inorganic colloids by inducing the formation of large flocs [5][36]. Clearly, the size of the polymer is relative: its chemical structure also plays a key role since the rigidity of the polymer will determine whether the polymer collapses onto the surface of the colloid or forms larges structures by bridging the inorganic colloids. In environmental sciences, the chemical and size heterogeneity of the biopolymer will thus have an important influence on the chemical reactivity. It is therefore essential to employ techniques that, whenever possible, provide a distribution of signals representative of the isolated molecules rather than a bulk or average signal. This final consideration has guided our choice of atomic force microscopy (AFM), transmission electron microscopy (TEM), FCS and capillary electrophoresis as our primary experimental techniques.

One aspect of this work has been the development of techniques for the quantitative physicochemical characterization of environmental biopolymers using atomic force microscopy. In this respect, atomic force microscopy is often semi-quantitative at best and non representative at worst. On the other hand, the technique has the undisputed advantage of single molecule detection. In order to obtain useful and quantitative information on environmental biopolymers, a systematic and non-artefactual sample preparation step is essential. Therefore, much of the work of the group has examined the role of the preparative conditions on the observed molecular structures. For example, the validity of performing AFM on biopolymers under ambient conditions has been studied. In this case, samples are put into contact with a mica substrate and then allowed to reach equilibrium with ambient or humid air. Under these conditions (hygroscopic biopolymers, relative humidity >50%), the polymers have been observed to retain their hydration water. Indeed, height measurements of the model environmental biopolymers schizophyllan and a humic acid were constant across a wide range of humidities [37].

Three preparative techniques are generally employed prior to AFM observation: drop deposition, adsorption and ultracentrifugation [38]. The techniques are complementary since each technique has its advantages and disadvantages. For example, while the drop deposition technique is the most representative of all of the molecules in solution, it has the disadvantage of allowing the formation of molecular aggregates and gels on the surface of the substrate during the drying step. On the other hand, the macromolecular structure of the individual molecules can be most easily quantified using an adsorption technique. In this case, however, only molecules which are sufficiently adsorbed to the surface after a specific time are quantified, potentially biasing the technique to the strongly adsorbing or fast diffusing species. Finally, the ultracentrifugation technique allows the direct comparison of AFM results with those obtained by transmission electron microscopy [38]. Our work has indicated that in many cases, the microscopic techniques, TEM and AFM, provide similar or complementary results.

This previous point should be emphasized: with any of the microscopic techniques, it is essential to use two or several techniques in parallel. Furthermore, in order to obtain statistically valid results, especially for polydisperse environmental biopolymers, the sample size must be large. Even then, results may often be technique dependant. For example, in the case of the humic biopolymers, AFM heights were compared to hydrodynamic diameters measured by fluorescence correlation spectroscopy, pulsed field gradient nuclear magnetic resonance and flow field flow fractionation [39]. Hydrodynamic diameters were consistently larger than AFM height measurements. This result could not be solely attributed to differences between the physical height measurement obtained by AFM and the hydrodynamic diameters determined by the other three techniques. Much of the difference could be attributed to the role of the mica substrate: interactions between the humic macromolecule and the mica resulted in a flattening of the humic macromolecules.

The role of the substrate on biopolymer conformations in AFM was also observed for a fibrillar polysaccharide, succinoglycan. Persistence lengths, end-to-end lengths and contour lengths can all be determined using atomic force microscopy for a sufficient number of observations. In the absence of added ionic strength, the co-existence of two populations of macromolecules is observed (Table 2, [40]): a rigid chain with a larger persistence length, end-to-end distance and diameter (Fig. 4) and a more flexible chain. It is clear that in this case, bulk measurements providing an average value would not be representative of the actual conformations of the macromolecules in solution. On the other hand, comparisons of measured and calculated end-to-end distances for the flexible chains indicated that the molecules of succinoglycan did not attain equilibrium with the mica surface. In this case, the flexible macromolecules are likely to adopt a more extended conformation than that occurring in solution due to a charge repulsion with the negatively charged mica.

On the other hand, in the presence of 0.01M added KCl, succinoglycan molecules appeared primarily as flexible individual chains (Fig. 5). Under these conditions, charge repulsion between the mica and succinoglycan was expected to be less important than in water due to screening effects. In this case, the number-average contour length of the molecules was 563 ± 278 nm (Table 3) while the mean end-to-end distance was 178 ± 100 nm. This observation was consistent with a conformational transition from a disordered chain in water to an ordered helical structure in salt. Note that the heights of the flexible chains in both water and 0.01M KCl were smaller than those observed for the rigid chains in water. The thickness of the flexible macromolecules

Table 3. AFM characterization of the different succinoglycan chains [40].

	contour length [nm]	end-to-end distance [nm]	persistence length [nm]	height [nm]
rigid chains in water	770±460	496±257	105±21	0.64±0.05
flexible chains in water	704±279	279±120	19±7	0.32±0.05
flexible chains in 0.01M KCl	563±278	178±100	36±13	0.44±0.09

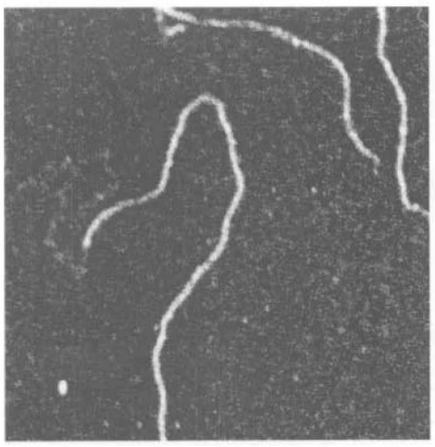


Fig. 4. AFM image of succinoglycan (10 mg l^{-1}, dissolved in water) deposited on mica. Scan size is 500 nm X 500 nm [40].

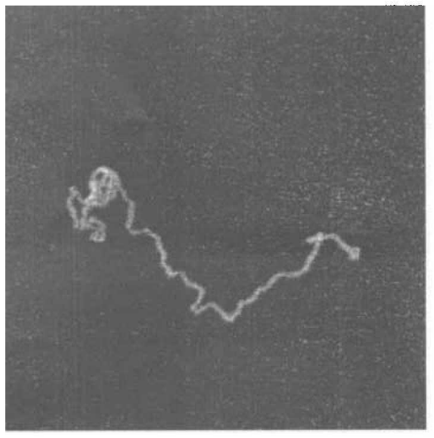


Fig. 5. AFM image of succinoglycan (10 mg l^{-1}, dissolved in 0.01M KCl) deposited on mica. Scan size is 705 nm X 705 nm [40].

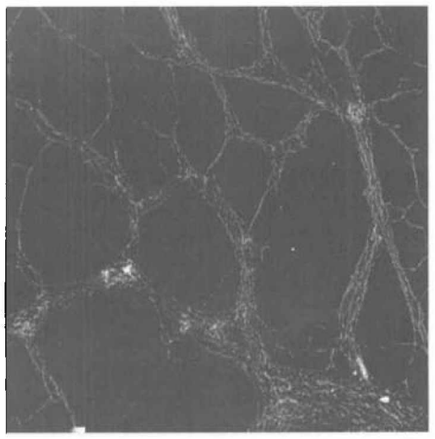


Fig. 6. AFM image of succinoglycan (10 mg l^{-1}, dissolved in 0.5M KCl) deposited on mica. Scan size is 8.0 μm X 8.0 μm [40].

corresponded well to the thickness of single glucose unit, suggesting that the rigid chains were due to the association of at least two individual chains (dimer).

Finally, in 0.5M KCl, succinoglycan molecules are observed to form a gel-like structure on the surface of the mica (Fig. 6). In these images, the height of the branches varied between 0.6 and 1.2 nm, indicating that the gel network was not formed with individual chains, but with aggregates of the succinoglycan chains.

Further studies are now underway in the group in order to determine the role of solution physicochemistry on biopolymer conformations. As expected neutral, rigid polysaccharides such as schizophyllan have shown no variability as a function of the solution conditions while carboxylate containing xanthan has shown a similar tendency to succinoglycan, *i.e.* an increased screening of charges promoting a decreasing rigidity of the macromolecule with increasing ionic strength. Clearly future measurements of individual macromolecules made with the single molecule based techniques of AFM, FCS or other sensitive techniques will only increase our knowledge on the macromolecular conformations of the biopolymers.

Acknowledgments

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