Introduction

Since the early years after its development [1], atomic force microscopy (AFM) has been employed not only to inspect sample topography, but also to probe local forces at high resolution as a function of tip–sample separation. Because the AFM probe is very small – the typical curvature radius of the tip is 40 nm – the force due to the interaction of a relatively small number of molecules is measured.

The force as a function of tip–sample separation (force–distance curve) provides information on the nanomechanical properties of hard and soft samples such as graphite [2], gold [2–4], bone [5], living cells [6, 7], gelatin [8] and other polymeric materials [9, 10].

Surface forces such as those due to electrostatic [11–13] and van der Waals interactions [2, 14] have been identified on the basis of their distance dependence. Furthermore since self-assembly techniques provide methods for functionalising the tip, adding chemical sensitivity to the system, the forces between ligands and receptors [15–18], antigens and antibodies [19–21] and complementary DNA strands [15, 22] have been measured.

Notwithstanding the fast development of this field, the way the physical and chemical properties of the interacting surfaces affect the curve shape has not yet been understood in detail. Especially, for chemically active surfaces made of biological molecules a general explicative picture of the measured forces cannot yet be made. The main difficulty is that the interfacial phenomena between biological macromolecules are very complicated and can occur on many different levels of molecular complexity.

Having in mind that a correct physical interpretation of the forces measured by AFM is based on the knowledge of the structure of both the interacting molecular systems and the surrounding environment we chose to study simple and well-defined model systems based on the building blocks of proteins.

In this article we address the effect of hydrogen bonding between tip and sample on the shape of the
force–distance curve and report the results of AFM force measurements performed with functionalised tips and self-assembled monolayers. The tip was gold-coated and functionalised with a mercaptoacid self-assembled monolayer. The system investigated was a hexapeptide film deposited by self-assembly on crystalline gold. The oligopeptide had a cysteine and an alanine amide as end groups. Cysteine provides the thiol group which binds gold. We also carried out control measurements on self-assembled films of two different thiols, one having a terminal carboxyl and the other having a terminal methyl.

**Experimental**

The oligopeptide (Cys-Gly-Ala-Ala-Ala-Ala amide, more than 95% pure) was synthesised for us by Tib-Molbiol (Italy). Ethyl alcohol (99.9% pure), 1-decanethiol (98% pure or greater) and urea (analytical reagent) were purchased from Fluka and were used without further purification. 11-Mercaptoundecanoic acid was prepared from the corresponding bromide (Aldrich, 99% pure) according to a method described in the literature for similar compounds [23]. The product, after purification by flash chromatography on silica gel (eluant: ether:petroleum ether 1:1), was characterised by $^1$H NMR spectroscopy. Water was Milli-Q grade.

For self-assembly, 1 mM solutions of the previous compounds were employed. The oligopeptide was dissolved either in a 9:1 mixture of water and ethyl alcohol or in pure water, while 1-decanethiol and 11-mercaptopundecanoic acid were dissolved in ethyl alcohol.

The gold substrates were prepared by vacuum evaporation of 120–150 nm gold (99.99% purity, Corradi, Milan, Italy) onto cleaved mica sheets (Lot Oriel Italia, Milan, Italy). The evaporations were carried out at a base pressure of 2 $\times$ 10$^{-6}$ mbar, at a substrate temperature of 600 K, and were followed by 1–2 h annealing in vacuum at the same temperature. Before use, the gold films were flame-annealed in a butane flame to red glowing, quenched in ethanol (p.a., Fluka) and dried in a stream of nitrogen.

The samples for the self-assembly experiments were transferred into the suitable solution immediately after quenching in order to minimise air exposure. The samples were kept in the solution overnight at room temperature and after extraction they were thoroughly rinsed with solvent.

For the AFM measurements a Dimension 3000 equipped with a “G” scanner head (92.8-µm scan range) and controlled by a Nanoscope III (Digital Instruments, Santa Barbara, Calif., USA) was used. Force–distance measurements were made using microcantilevers from Digital Instruments (“V”-shaped, length = 193 µm, width = 20 µm, spring constant = 0.06 N/m), gold-coated and modified by Bioforce Laboratory with a carboxyl (hydrophilic) surface.

**Results and discussion**

The force acting on the tip was measured as a function of the sample position. This measure produced a “force–distance curve” that was obtained by allowing the tip to approach the sample along the vertical axis (z-axis) and the resulting cantilever deflection, $\Delta s_c$, and piezo displacement were recorded. The force acting on the cantilever is provided by Hooke’s law,

$$F = -k_c \Delta s_c,$$

where $k_c$ is the elastic constant of the cantilever.

The piezo displacement, $\Delta Z$, which is the parameter directly controlled during the measurement, is related to the tip–sample distance, $D$, according to

$$Z_0 - \Delta Z = D + \Delta s_c + \Delta s_s,$$

where $\Delta s_s$ is the sample deformation and $Z_0$ the position of the sample surface with respect to the cantilever rest position before the approach. The force was recorded while approaching and withdrawing the tip and is reported as a function of the piezo displacement on a scale whose origin was chosen arbitrarily. Each curve has three distinct regions: the zero line, where the cantilever remains in its resting position because the tip/sample distance is too large for any interaction to occur, the noncontact region, where noncontact forces appear and the contact region, where, in the absence of sample (and tip) deformation, the piezo displacement and the cantilever deflection are equal. The noncontact forces can be repulsive and attractive. The attractive forces can cause a tip jump onto the sample in the approach phase and a jump off the sample in the withdrawal phase.

A typical force–distance curve obtained at pH 2 by using a tip, functionalised with carboxyl groups, on an oligopeptide film deposited on a gold substrate is shown in Fig. 1. The jump-to-contact region, in the approaching curve, and the jump-off-contact region, in the retracting curve, have two relative minima. Usually, when nonspecific interactions occur, these parts of the curves have only one minimum. In our case, while the tip approaches the film, it encounters a first attractive force causing the first jump. This force is followed by a repulsive force that acts for a few nanometres and, after this one, by another attractive force causing the second jump, which is usually larger than the first one. Subsequently the tip undergoes a repulsive force roughly proportional to the piezo displacement, indicating that the tip is in contact with the film surface. The retracting curve indicates similar behaviour, suggesting the presence of two kinds of attractive forces. The big hysteresis in the jump-off-contact region is a common feature of the retracting curves and is likely to be due to the fact that the number of interactions increases after tip and sample have been in contact. In fact, while the tip approaches the sample, at small tip–sample separations, only the few molecules at the outermost tip interact with the surface molecules. However, once the tip encounters the sample, the contact area of the touching tip is sufficiently large for additional molecules to interact.

The reproducibility of the force–distance curves is very high. Similar curves were obtained in different