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Protein Interactions on Phospholipid Bilayer, Studied by AFM Under Physiological Conditions

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1. Introduction

In this Chapter we would like to share our experience using the atomic force microscope (AFM) within the field of biochemistry. We present the preparation, observation and study of solid supported phospholipid bilayers with AFM. Because phospholipid surfaces are one of the most important places for the interaction between different molecules in the living beings, the knowledge of their preparation and study is essential. Second, we present three examples of protein molecules interacting on the aforementioned phospholipid surfaces: annexin A5, β_2-glycoprotein I, and antibodies. Each section of the Chapter contains all the details that allow the reproduction of: solid supported phospholipid bilayers; annexin A5 crystallization on solid supported phospholipid bilayers; β_2- glycoprotein I agglomeration on solid supported phospholipid bilayers; binding of antibodies to mica; and study of effects and interaction of specific antigen-antibody pairs. Practical instructions at the end of each section present some very useful suggestions when performing the same or similar experiments.

2. Introduction to use of atomic force microscopy in biochemistry

Since the invention of AFM in the year 1986 (Binning et al., 1986), the AFM has become very important tool in the field of biochemistry (Bippes & Müller, 2011). AFM has been used for 3D imaging of molecules (DNA, proteins, polysaccharides), structures (membranes, phospholipid structures, bacteria, cells) and their interaction (Shao et al., 1996; Gadegaard, 2006). Beside imaging, force spectroscopy has been used to reveal biochemistry processes on a molecular level (Rief et al., 1997; Oesterhelt et al., 2000). AFM has also been used for manipulation of biological material (Fotiadis et al., 2002; Thalhammer et al., 1997). High nanometer resolution, simple sample preparation (no need for vacuum), the ability to measure forces between 10^{-4} N and 10^{-12} N and possibility of the »in situ« study in dry or in liquid environment are the most straightforward advantages of AFM.

2.1 Basic principles

Figure 1 presents basic working principle and key elements of the AFM: sensitive cantilever with the sharp tip (AFM probe), piezoelectric scanner which moves the sample in all directions and an optical detection system which is measuring the cantilever bending. AFM can operate in different imaging modes, where the tip is scanning the sample surface to obtain the surface topography, or in force mode where the tip is approaching and retracting the sample to measure the force between the tip and the sample. The key element of the AFM is the probe, since the sharp tip of the probe is interacting with the sample and it measures and controls forces with the surface. Different probes have tips with different chemical and physical properties, which govern the adsorption of the sample components on the tip, what could have big impact on the imaging (modification of the tip apex) and measuring forces. The tips can also be functionalized in order to achieve different chemical activity of the tip (silanization) or particular biological specificities (functionalization with the antigen, antibody, etc.). Big advantage of the AFM comparing to other imaging techniques is in the fact, that samples could be studied in the ambient conditions or in different buffer solutions, which can mimic natural conditions for biological samples. Biological samples are usually studied on a particular platform also called a substrate (more details on the substrates further in the text). The right choice of a substrate is very important, since the substrate can governs the samples properties.

2.2 Modes of operation

AFM can be operated in different imaging modes, where the most common are contact and oscillation mode, or in force mode. Decision about which mode to use is dependent on the sample characteristic and the properties of the sample we want to study.

2.2.1 Contact mode imaging

Contact mode is the original and the most accurate imaging AFM mode, where the tip is brought into the hard contact with the sample surface and it is scanned across the surface. The cantilever deflection, and thus the contact force, are kept constant during the scanning by using a feedback loop. Images are generated by mapping the vertical position of the sample (or cantilever) as it moves up and down to maintain the constant force between the tip and the surface. The deviation of the cantilever deflection from its constant value is recorded as well and provides the error signal, or deflection image, which represents the slope. In contact mode the tip is dragged with relatively high velocity across the studied surface. This results in a friction force between the moving tip and the surface of the sample, which exerts shearing force on the sample. The result is a shear deformation of the sample surface. Therefore, the contact mode is more appropriate when examining the samples with relatively rigid surfaces (crystalline samples) or samples with high self-healing characteristics (phospholipid bilayers). For soft biological samples the contact force should be maintained as low as possible.

2.2.2 Oscillation mode imaging

In the oscillation, or dynamic, mode the cantilever is oscillated with very high frequency, close to the resonant frequency of the cantilever. The most popular oscillation mode is the

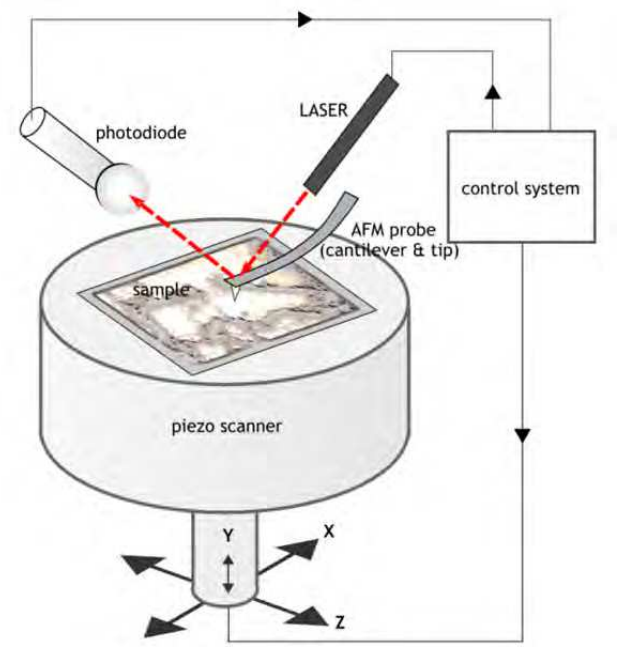

Fig. 1. Basic components and work principle of the atomic force microscope. The basic component of the microscope is a very sharp tip, which is mounted to cantilever. When measurement takes place, the forces between the sample and the tip cause deformation of the cantilever that bends according to the scanned surface. The bending of the cantilever is the most frequently detected optically by a reflected laser beam. The laser beam is directed to the upper side of the cantilever and reflected to the photo-detector (photodiode). The signal from photo-detector is then directed to the computer controlled hardware unit where the feedback electronics moves the sample, which lies on the piezoelectric scanner. In contact mode the laser beam is directed all the time to the same position on the photodiode, so the tip is following the sample surface. The computer transforms the information of scanner movement to the image shown on the monitor.

intermittent contact, or "tapping", mode, where the tip touches the sample surface only once during the oscillation cycle. When the tip is in the contact with the surface, the amplitude of the oscillation is slightly decreased and it is usually used as a feedback parameter. Images are generated by mapping the vertical distances that scanner moves to maintain the constant amplitude during scanning. This mode was developed in order to eliminate the lateral forces, which can damage samples, especially the biological ones.

For even more delicate samples one can use the non-contact mode. Here the tip during oscillation does not touch the sample surface. It oscillates above the sample surface with small amplitude and the distance between tip and sample is controlled by cantilever's resonance frequency which is maintained by the feedback loop. However, this mode does not reach the resolution of contact and tapping mode due to the relatively big separation between tip and sample.

2.2.3 Force spectroscopy mode

In force mode the tip is approaching and retracting from the sample above the same point and it measures the interaction between the tip and the sample. The result of this measurement is distance dependent force curve and not the 3D topographical image of the surface. High resolution of this measurement (piconewton range at nanometer distances) can provide accurate information about intra- and intermolecular interactions and mechanical sample properties.

2.3 Atomic force microscope used in the presented experiments

All our AFM measurements were performed in a liquid environment using a Nanoscope IIIa-MultiMode AFM (Digital Instruments, Santa Barbara, CA) equipped with E (15μm range) scanner. Mica substrates were installed into a contact mode fluid cell. Using AFM we have measured forces and acquired images in the contact mode using oxide-sharpened silicon nitride tips (MSCT-AUNM silicon nitride tip, Veeco, Camarillo, CA) mounted on cantilevers with nominal force constant of 0.01 N/m or 0.03 N/m, with typical curvature radius of 15 nm. We used different scanning rates, most often 5 Hz. The scanning force was kept at the lowest possible value by continuously adjusting the set point during imaging. All experiments were performed at 25°C (Irman et al., 2009, 2010, 2011; Žager et al., 2011).

3. Preparation and study of solid-supported phospholipid bilayers by atomic force microscope

During the past decade a large number of different membrane system have been described, including solid-supported phospholipid bilayers (SPBs), polymer-cushioned lipid bilayers, hybrid bilayers, tethered lipid bilayers, suspended lipid bilayers or supported vesicular layers, all of them having a potential bio-technological applications (reviewed in Richter et al., 2006). However, for our experimental purposes we chosen the *in vitro* model of SPBs because: a.) SPBs are easy to prepare and b.) together with atomic force microscopy this *in vitro* model offers measurements in real time in liquid environment, and therefore *in situ* observation and evaluation of interactions of particular molecules on phospholipid surfaces. SPBs are well suited to analyze lipid domain formation, intermembrane interactions, or membrane processes such as protein adsorption, protein self-assembly, protein localization at lipid phase boundaries and protein function.

3.1 Formation of solid-supported phospholipid bilayers

The mechanism of SPBs formation from large unilamellar vesicles was pioneered by Brian and McConell (Brian & McConnell, 1984): adsorbed vesicles fuse among themselves until critical size is reached, and then rupture to form bilayer disks (Brian & McConnell, 1984; Lipowsky & Seifert, 1991; Seifert, 1997; Reviakine & Brisson, 2000). The one-step procedure allows creating SLBs of different lipid mixtures. Such SLBs form a fluid two dimensional space allowing free diffusion in translation and rotation of lipid molecules and lipid-associated proteins. It was recognized that several factors govern SPB formation: lipid vesicles used for their construction (their composition, charge, size and physical state); aqueous environment (its composition, pH and ionic strength); and nature of support (its surface charge, chemical composition and roughness) on which SPB is formed. A number of reports have revealed difficulties to form

SPBs on surfaces such as gold, $SrTiO_2$, TiO_2 or platinum, leaving mica and silicon-based materials, such as glass, Si_3N_4 or silica as the most common surfaces used for preparation of SPBs. It was recognized that the interactions between lipids and solid support strongly affect the properties (inter-leaflet distribution of lipids in the SPBs) and the quality (integrity) of the final SPB (reviewed in Richter et al., 2006).

3.2 Mica as a solid support

In our study mica was chosen as solid support. Mica is molecularly smooth and hydrophilic and consists of negatively charged layers bound together by positively charged interlayer of K^+. In aqueous solutions, a mica cleavage surface becomes negatively charged due to the dissociation of K^+. It was reported that mica causes asymmetrical inter-leaflet distribution of phosphatidylcholine and phosphatidylserine (PS) containing mixtures of phospholipids, with more PS on mica-facing leaflet of SPB. The asymmetry on mica was suggested to originate from a specific calcium-mediated interaction between the support and PS (Richter & Brisson, 2005). In general it was found that calcium promotes the adsorption and rupture of vesicles and subsequent SPB formation. Effects are particularly strong on mica, since only mmol/l concentration of the ion is sufficient to generate significant effect.

3.3 Mica-supported phospholipid bilayers simulate the physiological conditions of the cell surface

3.3.1 Recipe for preparation of mica-supported phospholipid bilayers

Planar, mica-SPBs are formed from 0.5 g/l suspension of large unilamellar sonicated phospholipid vesicles in HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl), pH 7.5, with 1.5 or 2 mmol/l of $CaCl_2$. For preparation of HBS Water for injections from Braun (Melsungen, Germany) is used. Large unilamellar sonicated phospholipid vesicles are prepared as follows: Appropriate amounts of lipids either synthetic (30 % dioleil-phosphatidyserine in the mixture with dioleil-phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA)), or naturally derived phospholipids (30 % L-α-phosphatidylserine in the mixture with L-α-phosphatidylcholine (Sigma- Aldrich, St. Luis, MO, USA)), are dissolved in chloroform. The solvent is evaporated under low pressure in the presence of N_2 for at least 30 min. HBS with calcium is added to the newly generated lipid film, yielding a 0.5 mg/ml multilamellar vesicle suspension. Unilamellar vesicles are obtained using a bath sonicator (UZ 4R, Iskra, Kranj, Slovenia). Prepared vesicles could be studied by light scattering (Zeta Sizer 3000, Malvern Instruments, England, UK) and/or direct observation with AFM.

SPB is formed by deposition of 120 µl of freshly sonicated vesicles onto a cleaved mica surface (Provac AG, Balzers, Liechtenstein) followed by incubation for 60 min at room temperature. Further treatment of the sample to form the SPBs on mica is however dependent on the phospholipid mixture that is used for preparation of vesicles and the properties of vesicles themselves. If the vesicles are prepared from phospholipids, and the average Tm (Tm is a gel to liquid crystal transition temperature) of the phospholipid mixture is over the room temperature, then sample should be further heated to the Tm or above (in our experimental model the Tm for mixture from naturally derived phospholipids was designated 41°C) for 10 min. When using the phospholipid mixture with the Tm that is below the room temperature, no further heating of the sample is necessary (as was in our

case with the vesicles from synthetic phospholipids). The excess lipids are removed by exchanging the solution covering mica with buffer, and the sample is then installed in the contact mode fluid cell in the AFM. The microscope is allowed to thermally equilibrate for a minimum of 15 min before imaging.

3.3.2 Our comments to mica-supported phospholipid bilayers

The developed *in vitro* model of mica-SPBs starts by preparation and characterization of phospholipid vesicles, we found that by slightly changing only two conditions when preparing the vesicles, the strength of the vacuum and pressure under which N_2 was blown in the system, we were able to measure vesicles of different sizes, from 60 nm to 300 nm. An increase in vacuum coupled with a decrease N_2 pressure decreased the sizes of vesicles. An increase of the sonification time decreased the sizes of vesicles. Polydispersity of the vesicles was strongly dependent on the quality of chemicals used for preparation of vesicles and the time of sonification. In general, the longer the time of sonification, the less polydisperse were the vesicles. But after certain time of sonification, poydispersity began to grow. For production of unilamellar vesicles we have also used the extrusion method, where multilamellar vesicles dispersion were extruded through polycarbonate membranes with an average pore size of 50 nm, 100 nm and 200 nm. The vesicles made by extrusion method were around 20-30 nm larger than the sizes of the pores used and more homogeneous in comparison with vesicles made by sonification. Nevertheless, for our formation of SPBs on mica, vesicles made with sonification were used, and sizes around 200 nm proved to be the most useful. When studying vesicles with AFM, contact and tapping mode were used for imaging (Figure 2A).

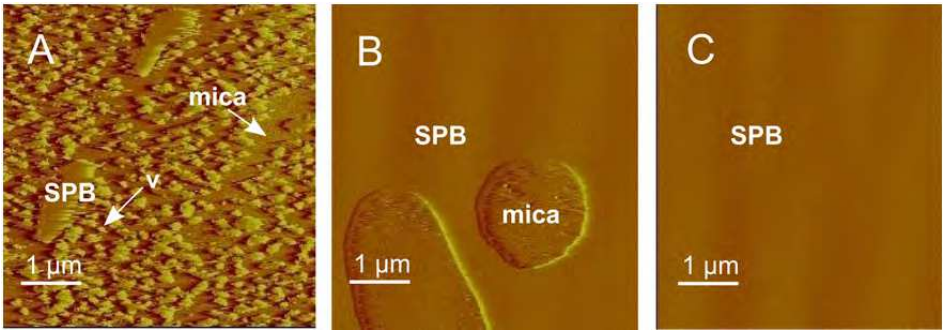

Fig. 2. Deflection images of vesicles, discontinuous and continuous SPBs, made in AFM contact mode. A.) 0.5 mg/ml sonicated unilamellar vesicles solution (30 % dioleil-PS in the mixture with dioleil-phosphatidylcholine in HBS with 1.5mmol/l of calcium) after 15 min incubation in AFM fluid cell. Phospholipid vesicles (v) with sizes from 60 nm to 250 nm can be observed. Some vesicles have already started to fuse among themselves to form SPB patches. B.) A discontinuous SPB. C.) A continuous SPB, where a completely flat surface can be observed.

The next step was preparation and characterization of SPBs. We found that formation of SPBs from naturally derived phospholipids was influenced by slightly different fatty acid compositions of both L-α-PS and L-α-phosphatidylcholine between the batches used. Several combinations of conditions (incubation time at room temperature, temperature and duration of heating) have to be experimentally tested to generate reproducible

formation of phospholipid bilayers. Phase transition temperature for the mixture of naturally derived phospholipids used in our experimental model was determined experimentally to be approximately 41°C. The work with synthetic dioleil-PS and dioleil-phosphatidylcholine was much more straightforward due to the fixed composition of synthetic phospholipids: we did not have to change anything, once the protocol for formation of SPBs was established. Thicknesses of phospholipid bilayers from naturally derived and synthetical phospholipids on mica measured by imaging with AFM in the contact mode were approximately 3.6 nm. The thicknesses were therefore lower than expected (around 6 nm). In AFM contact mode the tip is dragged with relatively high velocity across the surface of the SPB. This results in a friction force between the moving tip and the upper surface of the SPB, which exerts shearing force on the SPB. The consequence is a shear deformation of SPB, tilting of the molecules in the bilayer and a smaller apparent thickness of the SPB. Using force measurements we have determined the average force required for penetration into the phospholipid bilayers, which was approximately 0.4 nN. We also measured the thicknesses of the bilayers being penetrated and determined them to be approximately 5.3 nm (Irman et al., 2009). The presence of SPB was confirmed by imaging and force measurements as presented in Figure 2 and Figure 3. Figure 3 further supports the existence of phospholipid bilayer since two monolayers that together form phospholipid bilayer are clearly visible.

With the use of naturally derived phospholipids, we can mimic the conditions in the human body much better than with synthetic ones (Irman et al., 2009).

3.3.3 Practical instructions

1. AFM offers nanoscale observation. Therefore, selection of chemically, microbiologically and physically extra pure reagents (water, buffers and phospholipids) for preparation of SPBs is essential.
2. Since phospholipids are prone to oxidation and microbiological contamination one should make sure that phospholipids used are fresh and properly handled. When preparing the vesicles avoid: a.) overheating the phospholipid mixture during the sonification, b.) the contact of phospholipid mixture with air oxygen (use inert atmosphere: nitrogen, argon).
3. The amount of the phospholipids to form the bilayer should be sufficient. When using the 0.5 mg/ml phospholipid vesicles solution in particular buffer system (presence of mmol/l calcium is advisory), approximately 120 µl of the vesicles solution should be deposited onto a freshly cleaved mica surface.
4. In order to achieve the sufficient concentration of the vesicles on mica surface for their further fusion, the vesicles should be incubated on mica at room temperature for 60 minutes.
5. If the Tm of the used phospholipid mixture is higher than the room temperature, the sample has to be heated at the defined Tm for about 10 to 15 minutes. If the Tm of the mixture is at or below the room temperature no heating is necessary to achieve the fusion of the vesicles.
6. The removal of excess lipids is achieved by exchanging the solution covering mica with buffer.
7. The sample is then installed in the fluid cell (contact mode or tapping mode fluid cell) in the AFM. The SPBs in the fluid cell should be covered with the buffer all the time.

However, avoid flushing the SPBs sample with the buffer before the microscope thermally equilibrates which usually takes a minimum of 15 min.

8. Perform AFM measurements: imaging, force measurements.

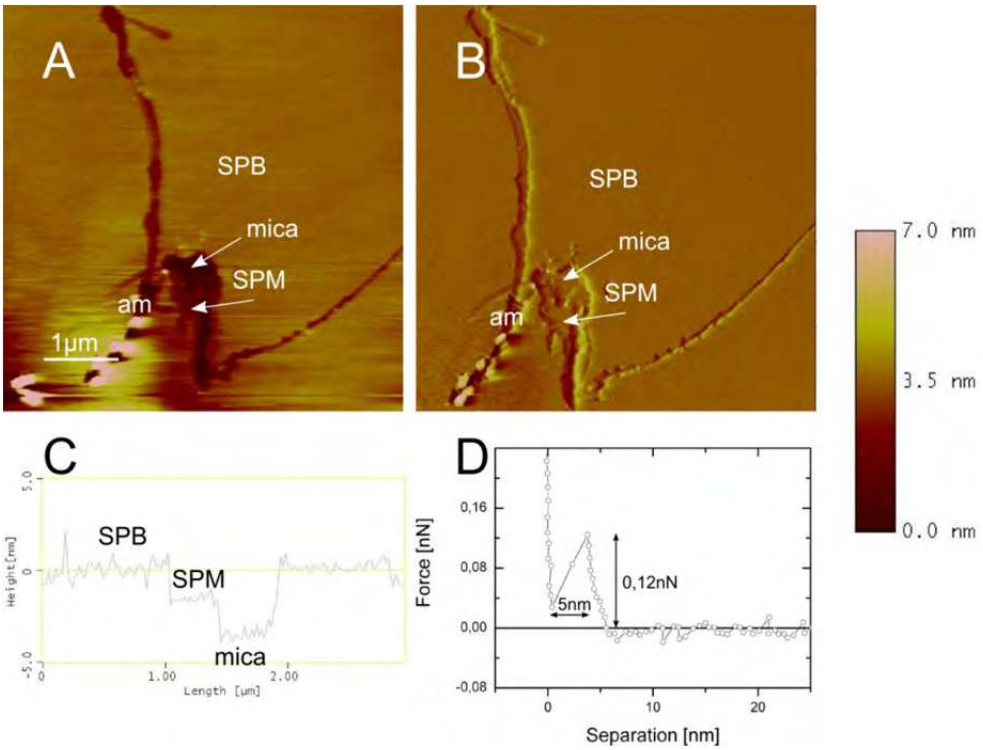

Fig. 3. AFM height and deflection images, height cross section and force measurement of SPB, disrupted with the AFM tip and measured in the contact mode AFM fluid cell. As a buffer above SPB, HBS with 2 mmol/l of calcium was used. This figure presents evidence that the phospholipid vesicles form phospholipid bilayer and not monolayer on mica. (A) AFM height image, where three layers: mica, first phospholipid monolayer (SPM) and second monolayer, which together form the SPB, are measured. Some accumulated material (am) can also be deduced. (B) AFM deflection image of the same surface as in image A, where all three layers are more clearly visible. (C) Height cross section of image A, indicating first phospholipid monolayer is 2 nm higher and the phospholipid bilayer is 4 nm higher than mica. (D) Force measurement showing that the tip is penetrating through SPB about 5 nm thick, with the required force for penetration of 0.12 nN.

4. Models of basic protein interaction - AFM study of annexin A5, β_2-glycoprotein I and antibodies

In this section we give three examples of the proteins that are physiologically present in human body. We present their interaction with phospholipid surfaces, mica, as well as their interactions with each other.

4.1 Crystallization of annexin A5 on phospholipid membranes

4.1.1 Annexin A5's characteristics

Annexin A5 (ANX A5) is an example of protein that forms crystalline lattice over the exposed phospholipid surfaces. ANX A5 is a 35.7 kDa member of the annexin family, which consists of water-soluble, structurally related and calcium-dependent phospholipid binding proteins (Benz & Hofmann, 1997; Gerke et al., 2005; van Genderen et al., 2008). ANX A5 is formed of four domains solely made of α-helices, and is shaped like concave disk with calcium and phospholipid-binding domains present on the convex side (Huber et al., 1990). ANX A5 molecules are monomeric in solution, however, after binding to phospholipid membrane, they spontaneously form tightly bound trimers. The membrane bound trimers self-organize into two types of 2D crystals, with p3 or p6 symmetry (Oling et al., 1998). The mechanism of ANX A5 crystallization on negatively charged phospholipid membranes in the presence of calcium has been thoroughly described (Reviakine et al., 1998, 2000, 2001; Richter et al., 2005; Irman et al., 2009) and it is here schematically shown in Figure 4.

To date, many physiological roles of ANX A5 have been proposed. It is generally accepted that its physiological significance is closely connected to the ability to bind in a calcium-dependent and reversible manner to negatively charged phospholipids, PS in particular. The anticoagulant properties of the protein are a consequence of ANX A5 crystallization on phospholipid membranes, resulting in a lattice of protein over phospholipid surfaces. This blocks the availability of phospholipid surfaces for coagulation reactions. The anticoagulant activity was connected also to more specific properties of ANX A5, such as down-regulation of surface-expressed tissue factor (Ravassa et al., 2005). Several other biological characteristics of ANX A5 have been described, including calcium-channel activities (Rojas et al., 1990; Berendes et al., 1993; Liemann et al., 1996), regulation of phospholipase A2 (van Heerde et al., 1995; Russo-Marie, 1990), cytosolic protein kinase C (van Heerde et al., 1995; Russo-Marie, 1990), inhibition of phagocytosis of apoptotic cells by both activated and inactivated macrophages (Callahan et al., 2000), inhibition of microparticle generation and induction of a novel endocytic pathway (Kenis et al., 2004).

4.1.2 Recipe for preparation and study of crystalline annexin A5 on mica-supported phospholipid bilayers

In order to study ANX A5 crystallization on SPBs, an appropriate amount of 10-40 mg/l ANX A5 (we used ANX A5 isolated from human placenta (Sigma- Aldrich, St. Luis, MO, USA)) in HBS with 1.5-20 mmol/l of calcium is injected into the fluid cell of the AFM with prepared SPB. HBS-Ca^{2+} is prepared in Water for injections (Braun, Melsungen, Germany). The SPBs could be formed of naturally derived: L-α-PS (Sigma- Aldrich, St. Luis, MO, USA) and L-α-phosphatidylcholine, (Sigma- Aldrich, St. Luis, MO, USA) or synthetic: dioleil-PS and dioleil-phosphatidylcholine, (Avanti Polar Lipids, Alabaster, AL, USA). The presence of ANX A5 crystalline shield is studied by imaging and force measurements (Irman et al., 2009, 2011).

4.1.3 Our comments on annexin A5 crystallization on mica supported phospholipid bilayers

We found that ANX A5 is able to self-assemble on planar phospholipid bilayers on mica, from synthetic or naturally derived phospholipids. It was found that 2D crystallization of

ANX A5 on phospholipid bilayers depends on concentration of calcium, PS and ANX A5 (Reviakine et al., 1998, 2000, 2001; Richter et al., 2005; Irman et al., 2009). By fixed weight share of the PS in the phospholipid mixture with the phosphatidylcholine, 30 %, and 21 mg/l of ANX A5 in HBS with 2 mmol/l of calcium, the p6 crystal form of ANX A5 was documented (unit cell: a = b = 20 nm, γ = 60°). Under identical conditions and 10 times higher concentration of calcium, ANX A5 self-assembled in two different crystallization forms, signed p6 and p3 (unit cell: a = b = 7 nm, γ = 60°). Figure 4 represents insight into gradual crystallization of ANX A5 on SPBs, as detected by AFM in the contact mode. In Figure 4 both, p3 and p6, crystal forms of ANX A5 are presented.

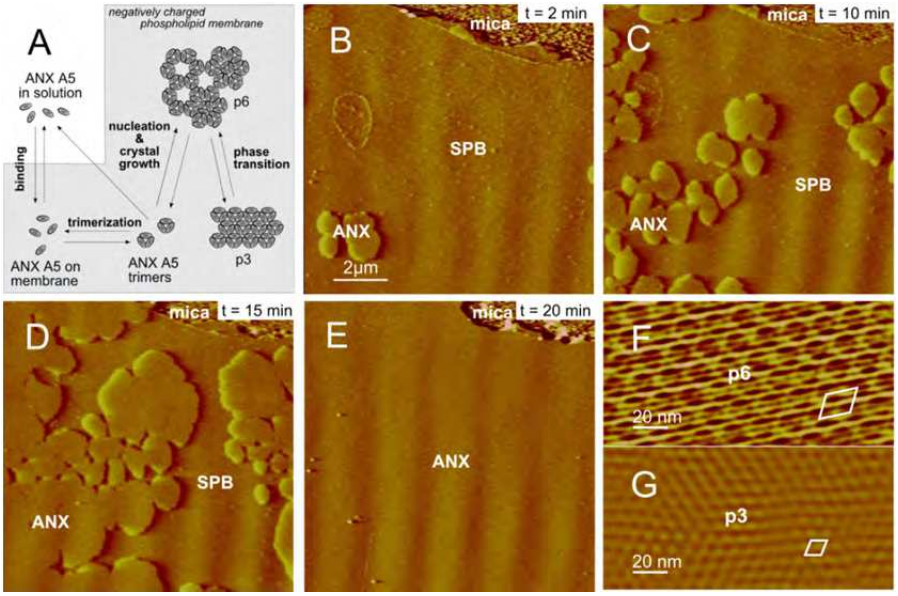

Fig. 4. Scheme and a time sequence of AFM deflection images of ANX A5 crystallization on SPB, measured in the contact mode. (A) Scheme of ANX A5 crystallization on negatively charged phospholipid surfaces in the presence of calcium ions (Govorukhina et al., 2003): Monomeric ANX A5 binds to membrane in a calcium-dependent manner where it forms trimers. The trimers crystallize in two dimensions. The holes in the honeycomb-like lattice of p6 symmetry can be filled with additional non-crystalline trimers, also called central trimers. At high protein coverage, a phase transition of the first order into a more densely packed crystal form with p3 symmetry can occur. When reducing the concentration of ANX A5 or calcium or both, the process is reversed and desorption takes place; (B) 10 mg/l ANX A5 in HBS with 1.5 mmol/l of calcium was used for observation of its crystallization on SPB. SPB contained 30% α-L-PS and 70% α-L- phosphatidylcholine. Image was measured 5 minutes, (C) 10 minutes, (D) 15 minutes and (E) 20 minutes after ANX A5 was administrated into the fluid cell of AFM. Growth of ANX A5 crystalline domains can be deduced. In image E the whole SPB is covered with crystalline ANX A5. ANX A5 crystalline domains are around 2.9 nm higher than SPB on which they grow. (F) p6 crystal form of ANX A5 (unit cell: a=b=20 nm and γ = 60°) and (G) p3 crystal form of ANX A5 (unit cell: a=b=7 nm and γ = 60°). Fourier transformation filtering (NanoScope Reference Manual, 1996) is applied in images F and G.

When studying crystallization using more physiological like conditions (10 mg/l of ANX A5 in HBS with 1.5 mmol/l calcium on phospholipid bilayers containing 30 % of L-α-PS), the p6 crystal form was predominantly found. The thickness of ANX A5 crystalline layer over phospholipids was determined to be around 2.9 nm. By performing force measurements we found that the crystallized ANX A5 layer on phospholipid bilayers could be elastically deformed, but could not be ruptured with forces up to 1nN. Demonstration that native ANX A5 is able to spontaneously crystallize on naturally derived phospholipids is supporting the putative role of ANX A5 crystal structures as a possible antithrombotic shield (in detail in Irman et al., 2009, 2011).

The process of ANX A5 desorption from crystalline ANX A5 on SPBs was also studied. Desorption can be achieved by reducing the concentration of ANX A5 above the studied surfaces. For observation of desorption of ANX A5 from the crystalline ANX A5 on SPBs we used pure buffer, HBS with 1.5 mmol/l of calcium. We started our observation with ANX A5 p3 crystal form and by allowing the degradation process to continue due to time, p3 gradually transformed into less dense p6 crystal form of crystalline ANX A5, and crystalline p6 domains then began to break under the minimum force of the AFM tip (Figure 5). Force measurement studies showed that during the process of desorption, the ANX A5 crystalline layer which was in p6 crystal form, with the holes (desorbed hexamers-signed as h in the Figure 5) could be penetrated. The AFM tip penetrated through a 5 nm thick layer, most probably representing the SPB. The desorption process made previously stable ANX A5 crystal structure on SPB mechanically very unstable (it could be ruptured by imaging the surface with minimum scanning force).

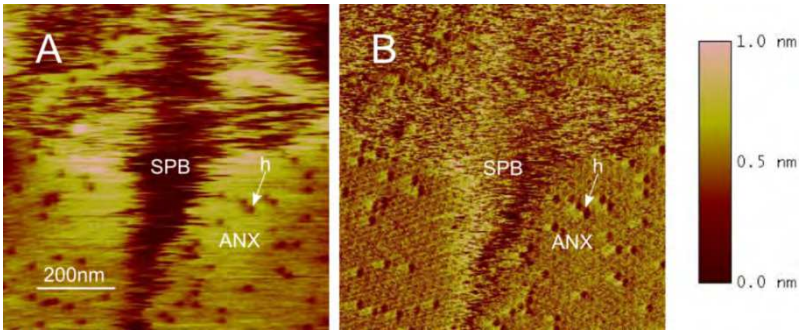

Fig. 5. AFM height and deflection images of breaking ANX A5 (10 mg/l in HBS with 1.5 mmol/l of calcium) p6 crystalline domains with the AFM tip when observing and studying the process of annexin desorption from SPB. Images were measured in contact mode at a minimum scanning force. (A) AFM height image presenting the p6 crystalline domains of ANX A5 on SPB, which are missing the hexamers (h) in the structure, due to the process of desorption. The AFM tip is breaking the crystalline domains during the scanning the studied surface, indicating the mechanical instability of the observed surface. (B) AFM deflection image of image A, where the topography of the surface can be more clearly deduced.

4.1.4 Practical instructions

1. ANX crystallization will occur with reagents (water, buffers, phospholipids, proteins) that are chemically, microbiologically and physically extra pure.

2. Different proteins need different composition of phospholipid surface in order to crystallize successfully. Make sure that phospholipid components that govern the crystallization are present and intact (phospholipids are prone to oxidation, microbiological contamination).
3. Make sure that other reagents (ions) that govern the crystallization process are present and in adequate concentration (in the buffer system).
4. For crystallization studies use the protein that is as highly purified and as fresh as possible. Impurities and degradation products may disturb the protein crystallization as well as observation of the crystallization by AFM (impurities may accumulate on the tip of the AFM cantilever and therefore cause worse resolution).
5. Prepare a phospholipid surface. Inspect it in the appropriate AFM fluid cell.
6. Inject the appropriate amount of the protein in the appropriate buffer into the AFM fluid cell.
7. Before measuring, the AFM should be thermally stabilized (approximately 15 minutes). When observing the crystallization of proteins, the AFM contact mode is appropriate.

4.2 Agglomeration of β_2-glycoprotein I on phospholipid membranes

4.2.1 β_2-glycoprotein I's characteristics

β_2-glycoprotein I (β_2-GPI) is an example of the protein that agglomerates on phospholipid surfaces. β_2-GPI is a glycoprotein of 54 kDa, with plasma concentration of about 150 mg/l. The crystal structure of the protein is composed of five domains that form a circular conformation or an elongated J-shaped conformation with overall dimensions of 13.2 x 7.2 x 2.0 nm^3 (Bouma et al., 1999; Scwarzenbacher et al., 1999; Agar et al., 2010). Each domain consists of 60 amino acids, except for domain V. Domain V consists of 82 amino acids due to C-terminal extension of 19 amino acids and an insertion of 6 amino acids, forming a hydrophobic loop. In addition, domain V carries a definite positive charge arising from 14 lysine residues. These specific structures of domain V are responsible for the binding properties of β_2-GPI to anionic phospholipids (Hamdan et al., 2007; Frank et al., 2009; Miyakis et al., 2004; Sodin-Šemrl & Rozman, 2007). Upon binding to negatively charged phospholipid surfaces, conformation of β_2-GPI changes and oligomerization (clustering) of the protein molecules on the phospholipid surface occurs (Gamsjaeger et al., 2005). Physiologically, β_2-GPI has been shown to be involved in the mechanisms such as inhibition of the coagulation pathway, platelet prothrombinase activity and platelet aggregation (reviewed in Lutters et al., 2003).

4.2.2 Recipe for study of agglomeration of β_2-glycoprotein I on mica-supported phospholipid surfaces

In order to study β_2-GPI agglomeration on prepared SPBs, an appropriate amount of the protein (in our experimental model we used 0.15 g/l of β_2-GPI purified from pooled human plasma (Čučnik et al., 2004) and dissolved in HBS with 1.5 mmol/l of calcium) is injected into the fluid cell of the AFM with preformed SPB. HBS is prepared in Water for injections (Braun, Melsungen, Germany). In our experimental model SPBs are prepared of 30% w/w L-α-PS (Sigma- Aldrich, St. Luis, MO, USA) in the mixture with L-α-phosphatidylcholine (Sigma- Aldrich, St. Luis, MO, USA). The agglomeration of β_2-GPI on SPBs is studied by imaging and force measurements (Irman et al., 2010; Žager et al., 2011).

4.2.3 Our comments on agglomeration of β_2-glycoprotein I on mica-supported phospholipid surfaces

β_2-GPI I clusters (0.15 g/l of β_2-GPI I in HBS containing 1.5 mmol/l of calcium) were detected approximately 3.1 nm higher than the phospholipid surface on which they were formed, measured in the contact mode. The measured heights of β_2-GPI clusters are lower than expected from the dimensions of the β_2-GPI molecule (13.2 x 7.2 x 2.0 nm^3) and confirm the horizontal-like orientation of the molecule on phospholipid bilayers (Hamdan et al., 2007). However, the specific nature of AFM measurements in contact mode should also be considered. The observed agglomeration of β_2-GPI on SPBs, as confirmed by our AFM experiments, was suggested as a reaction mechanism comprising two steps: i.) initial binding of the protein to the lipids, ii.) subsequent formation of protein clusters. Binding of the protein on the phospholipids was recognized to be mainly of electrostatic and also hydrophobic nature (Frank et al., 2009; Gamsjaeger et al., 2005). Greater binding of β_2-GPI to SPBs has been detected in the presence of more negatively charged net surface of SPBs within a reduced calcium environment (Gamsjaeger et al., 2005; Willems et al.,1996). When using discontinuous SPBs, β_2-GPI bound to mica patches when these are exposed. Because the mica surface is hydrophilic and slightly negatively charged, the interactions between protein and the support are likely to be mainly of electrostatic nature. Measured heights of the protein clusters on mica are approximately 8 nm. Since no insertion of the glycoprotein domains into mica is possible, vertical orientation of the β_2-glycoprotein I molecules on mica is expected (in detail in Irman et al., 2010; Žager et al., 2011).

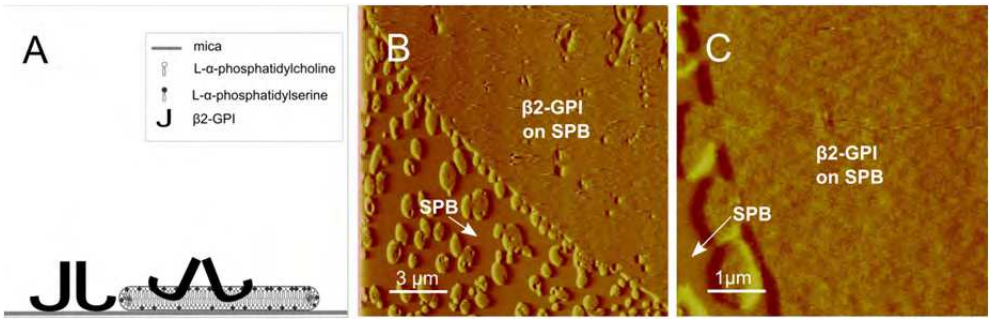

Fig. 6. Scheme and AFM deflection images of β_2GPI binding to SPBs and mica. A.) Scheme of β_2GPI binding to SPBs and mica. Notice a horizontal-like orientation on SPBs and vertical orientation of the molecule on mica. (B) AFM deflection images of binding β2-GPI (0.15 g/l in HBS with 1.5 mmol/l of calcium) to continuous SPB (no mica exposed). (C.) Magnified deflection image of the protein clusters on SPB.

4.2.4 Practical instructions

1. Select chemically, microbiologically and physically extra pure reagents (water, buffers, phospholipids, proteins).
2. Different proteins need different composition of phospholipid surface in order to agglomerate successfully. Make sure that phospholipid components that govern the agglomeration are present and intact (phospholipids are prone to oxidation, microbiological contamination).

3. Make sure that other reagents (ions) that govern the agglomeration process are present and in adequate concentration.
4. For agglomeration studies use the protein that is as highly purified and as fresh as possible. Impurities and degradation products may disturb the protein crystallization as well as observation of the agglomeration by AFM (impurities may accumulate on the tip of the AFM cantilever and therefore cause worse resolution).
5. Prepare appropriate phospholipid surface. Inspect the phospholipid surface in the contact or tapping AFM fluid cell.
6. Inject appropriate amount of the protein in the appropriate buffer system in the AFM fluid cell.
7. Allow to thermally stabilize (approximately 15 minutes). Measure.

4.3 AFM study of antibodies

4.3.1 The use of AFM in studies of antibodies and their interaction with antigens

Antibodies are a part of the specific humoral immune response, which target and neutralize the foreign objects (bacteria, viruses, tumors) in vertebrate body. Antibodies are glycoproteins belonging to the immunoglobulin superfamily. Immunoglobulins G (IgG) which are more thoroughly considered in this section of the Chapter are just one of possible antibodies isotypes. IgG is a 150 kDa globular molecule, 14.2 x 8.5 x 3.8 nm^3 (Silverton et al., 1977; Marquart et al., 1980; Deisenhofen, 1981), consisting of four polypeptide chains. These four segments are held together by disulphide bonds and noncovalent hydrophobic interactions, making the native state of the protein in aqueous solution relatively robust. Therefore, it is expected that the protein molecule on the surface should be largely a globular assembly, although some local deformations might occur because of protein-surface and protein-protein interactions (Xu et al., 2006).

AFM offers the study of antibodies on a molecular level and in an aqueous environment. Therefore, the possibilities regarding the use of AFM for the study of antibody-antigen interaction are numerous. AFM has been used: a.) to study the structure and binding characteristics of antibodies or antigens on a particular platform. In this section, the results of the study of binding characteristics of IgG molecules on SPBs and mica in aqueous medium (HBS with 1.5 mmol/l of calcium) are presented; b.) to directly study specific interactions between antibodies and antigens employed. Results are achieved by using the AFM probes functionalized with antigen or antibody molecules (Hinterdorfer et al., 1996; Allen et al., 1997); c.) to measure the effects of antibody-antigen interactions. AFM played very important role in visually presenting the possible pathophysiology in autoimmune diseases called antiphospholipid syndrome (Rand et al., 2008; Irman et al., 2010, 2011; Žager et al., 2011), as presented in the next section (Section 5) of the Chapter. As such, AFM could also be used in the evaluation of the biological drugs activity.

4.3.2 Recipe for studying the antibodies interaction on mica

In our experimental model IgG are purified from a sera of healthy blood donor with MAbTrapTM Kit (Amersham, GE Healthcare, Little Chalfont, UK), according to manufacturer's instructions. After purification, the preparations are dialyzed against HBS containing 1.5 mM of calcium, pH 7.5. HBS is prepared in Water for injections (Braun,

Melsungen, Germany). Concentrations of purified proteins are determined spectrophotometrically (Camspec M501 Single Beam Scanning UV/Visible Spectrophotometer, Camspec Ltd., Cambridge, UK), using the extinction coefficient of 14.0 for the 1% IgG solution. The binding of IgG in HBS with 1.5 mM $CaCl_2$ to mica substrates is measured in the AFM contact mode fluid cell: Mica substrates are installed into the fluid cell and sealed with the O-ring. Then the solution of antibodies (0.001 g/l or 10 g/l IgG in HBS containing 1.5 mM calcium) is administrated into the system. The results are obtained by imaging with continuously adjusting the set point to the minimum value of the scanning force. The binding of antibodies to mica and SPBs is studied also on discontinuous SPBs (SPBs that are not covering the whole mica surface).

4.3.3 Our comments on molecular and technical aspects

When imaging binding of 0.001 g/l IgG in HBS containing 1.5 mmol/l of calcium on mica, we found that IgG bind weakly and gradually to mica surface, as shown in Figure 7. The assemblies could be carried away by the AFM tip, with scanning force set just above the minimum (Figure 7 C).

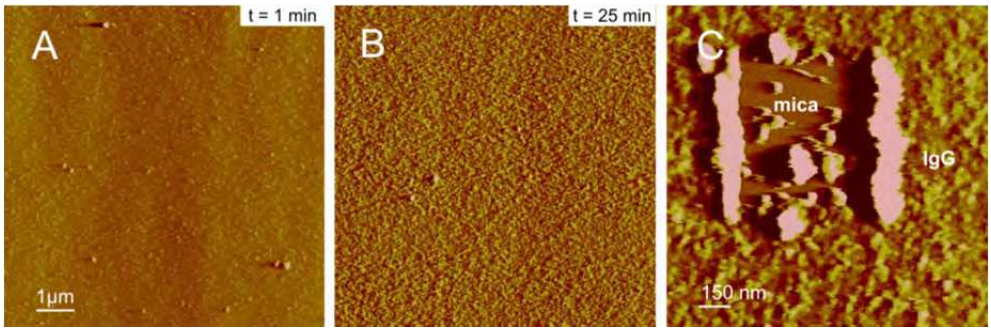

Fig. 7. Time sequence of AFM deflection images of IgG (1 mg/l in HBS containing 1.5 mmol/l of calcium) binding to mica, measured in the contact mode. IgG started binding to mica immediately after applying the solution into the AFM fluid cell. Image (A) was measured 1 minute and (B) 25 minutes after applying IgG to mica. More adsorbed or maybe even accumulated material can be deduced in image B. (C) When increasing the scanning force above minimum, the AFM tip carried away the IgG molecules. Globular assemblies can be deduced from image C.

IgG molecules had shapes of globular assemblies with diameters ranging from 30 to 50 nm and heights from 2 to 5 nm. In Figure 8 one such assembly is magnified. Dimensions of globular assemblies measured on mica led us to the assumption that we are observing most probably single antibodies or even clusters of IgG molecules together. In addition, the orientation of IgG on mica is most probably random: "flat on" (antibody lying flat on the surface), "side on" (Fab and Fc closer to the surface), "end on" (Fc closer to the surface) and "head on" (Fab closer to the surface). Great discrepancy in dimensions (overestimation of object size) of observed single molecules of IgG could be explained by the nature of AFM measurements with the tip (Ramirez-Aguilar 1998, Howald 1994). The sharper and more flexible the tip of AFM cantilever is used, the more precisely the surface can be described.

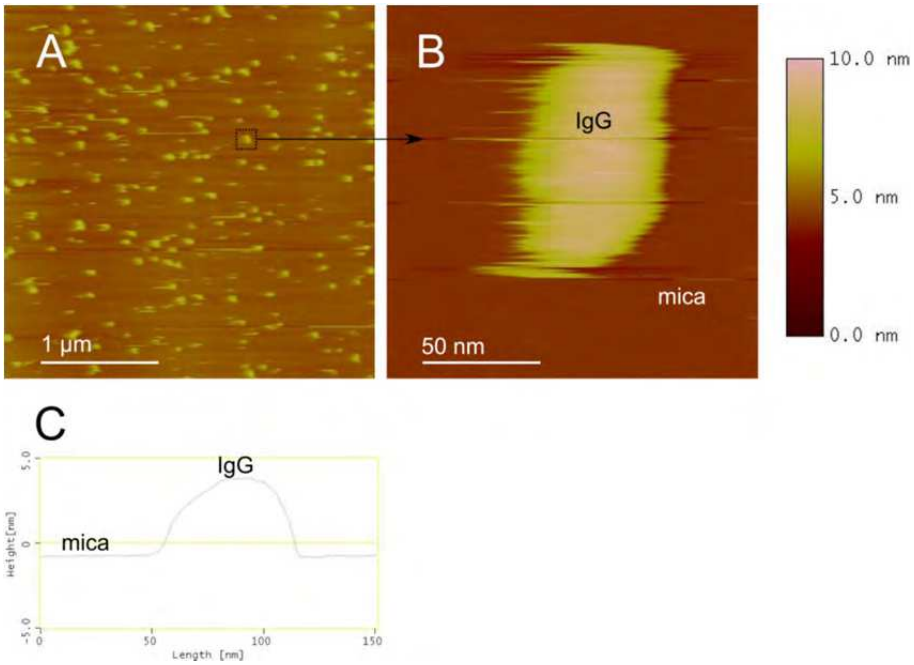

Fig. 8. AFM height images and height cross section of the IgG (1 mg/l in HBS with 1.5 mmol/l of calcium) assembly, measured in the contact mode. (A) Image of IgG assemblies on mica, with diameters ranging from 30 to 50 nm and heights from 2 to 5 nm. (B) Image represents single assembly with dimensions 40 x 80 x 5 nm^3. (C) From cross section profile of image B one can measure height of studied assembly of about 4.5 nm.

Because the mica surface is hydrophilic and slightly negatively charged, the interactions between IgG and the support are likely to be mainly of electrostatic nature.

When using the physiological concentration of IgG in HBS containing 1.5 mM of calcium, the observed globular assemblies randomly interconnected with each other, leading to the formation of loose antibody patches on mica, and they were measured to be 4.9 ± 1 nm higher than SPBs (Figure 9).

To conclude, we found that antibodies bound weakly, randomly and most probably electrostatically to the exposed mica surface. They further agglomerated into loose protein patches. No binding of studied IgG antibodies (from a healthy blood donor) directly to phospholipid bilayers could be detected.

4.3.4 Practical instructions

1. Study of antibodies structure and binding by AFM requires the use of at least microbiologically and physically extra pure antibodies. Chemical and biological purity is mandatory when studying the specific interactions. Impurities could make the observations far less specific. Microbiological, physical and chemical extra purity for all other reagents that are present in the experimental model is mandatory as well.

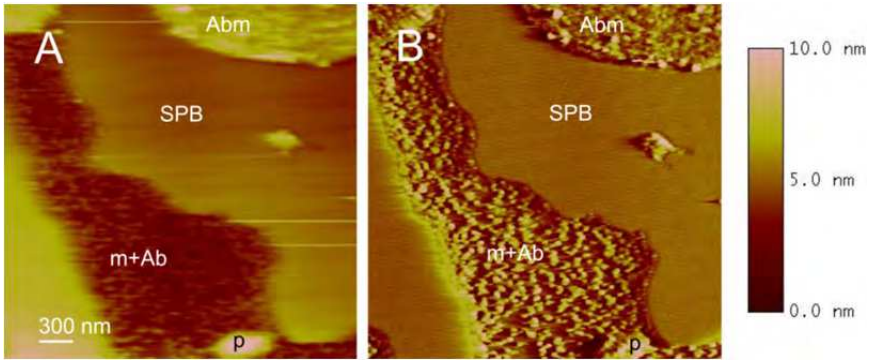

Fig. 9. AFM height and deflection images of IgG adsorption (10 g/l in HBS containing 1.5 mM of calcium) to mica patches of discontinuous SPB, measured in the contact mode fluid cell. (A) On the image, three surfaces can be observed: loose antibody layer on mica (Abm) that can be measured around 5 nm above SPB; the SPB layer (SPB), about 4 nm above mica; and mica with some antibodies that only started to adsorb or accumulate (m+Ab). One can deduce that assemblies on m+Ab, representing IgG, started randomly interconnect with each other to form the loose antibody patch on mica (p). On the Abm, the antibody patch is already formed. No binding of antibodies to the SPB was measured. (B) The deflection image of the same surface as in image A. All three surfaces can be more clearly observed. Globular assemblies can be deduced.

2. When studying the structure of antibodies one should consider the change in specificities and possibly the structure of antibodies as well, when using the antibodies that are being subjected to different isolation procedures (Omersel et al., 2010).
3. When focusing the study of the antibodies on their structure, one should use AFM imaging modes with less friction force interaction- oscillation modes.

5. Models of increased complexity – AFM study of specific antibody-antigen interactions on the *in vitro* model of phospholipid bilayer

AFM is a very useful method to study a variety of molecules, structures and the interactions between them, including an antigen-antibody and protein-membrane interactions. In the field of autoimmunity, knowledge about interactions among antiphospholipid antibodies and ANX A5 are of crucial importance to understand etiopathogenesis of antiphospholipid syndrome and to find new therapeutic targets. Antiphospholipid syndrome is an autoimmune disease which is characterized by the core clinical manifestation of thrombosis, venous or arterial, and recurrent foetal loss (Miyakis et al., 2006; Wilson et al., 1999; Ashersone et al., 2002). The family of antiphospholipid antibodies, when found in persistently high levels, present the laboratory criteria for the diagnosis of antiphospholipid syndrome. Antiphospholipid antibodies are very heterogeneous and combine antibodies directed toward: i.) phospholipids (cardiolipin, phosphatidylserine), ii.) different protein cofactors (β_2-GPI, prothrombin, ANX A5, protein S, protein C, low and high molecular weight kininogens and others), and iii.) complexes of protein cofactor-phospholipid (Meroni & Shoenfeld, 2008; Arnout & Vermylen, 2002; Shoenfeld, 2002). Many pathologic mechanisms of antiphospholipid antibodies associated with the antiphospholipid syndrome have been suggested so far. One of the possible

mechanisms was 1998 presented by Rand et al.: antiphospholipid antibodies interfere with a formation of ANX A5 crystalline shield over blood vessels and therefore diminish the proteins anticoagulant protective role (Rand et al., 1998, 2008).

In this Section we present the results of our study of ANX A5 and antiphospholipid antibodies (anti- β_2- glycoprotein I antibodies, anti-annexin A5 antibodies) interaction on a molecular level. The missing knowledge was acquired by the use of SPBs, simulating the activated cell phospholipid surface, and the use of AFM. Visualization of antigen-antibody interactions in their native aqueous environment with a nanometer resolution and the use of native reagents (phospholipids, ANX A5, β_2-GPI and antibodies) present the main advantages of our experimental model.

5.1 Study of interaction of annexin A5 and anti-annexin A5 antibodies on mica-supported phospholipid bilayer

5.1.1 Anti-annexin A5 autoantibodies

Anti-annexin A5 antibodies (anti-ANX A5) were first detected 17 years ago and were recognized as a subgroup of antiphospholipid autoantibodies (Matsuda et al., 1994). Since then, anti-ANX A5 have been associated with the occurrence of recurrent pregnancy losses, pre-eclampsia, *in vitro* fertilization failures and/or thrombotic events in patients with pregnancy complications and systemic autoimmune diseases such as systemic lupus erythematosus, antiphospholipid syndrome and rheumatoid arthritis (Matsuda et al., 1994; Rodriguez-Garcia et al., 1996; Kaburaki et al., 1997; Satoh et al., 1999; Nojima et al., 2001; Matsubayashi et al., 2001; Gris et al., 2003; Bizzaro et al., 2005; Zammiti et al., 2006, Božič et al., 2005). However, mechanisms through which these antibodies affect the mentioned pathological processes have not yet been elucidated. We have focused our study on the direct effect of anti-ANX A5 on ANX A5, a potent anticoagulant protein.

5.1.2 Recipe for study of anti-annexin antibodies and annexin A5 interaction on the mica-supported phospholipid bilayers

In brief, SPBs are prepared on mica, simulating the physiological conditions of the cell surface. On SPBs, ANX A5 is completely or/and incompletely crystallized. Effects of IgG are studied by imaging using AFM in the contact mode (in detail in Irman et al., 2011).

For the study of interaction of anti-ANX A5 and crystalline ANX A5 on SPBs, sera are acquired and selected from the sera bank of the Department of Rheumatology (University Medical Centre Ljubljana, Ljubljana, Slovenia). Hundred samples of sera are pre-tested by an *in-house* or commercial enzyme-linked immunosorbent assay for the presence of different subtypes of antiphospholipid antibodies. The antiphospholipid antibodies tested are: anti-cardiolipin antibodies (Božič et al., 1997); anti-β_2-glycoprotein I antibodies (anti-β_2-GPI) (Čučnik et al., 2004a); anti-prothrombin antibodies (Ambrožič et al., 2002); and anti-ANX A5 (Orgentec Diagnostica, Mainz, Germany). 6 sera are selected according to clinical features of the patient, immunologic tests and availability (adequate volume stored). The obtained samples are grouped into the group A (autoimmune venous thrombosis), B (autoimmune obstetric complications) and C (controls). Total IgG from our selected sera are purified with MAbTrap™ Kit (Amersham, GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions. After purification, the preparations are dialysed against HBS containing 1.5 mM calcium (HBS-Ca^{2+}), pH 7.5. HBS-Ca^{2+} is prepared in Water for injections

(Braun, Melsungen, Germany). Concentrations of purified proteins are determined spectrophotometrically (Camspec M501 Single Beam Scanning UV/Visible Spectrophotometer, Camspec Ltd., Cambridge, UK), using the extinction coefficient of 14.0 for the 1% IgG solution. Concentrations of IgG samples used in further AFM study are 10 g/l in HBS-Ca^{2+}.

The effects of particular IgG (patients' and healthy blood donor's) are studied on incompletely and completely crystallized ANX A5 on SPBs: SPBs are prepared of 30 % w/w L-α-PS (Sigma- Aldrich, St. Luis, MO, USA) in the mixture with L-α-phosphatidylcholine (Sigma- Aldrich, St. Luis, MO, USA). After the presence of SPB is confirmed, 10 mg/l ANX A5 isolated from human placenta (Sigma- Aldrich, St. Luis, MO, USA) in HBS-Ca^{2+} is injected into the fluid cell of the AFM. When the presence of ANX A5 crystalline shield, incompletely or completely covering the SPB is detected, IgG are added to the ANX A5 solution covering the SPB. ANX A5 concentration is kept at 10 mg/l. The effects of IgG on crystalline ANX A5 are measured by AFM for another 60 minutes. Results from the experiment where applying antibodies before the ANX A5 to SPBs, are also resolved.

Effects of the isolated IgG on the velocity of ANX A5 crystallization on SPBs are studied as well: ANX A5 (10 mg/l in HBS-Ca^{2+}) is injected into the fluid cell of AFM on the SPB, and the time needed for ANX A5 to cover all the SPB's surface is measured. The HBS-Ca^{2+} is then used in order to remove the bound ANX A5 of the phospholipid surface (desorption). After ANX A5 desorbed completely from SPB, a mixture of particular IgG and 10 mg/l ANX A5 in HBS-Ca^{2+} is added and the time needed for complete coverage of SPB's surface with crystalline ANX A5 in the presence of particular IgG is measured.

5.1.3 Our comments on technical and clinical aspects

Our study revealed two different effects of anti-ANX A5 IgGs (Irman et al., 2011): i.) a disruption of incompletely crystallized ANX A5 shield as presented in Figure 10 (IgG samples from patients with autoimmune venous thrombosis), and ii.) a decrease in velocity of ANX A5 crystallization (IgG samples from patients with autoimmune recurrent pregnancy losses). Therefore, our study confirms the heterogeneous effects of anti-ANX A5 on crystalline ANX A5 over planar mica-supported SPBs. This supports their distinct roles in pathogenic mechanism and also offers an explanation for different clinical conditions, such as autoimmune thrombosis or obstetric complications in the antiphospholipid syndrome. The observed different effects of anti-ANX A5 containing IgG samples on incompletely crystallized ANX A5 are due to the heterogeneity in epitope specificity and in avidity of anti-ANX A5. No disruptive effects of the studied IgG on the intact ANX A5 crystal shield (completely covering the SPB) suggests that anti-ANX A5 cannot be the primary cause but more likely potentiate the effect of other pathological factors. Pathological potential of anti-ANX A5 should therefore be considered as a "*second hit*", but for pathologies in human body to develop the "*first hit*" (genetic and environmental pathological factors influencing the expression, translation or structure of ANX A5 and therefore its binding to phospholipid surfaces and its 2-D crystallization) should also be present (Arnout & Vermylen, 2002).

To conclude, new approaches are necessary in laboratory diagnostics, based on the knowledge obtained by AFM studies, since the detection of anti-ANX A5 in the plasma by the routinely used methods is not able to identify their fine specificity and to differentiate between patients concerning their risk for annexinopathies.

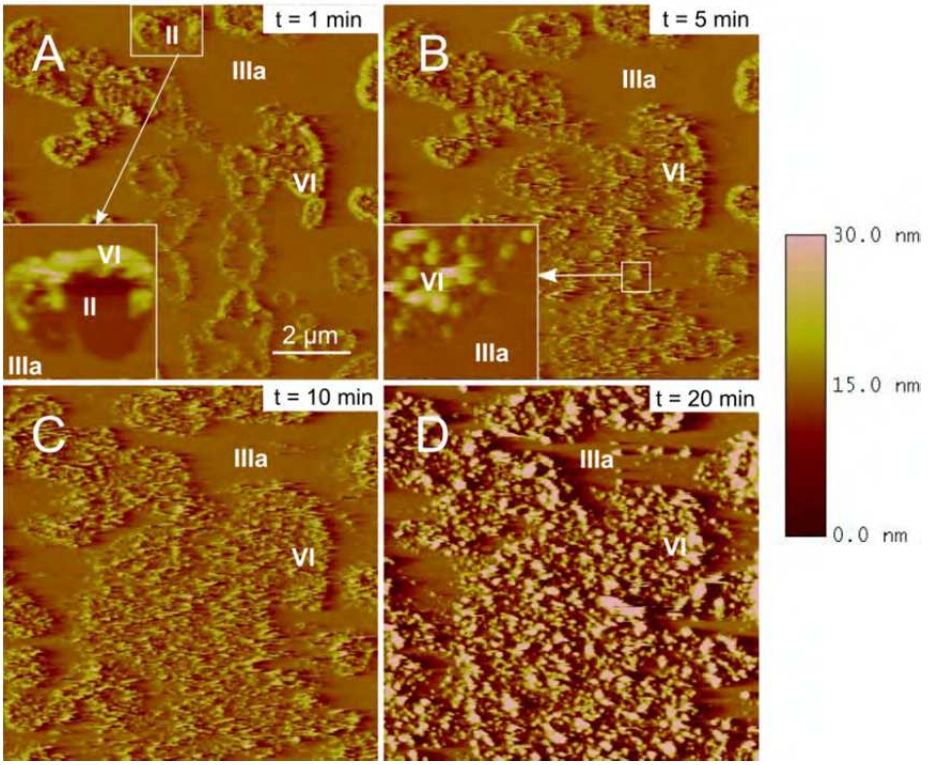

Fig. 10. Time sequence of AFM height and deflection images of the effect of sample IgG-A1(patient with autoimmune venous thrombosis) on ANX A5, not completely covering the SPB. After application of IgG-A1 to ANX A5 incomplete crystalline layer that is 64% covering the continuous SPB, antibodies start to bind to the lateral epitopes of crystalline ANX A5 and cause the disruption of ANX A5 protective shield. Image (A) is measured immediately, (B) 5 min (50% of SPB covered with crystalline ANX A5), (C) 10 min (33% of SPB covered with crystalline ANX A5) and (D) 20 min (28% of SPB covered with crystalline ANX A5) after IgG-A1 were applied. On the left lower corner of image (A), there is a magnified part of that image presented as 1.2 X 1.2 mm^2 height image. We detected that IgG-A1 bind to the lateral epitopes of crystalline ANX A5 (IIIa), which is around 2.6 nm higher that SPB (II). Antibody patches are around 3 nm higher than crystalline ANX A5. In the left lower corner of the image (B) there is magnified, 1.4 X 1.4 mm2, height image of ANX A5 crystalline (IIIa) and antibody patches (VI). It was measured, that antibody patches are in some parts approximately 1 nm lower and in some parts up to 12 nm higher, than crystalline ANX A5, suggesting that antibodies indeed disrupt the ANX A5 protective shield. (D) This height image shows the second layer of IgG-A1, most probably anti-ANX A5, 8-12 nm higher than the layer of antibodies that disrupted the crystalline ANX A5 (latticing of immune complexes of ANX A5 and anti-ANX A5). Reprinted and modified from Irman Š., Škarabot M., Muševič I., Rozman B., Božič B.. Effects of anti-annexin antibodies on annexin A5 anticoagulant shield, as observed by atomic force microscopy. Journal of Autoimmunity 2011;36:98-105, with permission from Elsevier.

5.2 Study of interaction between annexin A5 and β_2-glycoprotein I and anti- β_2-glycoprotein I antibodies pair on mica-supported phospholipid bilayers

5.2.1 Anti- β_2-glycoprotein I autoantibodies

Among the most studied antiphospholipid antibodies are anti-β_2-glycoprotein I antibodies (anti-β_2-GPI). Anti-β_2-GPI of different isotypes, directed toward different epitopes of the antigen molecule and of different avidity, have been associated with various clinical manifestations (Shoenfeld 2003): thrombosis and pregnancy complications in patients with antiphospholipid syndrome (de Laat et al., 2006a, 2006b, 2007; Rand et al., 2008; Čučnik et al., 2004b; Ioanou et al., 2007) described in patients with systemic lupus erythematosus (Čučnik et al., 2004b), in infectious diseases (Arvieux et al., 2002) and childhood atopic dermatitis (Ambrožič et al., 2002), in patients with stroke and acute coronary syndrome (Bizzaro et al., 2010). Disruption of ANX A5 protective crystalline structure by complexes of β_2-GPI and anti-β_2-GPI on phospholipid surfaces presents one of their possible pathological mechanisms of action.

5.2.2 Recipe for the study of the effects of β_2-glycoprotein I and anti- β_2-glycoprotein I antibodies pair on crystalline annexin A5 on mica-supported phospholipid bilayers

In brief, SPBs are prepared on mica, simulating the physiological conditions of the cell surface. On SPBs, ANX A5 is completely or/and incompletely crystallized. Effects of β_2-GPI and/or isolated IgG are studied by imaging using AFM in the contact mode (in detail in Irman et al., 2010; Žager et al., 2011).

For the purposes of the study, sera were selected from the sera bank of the Department of Rheumatology (University Medical Centre, Ljubljana, Slovenia). Sera are pre-tested by an *in-house* or commercial enzyme-linked immunosorbent assay for the presence of different subtypes of antiphospholipid antibodies: anti-cardiolipin antibodies (Božič et al., 1997), anti-β_2-GPI antibodies (Čučnik et al., 2004a), anti-prothrombin antibodies (Ambrožič et al., 2002) and anti-ANX A5 antibodies (Orgentec Diagnostica, Mainz, Germany). In the present study, sera were selected according to clinical features of the patient, immunologic tests and availability (adequate volume stored). Total IgG from selected sera are purified with MAbTrap™ Kit (Amersham, GE Healthcare, Little Chalfont, UK), according to manufacturer's instructions. After purification, the preparations are dialyzed against HBS-Ca^{2+}, pH 7.5. HBS-Ca^{2+} is prepared in Water for injections (Braun, Melsungen, Germany). Part of isolated IgG, after affinity purification with MAbTrap™ Kit, are dialyzed against phosphate buffered saline (PBS) and then further subjected to isolation of anti-β_2-GPI by an in-house affinity column (Čučnik et al., 2004b). Isolated anti-β_2-GPI are dialyzed against HBS-Ca^{2+}. Concentrations of purified proteins are determined spectrophotometrically (Camspec M501 Single Beam Scanning UV/Visible Spectrophotometer, Camspec Ltd., Cambridge, UK), using the extinction coefficient of 14.0 for the 1% IgG solution. Obtained samples are designated: i.) sample IgGA, 10 g/l, IgG fraction from a patient A with primary antiphospholipid syndrome who suffered from venous thrombosis and is positive for IgG anti-cardiolipin antibodies and anti-β_2-GPI; ii.) sample anti-β_2-GPIA, 0.4 g/l, isolated anti-β_2-GPI from a patient A, IgG isotype; and iii.) sample IgGB, 10 g/l, IgG fraction from a healthy blood donor without antiphospholipid antibodies.

The effects of particular IgG (patients' and healthy blood donor's) are studied on incompletely and completely crystallized ANX A5 on SPBs. SPBs are prepared of 30 % w/w L-α-PS (Sigma-Aldrich, St. Luis, MO, USA) in the mixture with L-α-phosphatidylcholine (Sigma- Aldrich, St. Luis, MO, USA). After the presence of SPB is confirmed, 10 mg/l ANX A5 isolated from human placenta (Sigma- Aldrich, St. Luis, MO, USA) in HBS-Ca^{2+} is injected into the fluid cell of the AFM. When the presence of ANX A5 crystalline shield, incompletely or completely covering the SPB is detected, IgG and/or 0.15 g/l β_2-GPI (purified from pooled human plasma (Čučnik et al., 2004a), in HBS-Ca^{2+}) are added to the ANX A5 solution covering the SPB. ANX A5 concentration is kept at 10 mg/l. The effects of IgG and/or β_2-GPI on crystalline ANX A5 are measured by AFM for another 60 minutes. Results from the experiment where applying antigen-antibody pair before the ANX A5 to SPBs, are also resolved.

5.2.3 Our comments on technical and clinical aspects

When experimenting with isolated anti-β_2-GPI (sample anti-β_2-GPIA), the β_2-GPI-anti-β_2-GPI pair grew on SPBs, but only in the presence of incompletely crystallized ANX A5 (Figure 11). No effect on crystalline ANX A5 was observed, when using the IgG fraction (which contained also anti-β_2-GPI) from the same patient. The observed *in vitro* effect only by isolated anti-β_2-GPI could be explained by different titers of anti-β_2-GPI. Anti-β_2-GPI in sample anti-β_2-GPIA were 10 x more concentrated than in sample IgGA (100 x above the cut off, determined by an *in-house* anti-β_2-GPI enzyme-linked immunosorbent assay (Čučnik et al., 2004b)). Avidity and epitope specificity of anti-β_2-GPI for β_2-GPI in the samples IgGA and anti-β_2-GPIA were identical as a result of using the same polyclonal antibody fraction. However, patient A has determined (by an *in-house* kaotrophic anti-β_2-GPI enzyme-linked immunosorbent assay) high avidity anti-β_2-GPI profile, which should not be neglected, when considering the reasons for ability for growth of β_2-GPI-anti-β_2-GPI pair in the presence of incomplete crystalline ANX A5 on SPBs (Božič et al., 2007; Žager et al., 2011). Since the antigen-antibody complex was unable to bind to SPB when the phospholipid surface was completely covered with ANX A5, we can deduce that the presence of pathological anti-β_2-GPI alone is not sufficient to cause disruption of ANX A5 crystalline shield, and therefore thrombosis. Our results correlate to clinical observations by which not all patients with positive antiphospholipid antibodies develop clinical manifestations and, in the ones that do, the thrombotic events occur only occasionally in spite of the persistent presence of antiphospholipid antibodies. Taken together, our results indicate that the hypothesis of antiphospholipid antibodies (more specifically, anti-β_2-GPI) mediated disruption of the ANX A5 protective shield is possible. However, the pathological potential of anti-β_2-GPI should be considered only as a necessary *"second hit"*, as is also the case with before disguised anti-ANX A5 (Arnout & Vermylen, 2002).

To conclude, we confirmed the proposed thrombomodulatory effect of β_2-GPI- anti-β_2-GPI pair to ANX A5 by *in vitro* model with higher physiological similarity to circumstances in human body, and AFM ability to visualize the interaction in real time and at a nanomolecular scale. Knowledge about molecular interactions between ANX A5 and antiphospholipid antibodies is prerequisite for the improvement of laboratory diagnostics of patients with anti-ANX A5 and a basis for possible new target therapy. AFM confirmed its role as very powerful tool for evaluation of pathological events "in situ", in aqueous environment and on a molecular level.

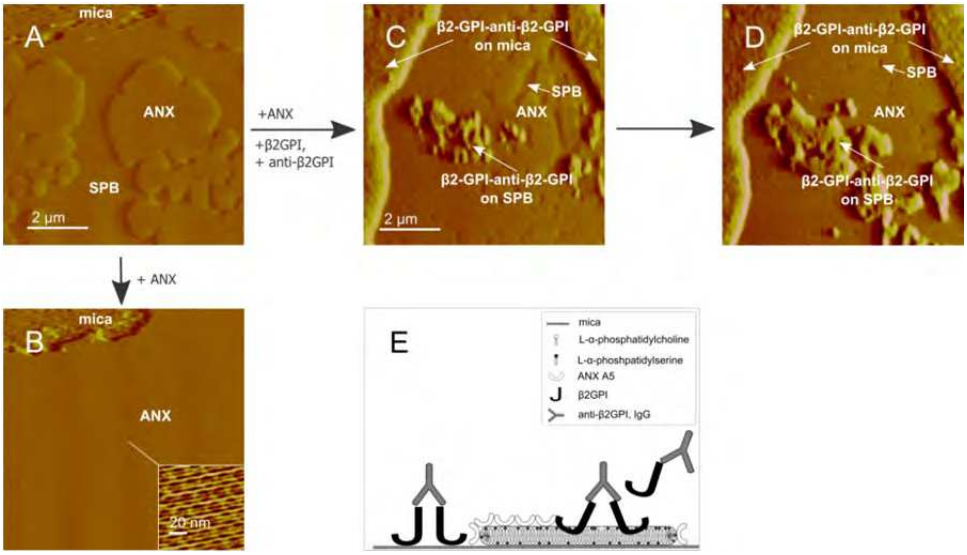

Fig. 11. AFM deflection images and a scheme of the effect of β_2-GPI and isolated anti-β_2-GPI (from patient A, suffering from primary phospholipid syndrome) on crystalline ANX A5 on SPBs. (A) Image of mica and crystalline ANX A5 (10 mg/l in HBS-Ca^{2+}) not completely covering the SPB; (B) after 15 min of incubating the ANX A5 solution (10 mg/l in HBS-Ca^{2+}) over the SPB crystalline ANX A5 completely covered the SPB. The right lower corner shows 100 X 100 nm^2 ANX A5 crystalline structure where p6 crystal form can be observed; (C) however, if β_2-GPI (0.15 g/l in HBS-Ca^{2+}) and anti-β_2-GPI isolated from sample A (0.4 g/l in HBS-Ca^{2+}) were added to the ANX A5 (10 mg/l in HBS-Ca^{2+}) solution covering the incomplete crystalline ANX A5 layer over the SPB, the complexes of β_2-GPI and isolated anti-β_2-GPI on SPB were measured as well. Antibodies and antigen formed complexes with apparently sufficient affinity to bind to SPB in the presence of crystalline ANX A5. The complexes of β_2-GPI and isolated anti-β_2-GPI bound to exposed mica surface as well; (D) image of further lateral growth of β_2-GPI-isolated anti-β_2-GPI patches on SPB in the presence of incompletely crystallized ANX A5 on SPB is shown; (E) scheme of binding complexes of β_2-GPI and isolated anti-β_2-GPI on mica and to SPB in the presence of incompletely crystallized ANX A5. Reprinted and modified from Žager U., Irman Š., Lunder M., Škarabot M., Muševič I., Hodnik V., Anderluh G., Čučnik S., Kveder T., Rozman B., Božič B.. Immunochemical properties and pathological relevance of anti-b2-glycoprotein I antibodies of different avidity. International Immunology 2011;23(8):511-8, with permission from The Japanese Society for Immunology and the Oxford University Press.

5.2.4 Practical instructions

1. In order to study the antibodies, their interaction with antigens, or the consequences of that interaction, antibodies should be microbiologically and physically extra pure. Chemical and biological purity is mandatory when studying the specific interactions. Impurities could make the observations far less specific. Microbiological, physical and chemical extra purity for all other reagents that are present in the experimental model is mandatory as well.

2. In order to ensure physiological relevance of the studied *in vitro* model, naturally derived reagents (phospholipids, proteins) should be used. However, one should consider the change in specificities of antibodies that are being subjected to different isolation procedures (Omersel et al., 2010).
3. When studying the specific interaction between the particular antibody-antigen pair one could use the force mode and different imaging AFM modes (contact, tapping, non-contact, ect.)
4. Monoclonality or polyclonality of specific antibodies should be considered for each experiment separately. Fine paratope heterogeneity and affinity heterogeneity may greatly influence results (in plus or in minus).
5. When studying the structure of antibodies or specific interaction between specific antibody-antigen pairs, concentrations of the reagents should be low.

6. Conclusion

In this Chapter we have described our experiences by AFM, studying protein-protein interactions on the phospholipid membranes in the *in vitro* simulated physiological conditions. Practical suggestions for preparation of SPBs are presented and upgraded with observations of single protein interactions – spontaneous crystallization of ANX A5, agglomeration of β_2-GPI, and antibody interactions. Some interpretation of AFM usefulness for research work with systems of increased complexity are shown – AFM study of specific antibody-antigen interactions on the *in vitro* model of phospholipid bilayer, supported by own experimental data and suggestions for successful work. Biological systems are hardly simulated in the *in vitro* conditions, due to their high level of complexity. However, AFM appears very usable tool in biochemistry which makes multimolecular interactions and micro environmental characteristics of the used models visible and therefore closer to our understanding.

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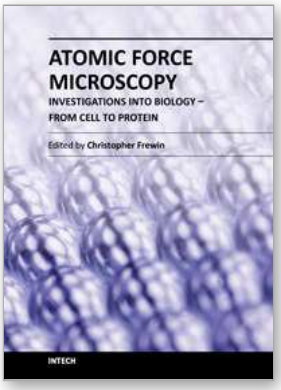

Atomic Force Microscopy Investigations into Biology - From Cell to Protein

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The atomic force microscope (AFM) has become one of the leading nanoscale measurement techniques for materials science since its creation in the 1980's, but has been gaining popularity in a seemingly unrelated field of science: biology. The AFM naturally lends itself to investigating the topological surfaces of biological objects, from whole cells to protein particulates, and can also be used to determine physical properties such as Young's modulus, stiffness, molecular bond strength, surface friction, and many more. One of the most important reasons for the rise of biological AFM is that you can measure materials within a physiologically relevant environment (i.e. liquids). This book is a collection of works beginning with an introduction to the AFM along with techniques and methods of sample preparation. Then the book displays current research covering subjects ranging from nano-particulates, proteins, DNA, viruses, cellular structures, and the characterization of living cells.

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