Biointerface analysis on a molecular level New tools for biosensor research

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Abstract

In the last decade various techniques have been developed to investigate biointerfaces on a molecular level. Here, their impact for biointerface analysis is reviewed with emphasis on biosensor research. In order to demonstrate the power and limitations of local probe methods the imaging and force spectroscopy on single molecules are presented in details.

Keywords: Biointerface; Analysis; Biosensor; Nanotechnology; AFM

1. Introduction

Biomaterials are of great importance for many medical and bioanalytical applications (see Fig. 1). Surface analysis and a better understanding of the interaction with tissues, cells and molecules are crucial in order to improve the functionality of biointerfaces.

Scanning probe microscopy (SPM) methods comprising atomic force microscopy (AFM), scanning tunneling microscopy (STM), scanning near field optical microscopy (SNOM) and related techniques are unique to address individual molecules on surfaces. Especially AFM has frequently been used for imaging immobilized molecules and also for functionality studies. Functional biointerfaces are especially important for biosensing. SPM-techniques can thus be very helpful in order to improve signal transfer, stability and selectivity of biosensors. However, it should be kept in mind that analytical and structuring methods on the nanometer scale are complementary to (bio)chemical methods and do not replace them.

In this paper, recent progresses in biointerface research are reviewed focusing on publications, which are relevant for biosensor research. Emphasis has been put on molecular and nanotechnological aspects, the ultimate point of view to inspect biointerfaces. In the first part (Section 2) micro- and nanoscale preparation and characterization methods for biocompatible surfaces are presented and as an example, in vivo biosensors are discussed. In the second part (Section 3) problems

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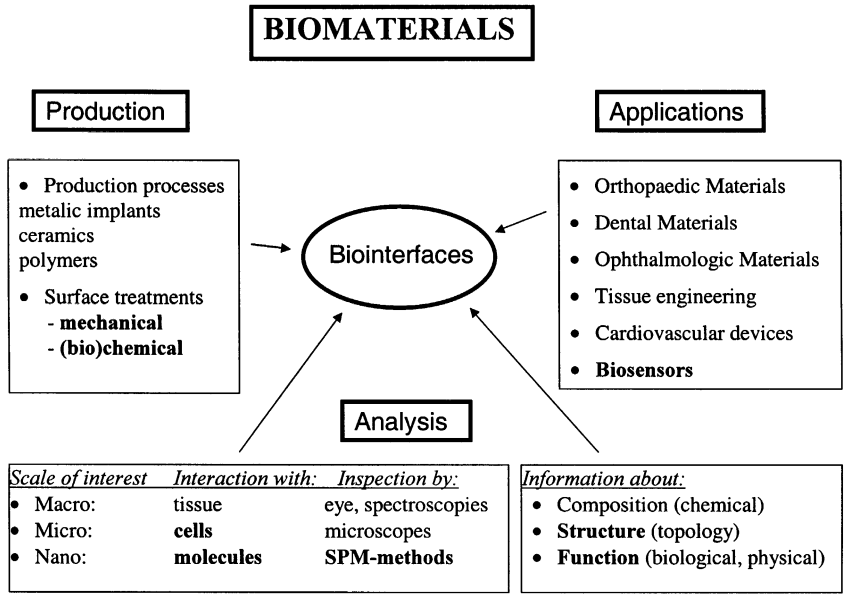

Fig. 1. Biointerfaces as an important aspect of biomaterials. Aspects addressed in this review are in bold.

relevant in biosensor research are addressed, the advantage of a molecular view and the benefit of applying nanotechnological tools are presented. In Section 4 an overview is given on state-of-the-art in molecular analysis using SPM-technique. After addressing technical questions, the achievements in the imaging of biomolecules are briefly reviewed (for more detailed information regarding molecular imaging see other contributions in this issue). Then, force spectroscopy is outlined in details in order to illustrate the power and limitations of SPM-techniques to characterize biorecognition processes by investigating single molecules. Finally, the contribution of these new methods for biointerface science and technology is shortly discussed.

2. Biointerfaces

The field of biomaterials comprises the production, analysis and applications of materials in bioscience, and mainly in medicine (Fig. 2). The surfaces of such biomaterials, the biointerfaces, are important in many applications. Biocompatibiliy in the narrow sense means prevention of adverse reactions, in a wider sense promotion of specific effects achieved by a suitable functionalization of the material. Especially for in vitro cell cultures and for in vivo implants — including biosensors — long-term stable functionalized biointerfaces are needed. Nanotechnological methods became important for the investigation of material surfaces and of processes occurring thereon. In this chapter we will first focus on preparation methods for functional biointerfaces with emphasis on biosensors. The current available methods for surface characterization will

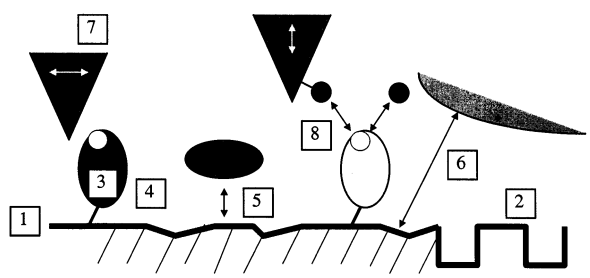

Fig. 2. Biointerfaces and processes at nanometer scale. (1) Topography, (2) Nanostructuring, (3) Molecular design, (4) Immobilization, (5) Biofouling, (6) Cell–material interactions, (7) Imaging, (8) Inter- and intramolecular forces.

then shortly be discussed, in order to allow a critical assessment of the contribution of SPM-techniques. Further, a review of micro- and nanostructuring is given to illustrate the potential of these preparation methods for research and future applications. Finally, a short overview on implantable sensors is given.

2.1. Biointerfaces preparation techniques

Since at least three decades immobilized ligands have been used for protein purification, and these experiences in affinity chromatography have turned out to be an important basis for biosensor research [1]. Whereas an intimate contact of the signaling biomolecule to the material surface is essential for electrochemical detection [2], the robustness towards regeneration procedures and a low non-specific binding (NSB) [3] are two important issues for all biosensor types. Physisorption of the recognition element onto surfaces may result in a low long-term stability and a loss of activity. Therefore, a stable functional surface can best be achieved by a direct covalent immobilization [4,5] of the molecules to a surface, by using self-assembling monolayers (SAM) or by a coupling to polymers.

Glass and silica are the most common materials for optical and in some cases also for electrochemical sensors and various silanization procedures have been reported [6–12]. Crosslinkers connect the biomolecules to the immobilized silane which are often functionalized by an amino- or a thiol-group. Bifunctional crosslinkers [13], combining two different reaction specificities in the same molecule, are more effective [14], since the intramolecular reactions of the target molecules to be conjugated can be prevented. Alternatively, photoactivable linkers [15] allow the immobilization on various materials in a homogeneous layer [16]. The chain length of the linker is very important to allow sterically unhindered biorecognition [17]; an increase of the chain length by two additional carbon atoms resulted in a higher flexibility concomitant with an increase of the sensor signal by a factor seven [18]. The importance of an oriented immobilization [19,20] of the first molecular layer has impressively been demonstrated: The photosynthetic reaction centers on a graphite electrode [21] yielded the extremely high quantum efficiency of 60% when using a conductive linker, but only if the protein complex was immobilized in the correct orientation.

Using gold as material for biosensors, a stable immobilization through thiol-groups is achieved [22]. A quasi covalent bond is formed and this simple technique can be utilized for a localized immobilization of proteins what is required in some cases [23]. An ordered and densely packed SAM is formed when molecules have an alkane chain length > 8 [24]. Based on STM-images it could be shown that the molecules are tilted and twisted with respect to the surface normal [25]. SAMs formed by thiolalkane endowed with a negatively charged end group protect the surface from non-specific binding and additionally provide a functional group for a coupling of antibody molecules [16,26–28], chelators for His-tag binding [29,30] or artificial recognition molecules [31]. Electric conductivity of individual protein molecules has been achieved by platinization of glucose oxidase, immobilized to a SAM film on gold [32]. Nano-objects of 10-nm diameter consisting of platinized photosynthetic active systems have been imaged by using scanning tunneling microscopy (STM) [33]. Binding of streptavidin molecules to SAM [34] provides a molecular anchor for any biotinylated molecule. The unique feature of the avidin–biotin system [35] has been utilized since about 20 years in bioanalysis [36] and is also suitable for electrochemical [37,38] and optical biosensors [39,40]. Furthermore, gold evaporation on a molecular sieve matrix [41], on a nylon membrane [42] and on PVC [43] yielded an easy production of electrodes. Gold on quartz surfaces, functionalized with a thiolated-ganglioside, allows the specific detection of cholera toxin by a quartz crystal microbalance sensor [44].

Another approach is to create a thin polymer layer on a sensor in order to increase the surface density of the signaling molecules. Conducting polymers (see a recent review [45]) such as polyvinylpyridin functionalized with Os-complexes [46], polypyrrole [47], polytyramine [48],

polythiophen [49], PEG-vinylferrocene [50], crosslinked avidin–biotin-enzyme [38] or silk fibroin [51] create wired networks and are suitable as matrices for redox enzymes. Using AFM, high-resolution images of dextran polymers have been presented [52]. Alternatively, enzymes can be entrapped in nanopores of gel-sol materials [53,54]. In all these cases the diffusion of small molecules is still possible whereas the docking of big antibody molecules to the corresponding immobilized antigen is hindered. For multistep enzyme sensors ordered crosslinked protein layers have been developed [55].

2.2. Characterization on a molecular level

Biosensor surfaces are characterized in order to get insights into molecular processes, resulting finally in improved procedures of production. Three kinds of information can be obtained (see Fig. 1): chemical information to confirm that the molecules are immobilized, structural information providing information of the surface topology, and functional information about biorecognition and signal transfer. Many different methods are available for surface characterization, which can be classified in ion (mass) spectrometry, electron spectroscopies, optical methods, X-ray techniques and proximal probes (SPM) [56]. Spectroscopic methods provide information about micrometer spot sizes and are based on ensembles of molecules. The presence of immobilized silane molecules for instance can be confirmed by X-ray photoelectron spectroscopy [57]. By using radiotracer methods a surface density of 1.2×10^{12} covalently bound molecules per cm^2 was verified [58] corresponding to one complete monolayer. However, as outlined above, characterization of the biointerface down to the molecular scale [59] is required in order to improve molecular interactions (Fig. 2). The potential of SPM methods to obtain information of molecular resolution was recognized already in the early years after their invention [60,61]. Comprehensive overviews on methods for biointerface analysis [62], especially on SPM methods [63,64] have been given. Thus, in the last years, SPM has been used to image immobilized antibody molecules on surfaces of biosensors [65–69] or microtiter plates [70]. A limit of 0.3 pg mm^{-2} for the micromechanical detection was reported [71]. When combining reflection interference contrast microscopy with AFM, a detailed understanding of protein–ligand interaction can be achieved [72]. Furthermore, electron transfer reactions in biomolecules have been studied in model systems using scanning electrochemical microscopy [73,74].

Using functionalized tips, information about the chemical properties at a resolution of about 0.5 nm [75] has been obtained when the so called lateral force scanning technique [76] is applied. These images allow discrimination of hydrophilic and hydrophobic molecular domains, respectively [77]. When protein A is adsorbed on mica at increasing concentrations, the density changes from a monolayer to a quasi-bilayer of protruding molecules as confirmed by AFM and surface force experiments [78]. Recently, very detailed information of surface properties were reported by using functionalized AFM tips: the chirality of immobilized molecules was identified [79] or the protonation state of immobilized amino-groups was investigated by a direct titration [80].

The presence of a molecule does not yet mean that it is still functional. Protein may lose their binding capacity upon adsorbing on a surface [81]. The quartz crystal microbalance is a sensitive device to monitor binding processes [82,83], but local information is obtained only in combination with SPM methods [84]. It has been shown that AFM allows measuring the functionality of single molecules [85] and the combination of imaging with the monitoring of binding processes is very promising [86]. The achievements in measuring the functionality of individual molecules, which go beyond biosensor applications, will be discussed below in Section 4.3.

2.3. Structuring in micro- and nanometer dimensions

Patterning of surfaces for DNA-analysis has been developed in the last decade by several groups and this technique has already reached a broad commercial level [87]. Patterning can also be relevant for studying the interaction of living

cells with surfaces or for biosensors and biointerfaces [88,89]. Thus, miniaturization of an optical immunosensor based on micropatterned antigen arrays has been proposed [90]. In that work, gold surfaces with micropattern of thiol-compound linked IgG have been analyzed using AFM. A further important application of chemically microstructured surfaces is the new field of neural networks. There, a biocompatible surface is mandatory for the neuron cultures. By generating lines of polylysine [91], lamine, RGD-sequence containing peptides [92] or neural adhesion proteins [93], neurite outgrowth can be guided into a predefined direction. Inkjet and micomechanical spotting devices are routinely used to deposit molecules in micrometer sized arrays. On a research level two further methods are mainly used to generate patterns on surfaces with nanometer scale: photolithographic patterning and micro contact printing.

Photolithographic methods have been developed to achieve guidance of neurons [94,95] and other cells [96]. Immobilized organic molecules or the robust DNA macromolecule [97] can tolerate solvents used in photolithographic processes. Proteins, however, have to be protected [98] in order to avoid their denaturation. The precise alignment of masks allows the generation of complex patterns of two proteins [99], also on surfaces, which are 3D microstructured. Immunofluorescence microscopy and AFM techniques are both useful [100] in order to verify the functionality of immobilized proteins. Patterns of biomolecules can directly be generated using the laser photoablation technique [101]. Electron beam lithography can achieve smaller structure sizes than light lithography [100,102].

Simpler than photolithography is the so-called soft lithography [103], a synonym for micro contact printing. By conventional lithographic techniques a negative of the intended microstructures is generated usually on a silicon wafer. The PDMS prepolymer is poured onto this negative microstructure and a positive stamp is made thereof [104]. To the microstructured PDMS stamp organic molecules [105,106], polymers [91] or proteins [107] are added which can be stamped even on curved surfaces [108]. A resolution below 100 nm can be achieved by micro contact printing [103], and as few as 1000 individual protein molecules, which still are functional, can be transferred by stamping [109]. An approach to produce nanostructured gold features on silicon is to pattern thiolalkanes on a thin gold layer by stamping, followed by a dry etching process [110]. Alternatively, nanoparticles have been utilized as a mask, followed by gold evaporation and lift-off process resulting in a thin gold layer with nanosized openings [111].

Inspired by nature [112], a step further towards molecular construction has been proposed already 10 years ago: molecular self-assembly for the synthesis of nanostructures [113,114]. Tri-block polymers spontaneously form nanostructures [115]. Alternatively, microstructures can induce controlled assembly of molecules [116]. STM can also be used as a nanotool for local etching or material deposition [117] or for the manipulation of atoms [118]. Methods for the manipulation and imaging of individual molecules by AFM [63] or STM [119] are currently developed and even the conformation of sugar molecules could be imaged [120] or induced [121].

The creation of nanomachines is a dream of today's nanotechnologists [122,123]. Together with the bottom-up approach of supramolecular chemistry [124] designed nanostructures on surfaces for sensing and further applications will probably become possible in the future.

2.4. Implantable sensors

Biocompatibility is especially important when long-term stability in the organism for months up to years is desired, as for implantable glucose sensors used for diabetes patients [125]. Ten years ago a breakthrough for in vivo sensors has been expected soon [126]. However, despite investigations of several problems such as sterilization [127], toxicity [128] and encapsulation by connective tissue cells [129], there is still no satisfactory solution for in vivo sensors. Adsorption of serum proteins is the first step in the inactivation process of a sensor surface. This can be prevented by polymers coatings [130–132] mainly poly(ethylene) glycols (PEG) [129,133,134]. Plat-

inum [135,136], glassy carbon and gold can serve as a material for electrodes. The later turned out to be best in a 45 months in vivo experiment [137]. Gold is also an unique material for a modification with short chain thiolated oligo(ethylene) glycol [138,139]. Analysis of gold surfaces on a nanometer scale [140] provides information about the roughness which correlates with the impedance. Rather than reduction of non-specific binding, biocompatibility will signify in the future the introduction of specific functions [141] to trigger intended biological responses as, e.g. healing pathways [142]. Analysis and improvement of biocompatible surfaces on the macro- and nanometer scale will also promote the development of implantable biosensors.

3. Molecular nanotechnology and biosensor research

Biosensors are constructs consisting of biomolecules, transducers and instrumental components designed for the effective detection of the substance of interest, which is usually present in aqueous solutions. Thus, the realization of biosensors is a complex and long-term endeavor and many technical barriers have to be overcome [143] for a successful commercialization [144]. The most challenging technical hurdle is not to find a specific recognition element, a sensitive detection method or to construct a suitable instrument; it is to combine all elements in an appropriate way to build up a functional analytical tool. The three major characteristics of sensors are specificity, sensitivity and stability. Specificity — which can also be a selectivity for a whole class of compounds — is given by the recognition element. This is in most cases a protein: an enzyme, an antibody or a membrane receptor. Sensitivity is firstly determined by the characteristics of these binding elements and secondly by the effectiveness of signal transfer and amplification. The transfer of the signal from the recognition element to the surface concerns the core of biosensing, the transducer. Thus, the interaction of the involved molecules with the material surface is of central interest in biosensor research.

3.1. A molecular view of biosensors

In order to improve biosensing interfaces, design and analysis on the molecular level have been proposed [145,146]. This basic approach is paraphrased by several terms: (a) molecular architecture or integrated molecular systems [147] to emphasize an appropriate arrangement of the building blocks in order to achieve an optimal interaction; (b) supramolecular interfaces [148], which focus on molecular assemblies and their interaction; (c) nanotechnology [149,150] when the surface structure [151] of the biosensor is of central interest. Depending on the point of view, one of the above expression may be used. All are addressing biointerfaces in molecular dimensions and thus can be summarized by the term 'molecular nanotechnolgy'. In the next sections an overview on the recognition elements, and their interaction with the surface will be given.

3.2. Design of recognition elements

The most prominent recognition elements are enzymes and antibodies. These functional proteins are extracted from biological material or are increasingly produced in vitro. Recent progress in biosciences, especially in genetic engineering, allows us to design and produce sufficient amounts of tailored antibody constructs for biosensors [152]. Thus, rational design of proteins [153] is very promising to generate recognition elements with improved properties, i.e. better specificity or sensitivity. The sensitivity of an assay is directly related to the affinity of an antibody towards its antigen or to the turnover of an enzyme. The mentioned improvements of molecular characteristics are relevant also in other analytical applications such as immunohistology, ELISA and in vivo radiodiagnostics. In practice, optimization of the recognition element is the first critical prerequisite in biosensor development and the efforts required are often underestimated. The redox enzyme glucose oxidase is very robust and thus a popular recognition element for a demonstration of improvements in biointerfaces and detection methods. In the future, artificial peptides, which are conjugated to redox active porphyrines as

prepared for energy conversion purposes [154], may be useful for electrochemical biosensing. Fusion proteins, which combine the recognition function with an enzymatic signal generation and/or amplification, could be considered as an integration of two complementary functionalities on a (sub)nanometer level. In summary, the optimization of molecules is of pivotal importance, since the critical length of electron transfer in proteins lies in the range of 0.1–1 nm [155], which can not be achieved by nanostructed materials alone.

3.3. Signal detection systems for biosensing

The importance of immobilization procedures for achieving a stable and functional interface has been outlined above. The immobilized recognition element recognizes a compound according to its specificity. In biosensors, a signal has to be generated which is related to the concentration of the analyte in the sample. The two major detection systems are based on optical or electrochemical methods. Alternatively, piezoelectric devices [156] and field effect transistors (FET) have been developed for detection purposes. Since molecular dimensions are most critical for electron transfer mechanisms, amperometric detection is discussed here in more details.

Electron transfer from the biomolecule to the surface is very complex [149] and relies not only on the conductive properties of the recognition element as mentioned above, but also on the immobilization [147], the sensor surface preparation [157,158] and the electrochemical detection method. Since the pioneering work of Hill's group [159,160] in the eighties, it is known that a direct contact of the redox group with the surface is required in order to achieve a direct electron transfer. In the last decade many groups developed model systems for biosensors [161–169] based on a molecular design of biosensor surfaces. Different biomolecules, immobilization procedures and electrode materials have been used, but in all cases the biomolecules involved in the electron transfer have been brought in close contact to the conductive surface. Commonly, nanotechnological methods produce regular features, which will not be required to facilitate electron transfer. Rather a high flexibility of the macromolecules and of the linked mediators [170,171] is necessary. In other words, free motion of critical moieties is often more important for functional supramolecular arrangements than nanostructured features.

3.4. Prevention of non-specific binding

A high specificity of the recognition element does not strictly result in an analyte specific response due to the interference of foreign molecules, in particular of proteins, with the sensor surface. In label-free optical sensors NSB of proteins results in an increase of the signal, whereas in electrochemical sensors the electron transfer is disturbed and the response signal becomes weaker. Biofouling is thus a serious problem for sensors when analyzing real samples, which contain a plethora of unknown interfering substances. Satisfactory solutions to this problem are difficult to find [172,173]. However, after preparation of a silanized surface NSB can be reduced by Tween 80 rinses [174]. Non-ionic detergents are commonly used in bioanalytical assays and can also decrease NSB in biosensors [132]. The total charge and the charge distribution on the molecule seems to be the major determinants for protein adsorption on surfaces [175] and short range interactions between molecules and surface have been simulated [176]. Negatively charged polymers as Nafion [177] and a polymeric mercapto-silane derivative [178] protect enzyme sensor surfaces from interfering molecules. Similarly, short chained negatively charged thiol-compounds immobilized on gold can drastically reduce NSB to gold electrodes prepared for electrochemical immunosensing [168]. Thiol-silanes used for coupling reactions can be converted to protein repellent sulphonated-silanes by UV irradiation in an oxygen atmosphere [179]. Alternatively, hydrophilic PEGs have been evaluated which are well known in medical applications to prevent protein adsorption. Ordered short chained PEG-derivatives immobilized on gold effectively prevent NSB [138,180], whereas unordered PEG-chains protect surfaces [181] against NSB effectively only when they are long [182].

The adsorption of a suitable protein on a surface can promote cell adhesion. Tailored surface has been generated, which are cell-attractant or cell-repellent, respectively [24]. Such surfaces are characterized by contact angle measurements [183] and adsorbed proteins have been visualized by using SEM [184]. In addition to topographic information [185], AFM can provide information about adhesive forces of adsorbed proteins [186].

4. SPM for molecular analysis

SPM techniques are frequently used to investigate surfaces prepared for biosensing or implantable materials as outlined above. In the second part of this review we will present recent achievements in the investigation of immobilized molecules while emphasis is put on the structure and function of individual molecules. SPM techniques can provide a direct insight into the molecular topography at physiological conditions. Furthermore, the measurement of the interaction between molecules becomes possible. The progress in the last years in nanoscience and nanotechnology activities allow us to judge the potential and the limitations for practical application of SPM techniques in the future. Therefore, the state of the art in imaging and force spectroscopy is reviewed. The preparation methods for samples and the modification of the tips are crucial and will be discussed first.

4.1. Preparative work

4.1.1. Sample preparation

A stable fixation of biomolecules on a flat surface facilitated the investigation by SPM in aqueous solution in most cases [187]. Surface roughness below the diameter of the molecules are helpful [188]. Different materials turned out to be suitable as substrates: mica [189], gold [190], glass or sapphire [191]. Mica is a natural mineral, which exhibits atomically flat sheets when cleaved. Covalent immobilization of proteins on mica [192] can be carried out using silanization methods analogous to silicon [151]. DNA is passively adsorbed on negatively charged mica whereby Ni (II) or Co (II) ions at a 1 mM concentration are needed [193]. Lipid vesicles spontaneously adsorb on mica forming a SAM of a lipid membrane [194]. Ultraflat gold surfaces can be prepared by using mica as a template [188]. The so-called mica template–stripped gold [195] has facilitated the immobilization of biomolecules since thiol-functionalized compounds bind strongly to gold allowing a quasi-covalent immobilization of molecules. Imminothiolane modified catalase [196] binds directly to gold and has been imaged by STM. Alternatively, a heterobifunctional crosslinker with a disulphide-group is first bound on gold followed by the addition of catalase molecules [197]. Analogously, chemically activated cysteine molecules bound on gold reacts with added IgG or cytochrome c [198]. Such small thiol-compounds do not form a dense and ordered SAM and binding of small molecules to gold is still possible [199]. Therefore, the long chain dithio-undecanoic acid, activated with *N*-hydroxy succinimide [200], was used forming a complete SAM on gold, which is able to react with amino-groups of added proteins or with other amino-group functionalized compounds.

In order to achieve a lateral resolution below 1 nm not only a stable immobilization of the protein molecules is required, but also a optimized buffer composition [201], which favorites short range forces [202].

4.1.2. Tip preparation

Cantilever and tip essentially determine the quality of the AFM data, i.e. the image or force resolution, respectively. Quality aspects of the cantilever concern micromechanical properties as well as surface chemistry of the tip apex, which comes into direct contact with the sample. Microfabrication processes of cantilevers made of silicon, silicon nitride [203] or polymers [204] are well established and cantilevers are commercially available in various shapes [205,206], spring constants and coatings [207,208].

Sharp tips are preferentially used for imaging, because the tip shape convolutes with the surface features [205] and determines the lateral resolution [151]. Furthermore, the measured interaction forces are depending on the surface chemistry of

the tips [209,210]. Analogously as described above, tips can be coated with gold in order to achieve a SAM with various functional end groups. For the investigation of biorecognition processes, tips have been functionalized with biotin [211], the antigen fluorescein [199] or DNA [212].

4.2. Imaging of biomolecules and cells

4.2.1. SPM methods

The invention of AFM [213] was a breakthrough for applictions of SPM techniques in biology. Several years ago it became possible to image samples in liquid environments with atomic resolution [214]. Soft samples like most biomolecules are deformed by applying a load of some nN what initially was assumed to be required to achieve a topographic resolution in the nanometer range [215]. Therefore, non-contact [216] or tapping mode [217] techniques [218–220] have been developed in order to prevent deformation or destruction of the biological samples with the aim of subnanometer resolution [221]. Atomic resolution could be achieved in the imaging of a calcite (bio)material [214].

Biological samples need an aqueous environment [222,223] in order to preserve their structure and — related to that — their functionality. It has been recognized early that the solvent composition strongly influences the image resolution of soft samples [224]. Imaging at different salt concentrations allows to filter out the topographic and electrostatic contributions, and the surface charge of bacteriorhodopsin molecules could be visualized [225]. If sophisticated sample preparation and the detection modes [224–228] are used, detailed information about the surface chemistry can be obtained. Finally, a combination of AFM with other methods like SNOM [229] or patch clamp techniques [230] could promote the understanding of processes on a molecular level.

4.2.2. Imaging DNA and lipids

DNA is a very large and robust molecule and it has therefore been investigated by AFM already a decade ago. However, the first experiments resulted in low resolution images mainly due to unsuitable immobilization techniques [231]. Covalent immobilization of modified DNA fragments on gold [232] or improved adsorption procedures on mica using cations [193] resulted in a much better resolution revealing substructures of the linear DNA molecules. When a bacteriophage DNA on aminosilane treated mica was dried [233], the length of the macromolecule did not change, whereas height and width appeared a factor of two larger in water than in air. Considering control experiments the authors concluded that the chemical factors involved in adhesion dominate AFM images quality [234]. All these findings show that the image information strongly depends on both, the preparation and the scanning method.

Recently, functional imaging was demonstrated combining height information with adhesion forces of specific DNA-interactions [235]. Intercalating ethidium bromide increase the flexibility in dsDNA resulting in altered force–distance (F/D) curves [236]. In the future the visualization of DNA–protein interactions [237] will be of interest in order to investigate genetic regulation processes. In these cases a loose binding to mica by using Mg^{2+} is indicated in order to allow dynamic binding processes.

Lipids are relative small and flexible molecules. In SAMs they stabilize each another and can thus be imaged by AFM [238]. Using Au (111) and a SAM of thiol-alkanoic acids, a well-defined hexagonal structure is seen for a C-18 chain length, whereas C-6 long molecules were less ordered [239]. SAMs of artificial thiolipid on gold appears as star-like domain structures [240]. Cardiolipids are more complex molecules and as a consequence, irregular but still repetitive features can be detected [241]. Thus, AFM can visualize defects or irregularities in micro- to nanometer dimensions in SAMs which can not be detected by analytical methods averaging over large areas as infrared spectroscopy or contact angle measurements.

4.2.3. Imaging proteins

Proteins are the most interesting molecules in biosciences, since they fulfill the essential functions in life: they form the intracellular and extra-

cellular structural backbones and are catalysts and switches for metabolic processes. Thus, the structures of ten thousands of different proteins are of central interest in biology and medicine and structural biology is a fast growing area. The most important tool to elucidate protein structures are X-ray diffraction of protein crystals and 2D-NMR. In the last 10 years AFM has alternatively been used to investigate protein assemblies at physiological conditions. This AFM information can confirm data obtained from protein crystallography and may also provide new insights.

In first attempts, biological samples were metalized before imaging using STM [242]. However, AFM instruments and methods were developed fast and a review early in the nineties already mentioned a long list of AFM investigations on proteins [243]. The pentameric structures of acetylcholine [244,245] and pertussis toxin [246] receptor known from crystallography and SEM studies were confirmed and bacteriorhodopsin on mica was shown to form a regular layer whereby individual molecules could clearly be delineated [247]. The bacterial cell surface layer (S-layer) forms a crystal like structure with pores of 4-nm diameter as expected from scanning electron micrographs [248]. Single large molecules of the intracellular protein transport system were covalently bound on mica and the Y-shaped structure was found as predicted by electron microscopy [189]. Attempts to estimate the molecular weight of proteins based on the dimensions determined using AFM has been reported [249]. Peptide [250] and peptide assemblies [251] on surfaces, the formation of insuline dimers [252] or hexameric insuline–Zn^{2+} crystals were visualized [253]. A lateral resolution of 5 Å is achieved for well fixed proteins [254] and single flexible protein loops in bacteriorhodopsin molecules were identified [255].

The uniqueness of AFM lies in the possibility to perform dynamic studies of single molecules at physiological conditions. Abalon is a protein, which is involved in biomineralization, and its adsorption mechanism was investigated [256]. In an other dynamic investigation, the growth of amyloid structures could be monitored in real time [257]. Structural changes induced by the addition of effector molecules can be seen in situ: the chaperon GroEL complex with the substrate protein looks different after addition of ATP [258] and the activity of single enzymes at work could be observed [259]. In the center of the huge nuclear pore membrane protein complex, openings appear by the addition of Ca^{2+} and this process was reversed by adding a scavenger for calcium [260].

4.2.4. Imaging living cells

Most fascinating is the observation of living cells at work with AFM: after the addition of a virus to kidney cells, protrusions appeared for several minutes and disappeared again [261]. By applying a micromechnical load through the AFM tip to a cell membrane the local viscoelasticity has been sensed [262,263]. Since glutaraldehyde-fixed cell membranes are more rigid, the image resolution is better than from living cells [264]. The cell membrane is damaged when relatively high forces are applied resulting in a biofouling of the tip [265]. However, with forces as low as 10 pN [266] a resolution of 10 nm can be achieved and granular features can be identified as single protein complexes [267]. Furthermore, intracellular organelles were localized by imaging through the membrane [268]. In order to localize the much smaller calcium channels in a chick ganglion cell membrane, toxin conjugated gold nanoparticles have been added which bind to the corresponding subunit of the channel [269]. Finally, human blood cells have been investigated: the shape of platelets at different stages of activation was imaged [228] and the spectrin meshwork of the cytoskeleton from erythrocytes ghosts after various treatments could be compared [270].

4.3. Force measurement using AFM

The power of AFM methods lies in the fact, that not only image of high resolution can be generated, but that actually forces can be measured [271] at a single molecule level. A surface force apparatus is available for probing surface interactions with high sensitivity [272–274]. However, the results are based on statistical calculations from a high number of molecules and inhomogenities are averaged. SPM techniques

provide local information from areas of some hundreds of nm^2 down to individual molecules [275].

After an overview on the interpretation of surface–surface interactions, some specific protein–ligand systems will be discussed, especially the avidin–biotin and antibody–antigen interactions. Finally, results of intramolecular unfolding processes and DNA–DNA hybridization forces will be presented.

4.3.1. Measurement of unspecific forces

Surface–surface interactions are complex and can be described by the DVLO theory [276]. This theory integrates the influence of two major long-range forces, the van der Waals and electrostatic double layer forces. For a hard sample experimental data and simulations are often in good agreement. However, force–distance curves from complex and soft biological samples are more difficult to interpret. For instance, short-range hydration processes below 1 nm show an oscillation of forces with a periodicity of 2.5 Å, which is beyond the DVLO theory [277]. In SPM-measurements the tip surface probes the surface of the investigated substrate and the following forces has to be considered [278]: van der Waals, capillary, capacitive, attractive, repulsive and magnetic. In air, capillary forces are dominating due to the present of moisture [279]. Attractive forces are observed when carboxyl groups are present on the tip as well as on the sample. When methyl groups are present on the tip and carboxyl groups on the sample or vice versa, the attraction is much weaker [76]. Depending on the material and the solvent composition both, attractive or repulsive forces, can occur. For instance, long-range interactions of colloidal polystyrene microparticles are attractive in distilled water and repulsive at 1 M KCl and a Debye length of 7 nm was obtained at a 1 mM salt concentration. Non-contact methods are best suited to sense attractive forces [280]. Since the investigated area can be very small, properties like elasticity [281], adhesion or charge density can be mapped with a high resolution [282]. When the friction mode is applied such properties are scanned and the generated image contrast is related to the chemical differences on the surface. In a similar way the surface charge density of lipids or membrane proteins can be mapped [282].

Unbinding force values are determined from F/D curves when the tip–surface interaction is ruptured and these values can be used for analytical purposes [283]. These curves provide additional valuable information, such as the rupture distance and stretching behavior of a polymer [284,285], which allows the investigation of biomolecular interaction processes on a single molecule level [286] as discussed below [287]. Hydrogen bridges play an important role in protein–ligand and especially in DNA interactions. Based on AFM measurements a force value of 16 pN was calculated for a single hydrogen bridge [288]. Finally, the measured unbinding force values correlate with the applied forces, via increasing the so-called loading rate [289]. When the cantilever is retracted from the surface very slowly in the range of the thermal dissociation time, the measured forces go to zero, since the unbinding process occurs then spontaneously. When the cantilever is retracted faster, the forces increase logarithmically with the pulling speed. The dependence of measured forces from the loading rate, the dynamic force spectroscopy, provides an energy landscape, which characterizes binding process [290].

4.3.2. Binding forces of avidin–biotin complexes

Avidin is a glycoprotein from chicken egg-white and has an unusually high affinity constant for its natural ligand biotin (vitamin H). The 3D-structure of the tetrameric protein is known [291] and biotin is bound in a deep binding pocket. The analogous protein streptavidin originates from *Streptomyces avidinii* and by genetic engineering techniques point mutants have been produced by rational design [292]. Due to their high binding 'strength' avidin and streptavidin together with biotin provide an ideal model system for force spectroscopy [293].

Unbinding force values can be compared directly only when the same loading rate was applied [290]. An unbinding force of 160 pN has been determined for a single avidin–biotin interaction [294]. Accurate measurements of individual interaction events require careful preparation: the

binding partners should be stable immobilized at a low surface density. In early experiments biotin has been conjugated to the carrier protein BSA, which was adsorbed to a glass microsphere glued to a tip and to the support mica, respectively. After addition of the bridging strepavidin, which binds both, biotin molecules attached on the sample and on the tip, the rupture forces were measured and the histogram of the force values showed a peak around 300 pN [295]. When streptavidin was adsorbed on a microtiter plate, forces of 409 ± 166 pN were measured [296]. The high standard deviations in both mentioned systems can be explained by the assumption that two interactions are frequently broken simultaneously. When Poisson statistical analysis methods are applied to the measured data, the biotin–avidin force value was calculated to 173 ± 19 pN, and for streptavidin–biotin to 326 ± 33 pN when applying relatively high loading rates [297]. For the biotin–avidin complex thermodynamic parameters have also been measured [298]. The unbinding force values were then measured using force spectroscopy and are 160 ± 20 pN for avidin, and 257 ± 25 pN for streptavidin. A good correlation of the unbinding force values to the energy change was found, indicating that the unbinding process is adiabatic and entropic changes may occur after the unbinding process is finished. Based on a series of mutant molecules from streptavidin, which differ in their enthalpic and entropic values, other investigators concluded that AFM force measurements directly probe the enthalpic activation barrier of ligand dissociation [292]. The unbinding process of the streptavidin–biotin complex has also been simulated [299] and a rupture mechanism was postulated which involves at least five steps. Hydrogen bridges stabilize the complex and steric restraints seem not to play a crucial role. It should be kept in mind that molecular dynamic processes are usually simulated for a time frame of picoseconds whereas the AFM-measurements occur in milliseconds. Therefore, experimental AFM data cannot be directly extrapolated to the time frame of molecular motions processes [300].

4.3.3. Binding forces in antigen–antibody systems and other biospecific interactions

More relevant and typical for ligand–receptor interactions than the avidin–biotin system is the binding of an antigen to the corresponding antibody. Although antigen–antibody complexes have about a 10^6 times lower affinity constant, unbinding forces should be detectable. The early investigations showed a broad distribution of force values up to 400 pN with some small peaks in between allowing to calculate a single rupture force to be 60 ± 10 pN [301]. When a flexible PEG linker for the attachment of a divalent Fab fragment is used, a symmetric distribution of forces was obtained allowing to determine more precisely the unbinding force value to 244 ± 22 pN [302,303]. However, this value seems to be too high for a single binding event; the analysis of the force spectrum for another antigen–antibody pair provide a periodicity of 49 ± 10 pN [304] when multiple binding events occur. A low detection sensitivity leads to high unbinding force values [305]. The best precision is achieved when antigen and antibody molecules are stable fixed. Monovalent single chain Fv fragment molecules are the minimum binding structure of an antibody, which still have the full binding capacity. They can be directly immobilized on protected gold via a C-terminal cysteine at a low surface density in the correct orientation. Using a tip which carries covalently attached antigen molecules via a long PEG linker, a Gaussian distribution of unbinding force with a mean of 50 ± 4 pN has been determined at a medium loading rate of 5 nN s^{-1} [199]. By genetic engineering several mutants have then been produced which varied in their dissociation constants. From all these mutant proteins kinetic and thermodynamic parameters have been determined and the unbinding forces were measured. It could be shown that the unbinding force correlates only with the off-rate of the antigen–antibody complex [306]. Since the reciprocal off-rate corresponds to the half-life time of a complex, force measurements can probe directly the expected lifetime of a complex.

Antibody molecules can be identified morphologically [68] in surface profiles and the use of conjugated gold nanoparticles improves the con-

trast significantly. It has been demonstrated that imaging and force spectroscopy can be combined [307]. Topography and force signals were simultaneously acquired by measuring the reduction of the oscillation amplitudes caused by binding events [308]. All these examples show the power of SPM methods for a better understanding of molecular processes, which also provide images of functionalities [302].

The adhesion of cells on target molecules is essential for many biological functions. Proteoglycans mediate cell interaction in marine sponges, a simple model for a multicellar organism. An adhesion force of 400 pN between two molecules has been determined [309], which was dependent on the presence of Ca^{2+}. Another typical cell–molecule contact occurs when leucocytes are stopped on activated endothelium before they can pass through the endothelial cell layer. The intrinsic molecular properties of the involved P-selectin/ligand interaction and the kinetic of this process could be investigated by AFM on a molecular level [310]. Adhesion forces of integrin to RGD-domains have been determined to be between 32 and 97 pN [311], which is within the range of antigen–antibody interactions.

4.3.4. Intramolecular forces in proteins and polysaccarides

Since AFM enables us to address single molecules, the elasticity of macromolecules has been intensively studied and also simulated [312]. The very large protein titin, which consists of about 300 repeats of IgG and related fibronectin type III (FnIII) domains [313], is an ideal naturally occurring model protein for the investigation of unfolding processes. When applying a force, domain after domain is unfolded resulting in sawtooth-like F/D curves [314]. The distance of the peaks provides directly the length of the unfolded domain. The unfolding force of a IgG domain is about 150–300 pN, whereas it is 20% lower for FnIII [315]. The force values depend on the loading rate [314], similarly as discussed above for ligand–receptor interactions. It has been shown that the mechanical unfolding as determined by AFM for single molecules reflects the same events that are observed in an equilibrium denaturation experiment; this is the proof that AFM methods are suitable to investigate unfolding processes quantitatively [316]. Refolding of the stretched domains occurs spontaneously which can be followed by laser tweezer investigations [317] or alternatively by AFM [314,318]. Simulations of a protein folding process for the very long time frame of 1 ms show that α-helix formation occurs first [319] what might also happen in IgG domains. When using a recombinant truncated titin polyprotein, a missing peak in the F/D spectrum indicates a single misfolding event [320]. The insert of five extra amino acids in a polyprotein results in a shift of the peak to peak distance of 2.0 nm, which can clearly be resolved [321]. By measuring the more complex structural protein tenascin, which also contains repeated FnIII domains, unfolding forces of 137 pN and distance between the peaks of 25 nm were determined [322]. Although essential contributions to the understanding of molecule properties can be made using AFM, these data are not sufficient to fully explain physiological functions [323]. This suggestion is supported by the fact that unfolding and refolding processes are very unlikely to occur in biological processes [324]. Finally, the elasticity of a cell adhesion proteoglycan [286] has been investigated by AFM.

The second class of important biomacromolecules are polysaccarides which do not have a defined 3D structure. When a low force is applied, entropic forces dominate, whereas at high forces a conformation change occurs [325]. It could be demonstrated that the glucopyranose ring is deformed from a chair-like to a boat-like structure at high forces [326]. AFM techniques could also determine altered mechanical properties of the denaturated bacterial polysaccaride xanthan [327].

4.3.5. Forces in DNA–DNA interactions

DNA macromolecules are stiff [328] consisting of a ribose phosphate backbone to which the four bases A, T, C, G are conjugated. The sequence of the bases determines, if a double strand is spontaneously formed or not, since A

matches only to T, and C to G. The formation of a 20 base pair (bp) DNA double strand in vitro takes 2 min, where one strand is fixed to a sample surface and the complementary strand to an AFM tip [329]. The force needed to separate a 20-bp long double strand has been determined to 1.52 nN [330] and it is logarithmically dependent on the applied load [331]. The relative force for one G–C interaction was determined to 20 ± 3 pN, for a A–T interaction 9 ± 3 pN [332]. Thus, it will be very difficult to probe one mismatch in a 20-bp long DNA.

5. Outlook

This article illustrates the impact of SPM on biointerface analysis. AFM and STM investigations provide structural and functional information from single molecules. As discussed above F/D-curves contain complex information and when combined with further analytical methods a comprehensive view of intermolecular force phenomena and molecular interactions can be achieved. Thus, in the future multifunctional analysis on the nanometer scale will allow us to understand and improve biointerfaces used for the different applications as mentioned. A tool-box of SPM methods, which combines one or more analytical techniques, will probably be available. The benefits and limitations of SPM techniques have been identified in the last decade. The challenge now is to develop multifunctional SPM tools, which are suitable for the analysis of biointerfaces. We suggest that the impact of SPM methods to this purpose is currently at the beginning and will become much more common and effective in the future.

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