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TRENDS

Scanning force microscopy based amperometric biosensors

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Introduction

Cantilever-based biosensing has developed into an important research area especially for biomedical and clinical analysis. In particular, the possibility to scan miniaturized amperometric biosensors with high-fidelity distance control across biomedically relevant sample surfaces enables the determination of pertinent analytes such as, e.g., adenosine 5′-triphosphate (ATP). ATP is involved in a wide variety of important regulatory cellular mechanisms, and quantification of ATP has therefore been the focus of extensive research in recent years.

Usually, scanning force microscopy-based biosensing relates to detection principles based on either mass-sensitive mechanical transduction, which can be detected as a frequency change of the cantilever, or a change in the force constant due to the absorption of molecules onto the sensor surface, as shown in Fig. 1a. Alternatively, a bimetallic cantilever can be used as a temperature sensing device for detecting calorimetric changes due to specific binding to an immobilized receptor, or due to absorption of molecules. Advanced microfabrication in combination with sophisticated surface functionalization schemes results in sensing interfaces tailored to specifically interact with analyte molecules, thus providing specificity and sensitivity of such miniaturized devices to the picomolar and even femtomolar concentration range [1]. Conventional cantilever-type biosensors do not provide a sharp tip, and are typically not applied for laterally resolved measurements in an imaging mode, which requires that the sensor is scanned across, e.g., a biological specimen at a controlled distance. Recently, the National Heart Lung and Blood Institute has published a report identifying demands for future developments related to diagnosis and therapy of cardiovascular, pulmonary, and hematologic diseases, which identifies in vivo nanosensors as a promising prospective application of nanotechnology for real-time monitoring of biological signals in response to cardiac or inflammatory events [2]. Hence, besides measurements in complex background matrices, the analytical challenges encompass the ability of biosensors to provide information on structural changes along with chemical information on, e.g., regulatory processes at single cells or cell ensembles, ideally at a nanometer scale. Consequently, miniaturization of the transducer and sensing interface, along with positioning of such miniaturized sensors in close proximity to the sample surface providing laterally resolved quantitative information is a prerequisite.

Alternatively to mass-sensitive cantilever-based sensing in bulk solution, atomic force microscopy (AFM) probes can be chemically functionalized, which enables detection and quantification of forces associated with single-molecule binding events at ambient conditions. Recently, this concept has evolved into a combined tool providing laterally resolved information along with force mapping, also known as topography and recognition imaging [3], which enables the visualization of single receptor binding sites at biological surfaces. The present article, however, focuses on the detection of chemical signaling events involving the secretion of transmitter molecules into the extracellular space, and associated efforts to detect such molecules and their concentration for enhancing fundamental understanding on disease-related cellular processes at a molecular level.

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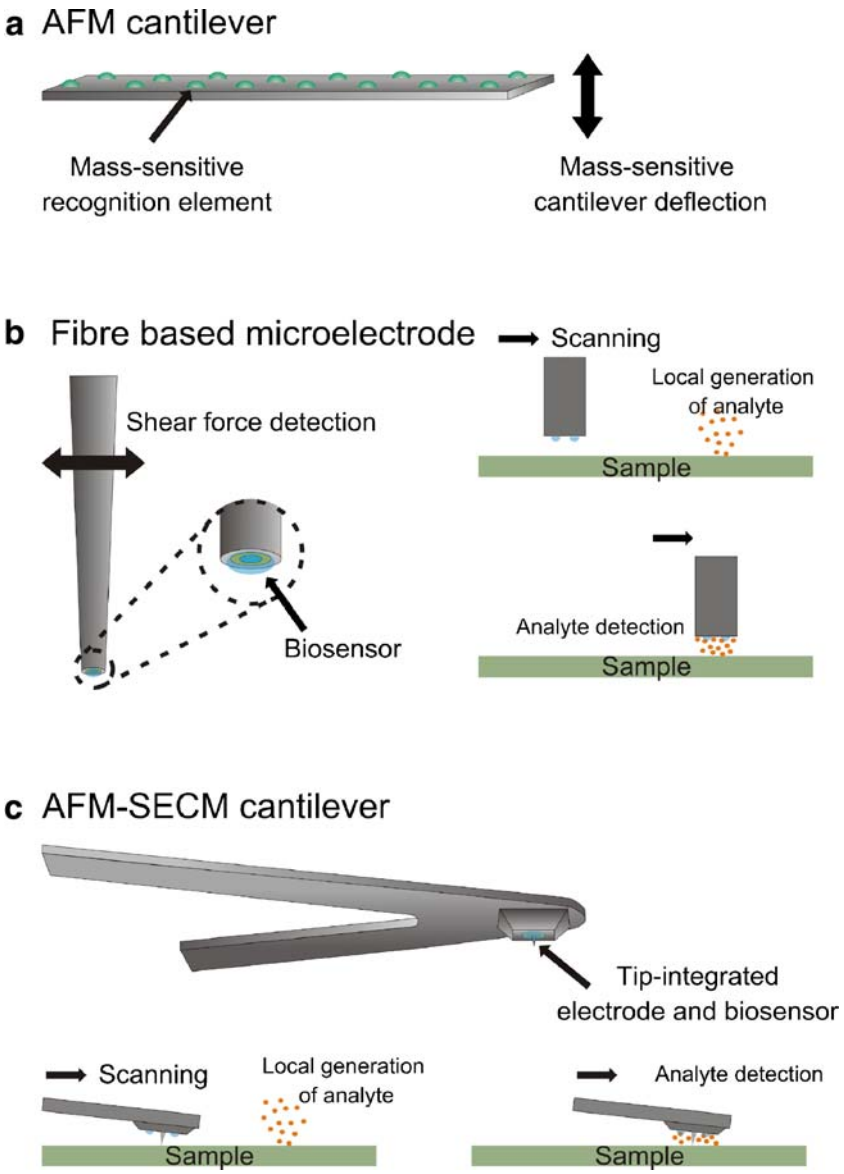


Fig. 1 Measurement schemes for scanning probe microscopy based biosensors: **a** mass-sensitive atomic force microscopy (*AFM*) cantilever; **b** shear-force based scanning electrochemical microscopy (*SECM*) biosensor; **c** AFM tip-integrated biosensor

In the past 15 years, AFM probes—or more general scanning probe tips—have been harnessed with the ability of (bio)molecular recognition by combination with amperometric biosensing schemes, provided that either the scanning probe is a miniaturized electrode, or an electroactive area is implemented into the SPM probe, as shown in Fig. 1b and c, respectively. By scanning this—possibly functionalized—probe across the sample surface, one can obtain spatial information on localized target analyte concentrations, ideally while simultaneously mapping topographical changes.

Amperometric enzymatic biosensors are among the most commonly reported class of biosensors, and are based on the catalytic conversion of electroinactive or electroactive species via enzymes—usually oxidoreductases—forming an electroactive by-product, which can be detected at the electrode surface. In particular, for real-time in situ monitoring of biomedically relevant analytes such as, e.g., lactate, hydrogen peroxide, acetylcholine, glutamate, and ATP, miniaturized amperometric biosensors provide the unique advantage of a detection scheme with suitable spatial and temporal resolution, as recently demonstrated during stationary experiments [4].

Imaging microbiosensors

Early approaches using microbiosensors for imaging of biologically relevant processes were based on amperometric biosensors serving as scanning electrochemical microscopy

(SECM) probes [5]. A difficulty herein is that surface modified electrodes cannot be positioned by recording conventional SECM approach curves monitoring the faradaic current, as the probe approaches the sample surface, which is the standard technique for probe positioning during SECM experiments. Hence, early imaging experiments with microbiosensors have focused on alternative positioning strategies such as, e.g., measuring changes in solution resistance when approaching the sample surface, or by preparing dual-microelectrode assemblies utilizing one electrode modified with the sensing layer as an electrochemical sensor, and the second unmodified electrode for positioning the dual-electrode assembly. For example, Horrocks et al. [5] have shown that a hydrogen peroxide microbiosensor could be positioned by applying a high-frequency alternating potential to the tip, and measuring the solution resistance between the tip and the auxiliary electrode, while the concentration profile of hydrogen peroxide around a micro platinum electrode was imaged.

In another example, ATP imaging was achieved with a dual-electrode assembly. Next to other functions, ATP is a key cellular messenger molecule; hence, determining ATP concentration profiles and distributions at biologically active surfaces such as cells, cell ensembles, or tissues is essential in clinical research [6]. Several ATP biosensing schemes have been developed [6–9], and have been applied to in vitro stationary measurements of ATP released from pigment epithelium, carotid body preparations, and from brainstem [10, 11] (J.-F. Masson, C. Kranz, B. Mizaikoff, and E. Gauda, unpublished results). Laterally resolved imaging of ATP transport through an artificial membrane was obtained by Kueng et al. [12] using a dual-electrode assembly, as shown in Fig. 2a. The sensor architecture involves the coimmobilization of hexokinase and glucose oxidase at the electrode surface of one platinum disk of the dual-electrode assembly, whereas the unmodified electrode is used for recording an approach curve, thereby enabling accurate positioning of the amperometric biosensor. ATP is indirectly monitored via the competitive reaction between glucose oxidase and hexokinase for glucose (substrate), with ATP as a cosubstrate and hydrogen peroxide as the electroactive by-product detected at the electrode surface. However, the dual-electrode approach does not solve the problem of constant-height imaging at samples that are not ideally flat, which is a common problem in conventional SECM. In particular, the targeted application of imaging the release of molecules above cells or cell assemblies is concerned with highly corrugated samples characterized by height variations at the micrometer scale [13]. Recently, several approaches have been presented focusing on positioning of chemically sensitive tips at constant distance, thereby enabling imaging of the sample topography with micro/nanoelectrodes or -biosensors at a deliberately selected distance.

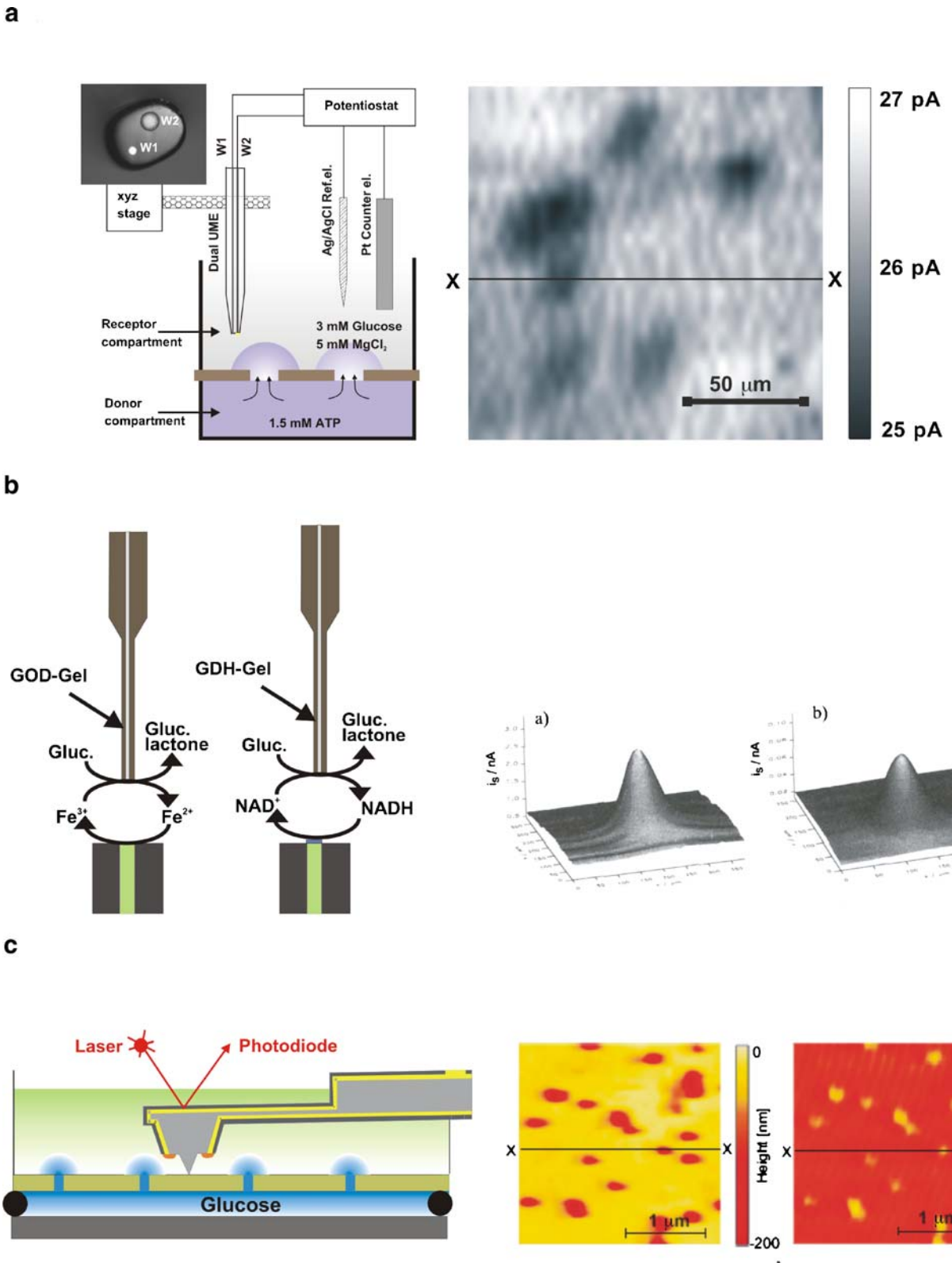
Shear-force-based amperometric biosensors

In analogy to noncontact mode AFM, shear-force mode SECM is based on monitoring hydrodynamic forces, if the micro- or nanoelectrode tip is vibrated in close proximity of the sample surface. By monitoring damping of the vibration amplitude either optically, by tuning-fork-type resonators, or via piezo-based actuator detectors, a constant distance of less than 300 nm between the tip and the sample surface can be maintained using a feedback loop, as schematically shown in Fig. 1b. Seminal studies by Hengstenberg et al. [14] have demonstrated shear-force-based constant-distance imaging with microbiosensors utilizing an optical read-out system. Glucose oxidase and glucose dehydrogenase, respectively, were immobilized within polymer hydrogels filled into fiber-shaped glass capillaries with a diameter of 10 μm. Thus obtained enzyme-filled capillaries were scanned across a 50-μm platinum electrode using the glucose oxidase biosensor, and across a poly(methylene blue)-modified microelectrode using the glucose dehydrogenase filled capillary. Identification of such reactive spots is shown in Fig. 2b. The group of Schuhmann recently also demonstrated imaging of nitrogen oxide (NO) release above human umbilical vein endothelial cells with a nickel tetrasulfonate modified microelectrode. The modified electrode was positioned above the center of an individual cell, and NO release was stimulated by bradykinin. NO is a relevant messenger molecule in the vascular system, and abnormally high or reduced levels of NO have recently been associated with vascular disease conditions [15].

AFM tip-integrated amperometric biosensors

Scanning force microscopies are ideally suited for directly imaging biological systems and biomaterials in buffered solution, in particular in intermittent operation reducing the force impact of the probe at soft biological samples. In combination with selective and sensitive detection schemes based on miniaturized electrochemical transducers, high lateral resolution along with localized (electro)chemical information can be obtained (Fig. 1c). Several approaches have recently been published reporting on the integration of microelectrodes and nanoelectrodes into AFM probes with either conical electrodes exposed at the apex of the AFM tip [16–19], or disk-, ring-, or frame-shaped electrodes [20–22] recessed from the apex of insulating AFM tips [23].

Since direct contact between the imaging microbiosensor and the sample surface should be avoided, to date only AFM tip-integrated biosensors based on probes with a recessed electrode have successfully been demonstrated, thereby enabling positioning of micro- and nanobiosensors at a deliberately selected, well-defined, and constant



distance above the sample surface. AFM tip-integrated biosensors for glucose [24], hydrogen peroxide [25], and ATP [23] have been demonstrated so far. The example in Fig. 2c shows imaging of glucose transport through the pores of a track-etched model membrane, along with the simultaneously mapped pore topography. Glucose oxidase was immobilized at the electrode surface of the integrated AFM–SECM probe by entrapment into an electrophoretic

◀**Fig. 2** **a** Imaging dual biosensor assembly. *Left*: The set-up with a dual-electrode assembly (*inset*). *Right*: SECM image of ATP transport through a track-etched membrane (pore diameter 20 μm). **b** Shear-force-based biosensor; mapping of glucose conversion by gel-immobilized glucose oxidase (*GOD*) (*a*) and glucose dehydrogenase (*GDH*) (*b*). The electroactive by-products of the enzymatic reaction were detected by a microelectrode, if the enzyme-filled fiber-shaped capillary was scanned in the shear-force regime across a microelectrode, and a poly(methylene blue)-modified microelectrode, respectively. **c** AFM tip-integrated glucose sensor. *Left*: Detection of glucose transport through the pores of a track-etched membrane (pore diameter 200 nm). *Right*: Simultaneously recorded height and current image. (**a** Reprinted from [12], copyright 2005 with permission from Elsevier Science. **b** Reprinted from [14], copyright 2000 with permission from Wiley-VCH Verlag. **c** Reprinted from [24], copyright 2005 with permission from Wiley-VCH Verlag)

polymer matrix [26], thereby locally catalyzing the conversion of glucose, which is diffusing through the membrane pores, to gluconolactone and hydrogen peroxide; the latter is detected at the integrated electrode (+0.65 V vs. Ag quasi-reference electrode), resulting in an increased current above the pores. Localized pore transport of glucose was determined during high-resolution AFM imaging of the sample topography, as shown by Kueng et al. [24]. In order to quantify the local analyte concentration, which is the ultimate goal of spatially resolved measurements above biological specimen, tip-integrated biosensors have to be calibrated prior to and after the measurement to compensate for loss of activity and interferants. With precise knowledge on the electrode geometry, and on the distance between the electrochemical probe and the sample surface, the glucose concentration can be determined using a single pore model and assuming hemispherical diffusion [27].

Outlook

Miniaturized biosensors are an emerging field in cell biological research, with the potential of elucidating fundamental mechanisms during the progression of diseases, thereby gaining insight into cellular signaling processes at a molecular level. By further stimulating interdisciplinary research on nanobiosensors involving the areas of nanotechnology, chemistry, and cell physiology, the potential for bridging the macroscopic world with the nanoscopic domain enabling localization, quantification, and imaging of correlated chemical and structural changes at cell surfaces is provided.

While to date only model systems have been imaged with AFM tip-integrated amperometric biosensors, it is anticipated that first results at biological surfaces such as epithelial cells will be achieved in the near future.

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