EXPERT OPINION

Applications of biosensing atomic force microscopy in monitoring drug and nanoparticle delivery

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Introduction: The therapeutic effects of medicinal drugs not only depend on their properties, but also on effective transport to the target receptor. Here we highlight recent developments in this discipline and show applications of atomic force microscopy (AFM) that enable us to track the effects of drugs and the effectiveness of nanoparticle delivery at the single molecule level.
Areas covered: Physiological AFM imaging enables visualization of topographical changes to cells as a result of drug exposure and allows observation of cellular responses that yield morphological changes. When we upgrade the regular measuring tip to a molecular biosensor, it enables investigation of functional changes at the molecular level via single molecule force spectroscopy.
Expert opinion: Biosensing AFM techniques have generated powerful tools to monitor drug delivery in (living) cells. While technical developments in actual AFM methods have simplified measurements at relevant physiological conditions, understanding both the biological and technical background is still a crucial factor. However, due to its potential impact, we expect the number of application-based biosensing AFM techniques to further increase in the near future.

Keywords: atomic force microscopy, drug delivery, force spectroscopy, molecular interactions, nanoparticle, single molecules, tip functionalization

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1. Introduction

Tailored drug-delivery systems have become increasingly important in the development of novel medical therapies and pharmaceutical products. Short-lived therapeutic molecules like peptides or nucleic acids, and also highly active drugs with potentially severe side effects, such as chemotherapeutics, require site-directed transport to target specific sites. Nanomaterials with the capacity to carry combinations of drug molecules and active targeting agents are moving into the spotlight [1-3], as they are designed for controlled drug release. Their incorporation into pharmaceutical formulations may promise enhanced biodistribution and pharmacokinetic properties, increasing the efficacy of a therapeutic product and potentially reducing unwanted side effects. Analysis of the specific interaction of drugs and nanoparticle-based drug-delivery systems with isolated receptors, and moreover live cells on a molecular level, are key challenges in advancing new drug-delivery approaches into potential new therapies. In this context, atomic force microscopy (AFM) has emerged as a powerful characterization platform, providing valuable insight that may have major implications for drug design and development.

AFM was invented in 1986 as a further development of the scanning tunneling microscope [4]. It has successfully complemented scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as an instrument for visualizing cells and biological membranes on the micrometer-scale level and down to the

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Article highlights.

- Atomic force microscopy (AFM) imaging enables us to monitor drug and nanoparticle effects on membranes and live cells under physiological conditions and with high lateral resolution.
- Single-drug molecules and nanoparticles can be tethered to the outer apex of an AFM tip to follow the interactions with membranes and cells.
- Force spectroscopy is a useful technique for determining the binding efficiency of targeted drug-delivery systems in order to establish optimal configurations of targeting agents and guide design of next-generation drugs.
- Though AFM experiments are generally straightforward, careful sampling, AFM tip preparation and critical data evaluation are all of paramount importance.
- A standard in most AFM setups is a combination with powerful optical (fluorescence) microscopy that greatly benefits live-cell applications.

This box summarizes key points contained in the article.

subnanometer range. Textures and shapes of biological specimens can be visualized as three-dimensional maps with an exceptionally good vertical resolution when scanning the surface with a sharp tip attached to a sensitive cantilever and monitoring the force between the surface and the tip surface. Though SEM routinely achieves a resolution of 5 nm, this technique depends on vacuum conditions and extensive sample preparation, which also includes steps such as freeze drying, staining or metal coating that can result in artifacts. AFM, by contrast, does not require laborious sample preparation, staining or labeling. Most importantly, AFM has the capability to operate in liquids and under ambient temperatures, and thus allows visualization of biological samples in near physiological conditions. It thus preserves sample structure and function over an extended period of time, such as nanoscale substructures of individual membrane proteins and native membranes [5-7]. When applied to live cells, new cellular surface structures and their physiological functions can be identified [8-10], and dynamic processes, such as structural changes caused by growth or drug interactions at the nanoscale, can be revealed in real time [11,12].

In addition to topographical imaging, AFM offers the possibility to measure elastic properties like the elastic modulus of biological samples using the AFM tip as nanoindenter to probe the stiffness of tissues and cells [13,14]. Moreover, the AFM cantilever can be converted into a specific biosensor to probe interactions with isolated receptors, reconstituted and native membranes and the surface of live cells [15-17], thereby measuring inter- and intramolecular forces at the nanometer scale with outstanding signal-to-noise ratio and piconewton resolution [18,19]. For this purpose, the AFM tip is functionalized with specific biomolecules, such as Abs, hormones, nucleic acid, etc., or replaced by a living cell. In this way, AFM-based force spectroscopy is used to measure interaction forces and dynamics between individual pairs of ligands and receptors (single-molecule force-spectroscopy [SMFS]) [15,20-22], ligands and cells [23-26], or to quantify cell adhesion forces single-cell force-spectroscopy (SCFS) [27].

AFM has evolved from a mere physical microscopy technique into a standard method in pharmaceutical [28] and life sciences [29]. In light of the ever-increasing diversity of biological applications, AFM has become a multifunctional molecular toolkit. It enables researchers to observe structural details of molecular assemblies and cell surfaces. It can monitor cellular responses to drug and nanoparticles interactions, and force-probe their receptor specific binding. In this review, we highlight the scope and advantages of AFM in pharmaceutical research to monitor drug and nanoparticles delivery. In recognition of the increasing amounts of literature in this genre on physicochemical characterization of nanoscale drug-delivery systems using AFM imaging [30,31], we will focus on imaging applications to visualize drug and nanoparticle interaction with model membranes and cells. A special emphasis will be placed on the chemical functionalization of the AFM tip with drug molecules and nanoparticles and their applications on isolated receptors, model membranes and live cells to monitor and assess drug and nanoparticles delivery on a single molecule level. Finally, we discuss challenges, future advances and new developments in AFM techniques and its prospective role in pharmaceutical research.

2. AFM principles

2.1 Physiological imaging

AFM can be operated in numerous modes, most of them are well established. Comprehensive literature concerning operation and instrumentation is available (e.g., [32-39]). Due to the limited word count, we kindly ask the readers to consult relevant literature.

2.2 Single-molecule force spectroscopy

AFM is well known for its ability to visualize surface topography with nanometer resolution under (near) physiological conditions. Nevertheless, SMFS, with biosensing-based techniques, gives additional insight into biological samples. SMFS allows exploration of the energy landscape that underlies ligand–receptor interaction. This bond energy landscape describes the dependence of the free energy on structural parameters. In addition, features of the energy surface define the kinetic parameters of the complex. The global minima represent the most stable states within the energy landscape, whereas local maxima appear at transition states. Unraveling this energy surface requires a method for overcoming the heterogeneity of ensemble measurements. This is realized by AFM single-molecule pulling experiments, where a force is applied on the complex. The application of increasing forces yields an increased tilt of the energy surface in the direction of the force. Thus, the energy barriers are lowered, making the dissociation easier due to thermal fluctuations (**Figure 1A**). The dependence of the most probable unbinding force on the rate at which force is applied

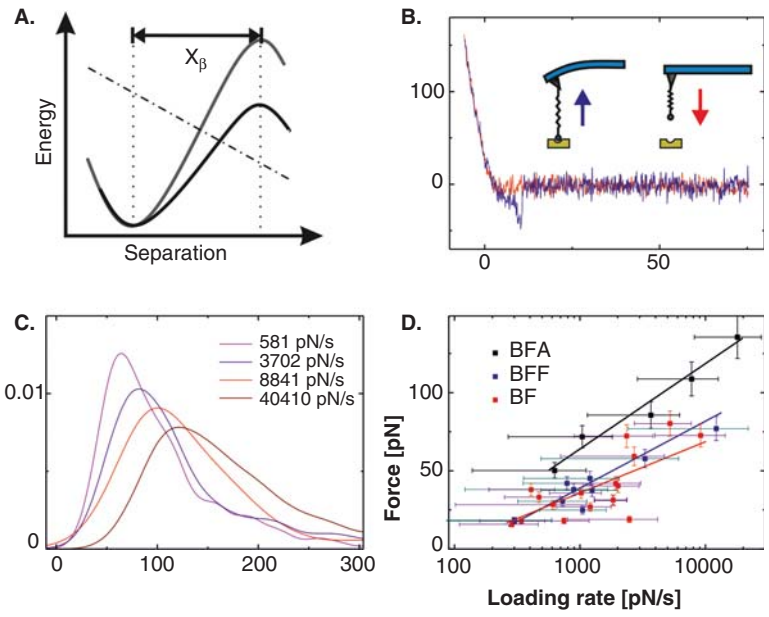

Figure 1. (A) Changes of a single barrier potential as result of an applied force. **(B)** Typical force–distance cycle (red curve: approaching, blue curve retracting). **(C)** Probability density functions of typically 1000 FDCs/per loading rate at different loading rates. **(D)** Loading rate dependence of ligand–receptor interactions (right).
Data of (**C**) and (**D**) taken from Neundlinger *et al.* [174].
FDCs: Force–distance cycles.

is the force spectrum that includes information on: i) the number of energy barriers; ii) their height; and iii) their position along the reaction coordinate. Subsequent to this, we very briefly describe the underlying basics of SMFS from the experimental approach to data acquisition.

2.2.1 *From force distance cycles to unbinding force distributions*

A ligand-functionalized tip has to be brought in contact with its corresponding receptor that is immobilized on a solid support (or vice versa) in order to measure the dissociation forces of a ligand–receptor complex. In practice, this is done in so-called force distance cycles (FDCs) (**Figure 1B**). At a fixed lateral position, the bio-functionalized tip approaches to the surface with a given velocity. At the point of contact, the cantilever starts to bend until a previously set bending limit is reached. After an optional resting time, the tip is then retracted from the surface again allowing the cantilever to reach its resting position. During the contact period, the tip-tethered ligand can form a complex with its corresponding receptor. In the case of complex formation a second, but this time downwards bending of the cantilever can be observed (**Figure 1B**, inset). In addition to these specific interactions, nonspecific attractive and repulsive forces can also appear but should be minimized (e.g., by nonadhesive tip functionalization). In case of a specific interaction, the observed force profile usually shows a typical nonlinear parabolic shape caused by the elastic properties of the (PEG) linker. Cantilever bending is monitored by AFM and translated into an applied force by multiplying with the spring constant according to Hook's law. The determination of the spring constant, which is essential when performing AFM force spectroscopy experiments, can be done by a calibrated reference [40], the added mass method [41], the thermal noise method [42] or by the Sadar method [43].

In order to get accurate results in SMFS, sufficient statistics have to be collected. Thus, at each pulling velocity 1000 – 3000 FDCs have to be performed yielding a distribution of unbinding forces. The distribution can either be plotted as histograms or as continuous probability density functions (**Figure 1C**) [44] where the values are weighted by their reliability. Here the maximum in the pdf reflects the most probable unbinding force, whereas the broadness corresponds to the standard deviation. Interested readers can find comprehensive literature on the evaluation of SMFS experiments, for example, here [45-47].

2.2.2 *From force distributions to the energy landscape*

The lifetime of a complex without applied force is defined as the inverse of the kinetic off-rate. Although this unforced dissociation process can take up to several days, a typical force–distance cycle is performed in seconds. There, the dissociation process is influenced by the applied (pulling) force. In the millisecond to second time regimen, representative for force spectroscopy experiments, thermal impulses govern the unbinding process. The thermal activation model allows us

to describe the lifetime of a bond by a Boltzmann ansatz, where τ_{osc} is the inverse of the natural oscillation frequency and E_{barr} is the height of the energy barrier of the dissociation process [48]

$$\tau(0) = \tau_{osc} e^{\frac{E_{barr}}{k_B T}} \quad (1)$$

If a force is now applied on the complex, the stability is lowered and the energy landscape of the interaction is deformed. The changed lifetime of the bond $\tau(f)$ at the applied loaded force is now

$$\tau(f) = \tau(0) e^{\frac{E_{barr} - fx}{k_B T}} \quad (2)$$

Here x represents the distance of the energy barrier from the energy minimum in the direction of the applied force. The reason for the forced rupture is the lowered energy barrier between the bound and unbound state. The effective force increase, also termed the force loading rate *r*, which is the change of force per time (r = df/dt), changes when varying the pulling velocity. Predictions of the unbinding force at different loading rates can be made by combining the Boltzmann ansatz with the stochastic description of the unbinding process. As a result, the most probable unbinding force at a given loading rate *f(r)* is

$$f(r) = \frac{k_B T}{x_\beta} In \frac{r x_\beta}{k_B T k_{off}} \quad (3)$$

If pulling experiments are now performed at different pulling velocities – resulting in different ramps of forces – a dynamic spectrum of bond rupture forces as a function of the loading rate *r* is generated. The semi-logarithmical plot of the loading-rate-depending unbinding forces depicts the force-driven pathway of dissociation (**Figure 1D**). Changes in the slope give a clear indication of additional, eventually hidden, energy barriers (**Figure 1A**).

2.3 Tip functionalization

A crucial requirement for performing successful biosensing AFM experiments are biologically functionalized cantilever tips. For this, the normally inert AFM tip has to be converted into a (mono)molecular sensor by attaching one or a few ligand molecules to the tip apex. Within this section, we detail strategies that enable creation of such sensing tips with sufficient success rates and chemical stability. Based on the nature of the tip surface material, two different approaches are generally possible. Although the use of gold-coated tips allows functionalization based on self-assembled monolayers (SAMs) of thiolated linker molecules, the common silicon or silicon nitride tips have to be chemically modified in the first step (e.g., by aminofunctionalization protocols). In addition, different strategies like simple adsorption or chemisorption have been developed providing for efficient coupling under suitable circumstances. A more detailed description can be found in [49].

Physisorption means nonspecific adsorption relying on noncovalent bonds like hydrophobic interaction, stacking, hydrogen bonds and electrostatic attraction. It offers an easy and effective coupling strategy for probe supports. Due to their positive charge, avidin molecules can be bound to freshly cleaved mica just by simple incubation of an avidin solution. In contrast, exchanging the surface net charge of mica by substitution of potassium to nickel ions allows stable binding of negatively charged DNA. However, when attaching ligands to the tips used in biosensing AFM experiments, very firm coupling is needed. Although this can also be realized by physisorption (e.g., with the help of crosslinking [50,51]), primarily covalent attachment protocols have been established for reliable AFM tip functionalization.

2.3.1 *Tip functionalization of silicon or silicon nitride tips*

The most common approach for converting inert silicon/silicon nitride materials into a chemically addressable surface is the aminofunctionalization [52]. Here, the most frequently used methods are the gas-phase protocol that uses aminopropyl-triethoxy-silane (APTES) and the liquid phase method based on the use of ethanolamine-hydrochloride in DMSO. Both protocols result in surfaces with a surface density of sufficient reactive sites (~1000 – 2000 amino-groups/μm^2), they are not sticky and avoid the formation of sponge like multilayers, which are not suitable for force spectroscopy experiments.

A clear understanding of the chemical process helps to perform successful aminofunctionalization: we would first like to point out that both silicon and silicon-nitride tip surfaces have an oxidized silicon-oxide layer with a high number of silanol (-Si-OH) groups under ambient conditions. APTES, the most commonly used aminosilanization reagent, now reacts with these silanol groups. The reaction itself has to be performed in an argon-flooded desiccator to control the amount of water. Typically 30 µl APTES and 10 µl triethylamine as catalysts are placed in a six liter argon-filled desiccator and allowed to react for 120 min followed by careful rinsing with argon gas. To ensure sufficiently strong binding of the generated APTES layer, 'curing' at room temperature for 2 days has to follow. Alternatively, the aminofunctionalization protocol based on ethanol hydrochloride is simpler but results in more undefined chemical coverage. In a nutshell, ethanolamine hydrochloride is dissolved in DMSO, molecular sieves are added and the cleaned tips are incubated overnight followed by a careful cleaning procedure.

In the next step, a heterobifunctional crosslinker is typically bound to the generated amino residues on the tip. PEG consisting of 10 – 30 ethyleneglycol units turned out to be best suited for this due to the properties of this polymer. PEG itself is an inert, water-soluble and distensible polymer with the advantage of being protein repulsive. In addition,

Table 1. Functionalization strategies for silicon/silicon nitride tips based on heterobifunctional PEG linkers.

Typical target molecule	Linker	Reactive site	Ref.
Biotin	Biotin-PEG-NHS	-	[49,52,53]
Protein, R-NH_2	Aledehyde-PEG-NHS	Lysine (R-NH_2)	[49,55]
Protein, R-NH_2	Acetal-PEG-NHS	Lysine (R-NH_2)	[49,56]
Protein, R-SH	PDP-PEG-NHS	Cysteine, R-SH	[49,57]
Protein, R-SH	Mal-PEG-NHS	Cysteine, R-SH	[49,173]
Aliphatic carbons (e.g., DNA)	Benzophenon-PEG-NS	Aliphatic carbon	[49]
His_6-tagged protein	Multi-step procedure	His_6-tag	[49,58]

denaturation of most of the bioligands is markedly lowered compared with coupling with a short linker. Only in very special cases does this linker already contain the ligand molecule itself. Biotin [53] or fluorescin [54] are such candidates that can be bound as part of the linker as their structures are stable at the typical coupling conditions like chloroform as solvent using triethylamine as catalyst. Most of the typical ligands are proteins and require aqueous coupling conditions.

Numerous linkers with different reactive sites are developed depending on the nature of the coupling group of the bioligand (**Table 1**). Most commonly, proteins are bound via their lysine residues. Here two linkers, NHS-PEG-aldehyde [55] or NHS-PEG-Acetal [56], are suited for direct coupling, whereas the second one is preferable as it allows avoids loop formation. As an alternative, ligands with a free thiol end (e.g., cysteine mutants) can be tethered to aminofunctionalized AFM tips using NHS-PEG-PDP [57] for cleavable or NHS-PEG-maleimide for noncleavable coupling. A very general coupling strategy is realized by the use of NHS-PEG-benzophenone (manuscript in preparation). After binding the linker to the aminofunctionalized tips via its NHS end, benzophenone can be activated by UV light (366 nm) and allows stable covalent bond formation with aliphatic carbons.

All of the abovementioned strategies admittedly do not allow site-directed binding as the ligand molecule usually offers more than one coupling sites (e.g., due to the fact that more than one lysine exists in the amino acid sequence of the protein). Site-directed coupling is possible for proteins containing a hexa- or deca-histidine tag, which is common for artificial expressed proteins, by the use of nitrile-tri-acetic acid functionalized linkers. Here the coupling protocol requires somewhat more effort as the synthesis of a NHS-PEG-NTA linker is not possible. A detailed description can be found elsewhere [58]. We would like to mention that the given coupling schemes reflect the most common strategies, but there are numerous alternative means that also yield stable und distensible coupling of ligands to AFM tips [59].

2.3.2 *Tip functionalization of gold-coated tips*

Gold is a highly inert noble metal that can be cleaned at harsh conditions without any troublesome effects like surface oxidation. Nevertheless, gold can be functionalized with an extremely high efficiency using the thiophilic properties of gold. Gold-coated tips are commercially available, but the drawback is that the gold coating includes a precoating with an adhesive layer (e.g., chromium) that increases the tip radius by typically 10 nm or more. To ensure sufficiently strong binding of the thiolated linker, either a SAM built up by bifunctional alkyl mercaptans or linkers containing a higher number of sulfur residues should be used. In general, the best success in SAM formation is achieved by the use of a mixed SAM. For this one component (e.g., a ω-mercaptoalkanol) is used as a nonadhesive part, whereas the other component affords covalent coupling of the ligand of interest. In contrast, the use of thiolated building blocks like thiolated DNA tetrahedra [60] allows generating reactive coatings with defined spacing within one single step.

In conclusion, tip functionalization with biomolecules can be done on both silicon (or silicon nitride) (**Table 1**) and on gold tips (**Figure 2**). For successful sensing experiments, the coupling has to yield in a nonadhesive coating with ligands bound via a distensible linker. This can be realized by mixed SAMs (gold) or be aminofunctionalization (silicon/silicon nitride tips) followed by coupling of heterobifunctional PEG crosslinkers. In a final coupling step, bioligands are bound and statistically yield one single molecule on the outer tip apex.

3. AFM imaging to monitor drug and nanoparticle delivery

Dynamic light scattering (DLS), TEM, SEM and AFM are commonly employed for physical characterization of nanoscale particles' size, shape and morphology. However, although DLS is a valuable technique to determine a mean diameter of a great number of particles, it is difficult to reveal a real size distribution especially when it comes to aggregates [61] or rod-shaped nanoparticles [62]. Also, in order to obtain single-particle dimensions, AFM imaging often facilitates faster results compared with SEM or TEM, as it does not require specific sample preparation and can be performed on submersed particles. In this regard, size determination by AFM imaging has become a standard technique in pharmacology to characterize, for example, delivery vehicles for tumor-targeting drugs [63-65], genes [66,67] and other therapeutic molecules [68] such as insulin [69]. Moreover, AFM imaging can yield important information about *in situ*

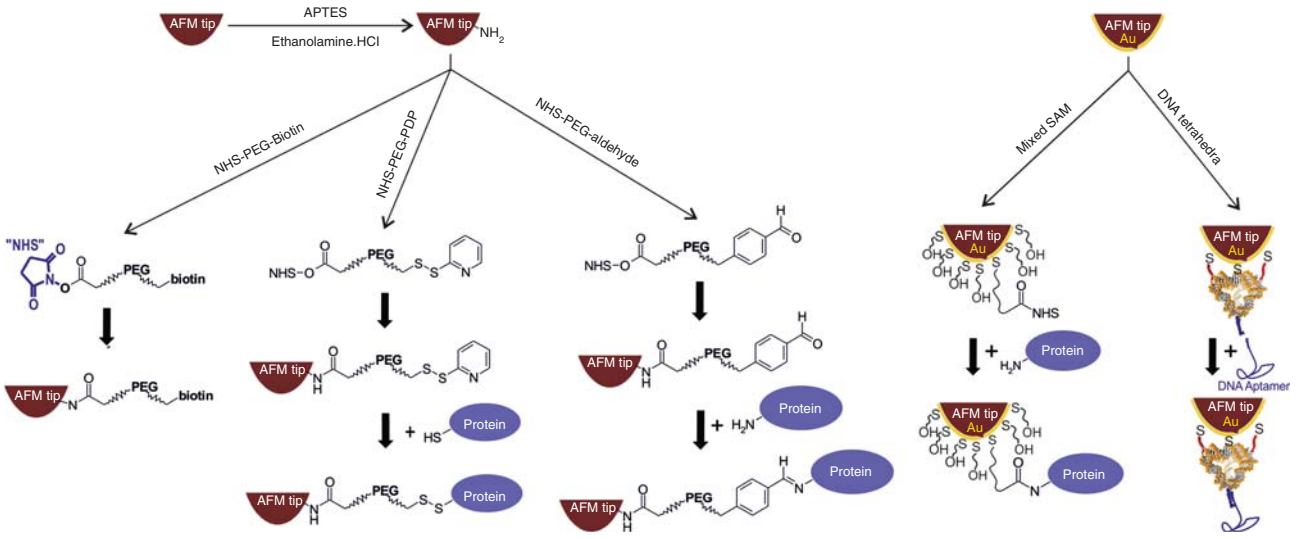

Figure 2. Functionalization of the AFM tip with biological ligands for silicon/silicon oxide and gold-coated tips.
AFM: Atomic force microscopy; APTES: Aminopropyl-triethoxy-silane; SAM: Self-assembled monolayers.

assembly of delivery vehicles [70-72], their surface modification with targeting agents [73,74] and loading with drug molecules [75,76], as well as the stability of drug and nanoparticle suspension, their degradation and release profiles [77-79]. For a review of the extensive literature in this field, the interested reader may refer, for example, to the comprehensive review by Sitterberg *et al.* [30].

3.1 Monitoring interactions on model membranes

The first step in drug delivery to the desired site of action is their translocation across biological barriers such as epithelia and endothelia is their interaction with biomembranes. Along with the aim of releasing a sufficiently large amount of drug into the target cell, strong interactions with the membrane also bear the possibility to disrupt and destabilize the membrane leading to potential cytotoxic effects. AFM imaging provides for the necessary spatial and temporal resolution to monitor structural dynamics and reorganization of model membranes in response to drug molecules [80-86] or nanoparticles and drug complexes (**Figure 3**) [87-93].

Berquand *et al.* [80] used time-lapse AFM imaging to study the effect of the amphiphilic antibiotic azithromycin on model biomembranes of phase-separated dioleoylphosphatidylcholine/dipalmitoylphosphatidylcholine (DOPC/DPPC), as well as bilayers of DOPC mixed with sphingomyelin (SM) and SM/cholesterol (SM/Chl). Images revealed that exposure to azithromycin leads to membrane disruption due to progressive erosion of DPPC domains, whereas SM and SM-Chl domains remained unaltered. This study, as the first of its kind, could directly visualize the perturbation of a membrane due to interaction with a drug demonstrating the potential of AFM real-time imaging for developing new applications in membrane biophysics and pharmacology.

Franquelim and coauthors [86] recently presented their study on HIV-1 fusion inhibitor peptides enfuvirtide, an FDA-approved drug and T-1249, as a possible future alternative agent to treat drug-resistant HIV-1 variants. The researchers used time-lapse AFM imaging to visualize the interaction of both drugs on supported lipid bilayers of POPC:DPPC. The results demonstrated an increased affinity of T-1249 for lipid membrane POPC compared with enfuvirtide as evident in an increase in membrane surface roughness, disruption of the domain borders, decrease in membrane fluidity and bilayer thinning of POPC domains. The later finding was also supported by AFM force spectroscopy, where the penetration force of the AFM tip through a POPC bilayer decreased by a factor of 3 under addition of T-1249, but remained unchanged under the influence of enfuvirtide. The observations could be correlated to the presence of both a tryptophan-rich domain (TRD) and an additional pocket-binding domain, which enhances the amphipathic characteristics of T-1249, whereas enfuvirtide possesses only a TRD. These findings provide valuable insight on the mode of action of HIV-1 fusion inhibitors and allow for a correlation between their structural properties and membrane interactions.

Considering the interaction of nanoparticles with model and isolated membranes, Hong *et al.* [87] were able to show in time-lapse AFM imaging how amine terminated poly(amidoamine) (PAMAM) dendrimers, which are considered promising candidates as gene delivery vehicles can induce pore formation in certain phospholipid regions of a supported lipid bilayer of phosphatidylcholine (DMPC). The height images of DMPC bilayers at different time points revealed that those dendrimers induced ~30 nm diameter holes that grew via removal of lipids from the initial defect area, whereas uncharged dendrimers had no effect. The results point

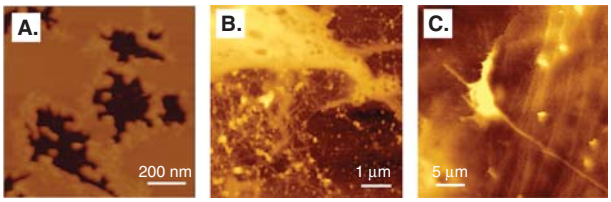

Figure 3. A. Interaction of cationic G5-NH_2 dendrimer nanoparticle with DMPC-supported bilayer. G5-NH_2 nanoparticles do not cause new hole formation in the lipid bilayer but instead expand pre-existing defects. **B.** Topography image (logarithmic contrast) of RNA coated single-walled carbon nanotubes on HeLa cells acquired in contact mode under dry conditions. **C.** Contact mode topography image of fixed T24 cells captured in buffer solution showing a 100 nm thick bundle of BSA-coated carbon nanotubes. The underlying filamentous structures belong to the cell cytoskeleton.
A: Adapted with permission from [89].
B: and **C**: Adapted with permission from [92].

towards a possible application of cationic dendrimers with a dual purpose of gene delivery and enhancement of membrane permeation if dendrimer concentration is kept sufficiently low in order to prevent mitigation of cell stability.

Huang *et al.* [93] extracted the nuclear envelope of *Xenopus laevis* oocytes and prepared the membrane according to a special method that kept the membrane pores, the so-called nuclear pore complexes (NPCs), functional. This could be documented by time-lapse AFM imaging of the reversible calcium-mediated opening and closing of the iris-like ring of the nuclear side of the NPC basket structure. Afterwards, they monitored specific binding of monoclonal antinucleoporin antibody QE5 tagged with 16 nm gold nanoparticles to individual NPCs *in situ*. In the near future, the researchers plan to use their new preparation method to follow transport of single gold-conjugated cargos across individual NPCs by time-lapse AFM.

3.2 Imaging drug and nanoparticle delivery to cells

In the past years, AFM topographic imaging has been successfully integrated into life science to complement fluorescence and electron microscopes, offering new possibilities for visualizing the organization of cell surfaces [94,95]. Under physiological conditions, imaging resolutions on live cells can typically reach 50 – 100 nm, and substructures of native cellular surfaces like the cytoskeleton or endosomal vesicles beneath the cell surface are routinely resolved [73]. Enhanced AFM resolution may be obtained with fixed cells, which allow a more detailed subcellular analysis and visualization of drug effects on cell morphology [96-98] and nanoparticle interaction with the cell surface [73,99-104]. Compared with animal cells, investigations of living microbial cells and their response to antibiotic and antifungal drugs have proved to be less intricate [105-110]. For example, El-Kirat-Chatel [110] and coworkers were able to visualize major morphological and structural alterations of the cell surface of *Candida albicans* induced by caspofungin, a novel antifungal drug of the group of echinocandins. AFM measurements were performed at room temperature in sodium acetate buffer, and yeast cells were immobilized by mechanical trapping into porous polycarbonate membranes.

However, today's AFM instrumentation also provides for investigation of biological membranes, cells and tissue of mammalian origin in a controlled environment in buffer solution or medium at physiological temperatures (e.g., 37°C), with the possibility to add liquids or even change the gas composition (e.g., CO_2 level of 5%) in the sample chamber *in situ* during the process of imaging. Monitoring a cell's reaction to exposure of drug molecules and nanoparticles under appropriate physiological conditions *in vitro* and on the single-cell level may reveal new insight in understanding the mode of action and improve screening for potential new therapeutical agents [111-114].

In their pioneering study, Rotsch and Radmacher [111] investigated the effect of several f-actin-disassembling drugs (cytochalasin B, cytochalasin D, latrunculin A and jasplakinolide) and microtubule depolymerizing agents (colchicines, colcemid and taxol) on the morphology and elasticity of two fibroblast cell lines with an AFM that was mounted on an inverted fluorescence microscope. In time-lapse contact mode imaging, they recorded changes of the cytoskeleton fibrous structures on life cells that were maintained under physiological environmental conditions (5% CO_2, 37°C) throughout the experiment in a custom-built sample chamber, which is now standard equipment of commercial bio AFM setups. The researchers further employed point-by-point force spectroscopy data that were used to determine the Young's modulus at each point to create elasticity maps of fibroblasts that enabled them to assign stress fibers by correlation of AFM data with fluorescence images of the same cells.

Oberleithner *et al.* [112] showed that aortic endothelial cells swell and soften when exposed to high-level concentrations of extracellular potassium (K^+) by monitoring the cell height and recording elasticity maps. High physiological sodium concentrations, in the presence of aldosterone, prevented the physical changes of cells. The effect of high (K^+) was shown to be associated with an increase in the release of nitric oxide (NO) and could be mimicked by actin depolymerization induced by cytochalasin D, hinting at submembranous cortical fluidization depending on potassium concentrations. The researchers suggested that (K^+) concentration could control endothelial deformability and NO release as a mechanism that influence systemic blood pressure.

In a similar approach, Jeong *et al.* [113] investigated the effect of telmisartan on preventing contractive action of glomerular mesangial cells (MCs) induced by Ang II. Telmisartan was reported to prevent glomerular injury in models of experimental renal disease by specifically blocking the Ang II receptor (ARB). Real-time AFM imaging of live MCs in cell culture medium in contact mode was used to

monitor single live-cell morphology, dynamics and mechanical changes under the influence of telmisartan. The images showed counteraction of telmisartan against Ang II-induced contraction of the cell towards their center. Measurements of MC cell elasticity and adhesion properties demonstrated that telmisartan prevented MC stiffening due to Ang II exposure and allowed investigation of the role of ARBs and the mode of action for therapeutics in renal protection.

4. Quantification of elastic properties and adhesion to monitor cell response

Through membrane receptors cells can not only sense their local chemical environment, but also forces and the micro- and nanoscale patterns of the surrounding topography. They have profound impact on cellular reorganization, signaling, proliferation and differentiation. AFM force spectroscopy provides an unparalleled opportunity to study and analyze those kinds of interactions *in situ* in live cells employing the AFM tip as a nanomechanical probe, for example, to measure differential adhesion to (bio)functionalized surfaces [12,13,115]. By pressing the AFM tip onto the soft cell membrane, cytoskeletal structures and mechanical properties can be accessed in real time [116] and displayed in elasticity maps, as already mentioned in the previous section [111,113].

The investigation of mechanical properties of cells in the area of pharmaceutical research is a continuously growing field, where studying the effects of molecules such as enzymes and hormones [117,118], therapeutic drugs [119] or nanoparticles [120] on cell mechanics can reveal interesting correlations between actions of the agent and membrane alterations or cellular remodeling. For instance, Zuk *et al.* [119] examined the effect of different asthma medications on morphology and elastic properties of red blood cells. When using high concentrations of these drugs, they found evidence that the intended therapeutic effect of increasing opening of breathing arteries is counteracted by a lowered ability of red blood cells to bind and transport oxygen in the human vascular system.

In medical applications, cell elasticity and AFM force measurements may prove useful for detection and diagnosis of diseases, for example, carcinosis. Cross *et al.* showed that cancer cells are substantially softer than benign cells [121], which was confirmed for a number of different human tumors and cancer cell lines [122-124]. It has been suggested that stiffness measurements of tumor biopsies and extracted cells can assist in identifying different cancer stages [125].

5. SMFS to measure binding of drugs and nanoparticles

Measuring nano- and microscale mechanical changes of cells in response to drug and nanoparticle interactions under physiological conditions in adhesion maps have proven to be a powerful approach. However, the most basic level of drug–cell and nanoparticle–cell interaction is at the intermolecular level, and analysis of the binding process on a single-molecule and single-particle level promises new insight into initiation of drug responses in living cells. Over the past 20 years, a range of powerful manipulation techniques have been developed that permit measurement of force and interaction of single molecules ranging from cells to proteins [126-128], where AFM-based SCFS and SMFS are among the most prominent tools [128-130]. The advantages of single-molecule experiments as opposed to ensemble methods (e.g., surface plasmon resonance) are notably the direct visualization and quantification of drug or nanoparticle binding to receptors and intact cell surfaces. The distribution of molecular properties of inhomogeneous systems can be determined by conducting many sequential measurements, which would otherwise be obscured by averaging. Finally, the analysis of rupture forces and dynamic force spectra provides a measure of kinetic rates such as bond energies and lifetimes, and offers a window to explore the entire energy landscape of molecular interactions [15,18]. Thus, SCFS and SMFS techniques provide a valuable new scope to pharmaceutical research in studying the intermolecular mechanisms of drug molecules and nanoparticle-based drug-delivery systems (see [131] for another excellent review).

5.1 Investigations on isolated molecules and nanoparticles

In an interesting approach, Rogueda *et al.* [132] employed force spectroscopy as a tool for characterizing the drug–drug interaction in two commercial inhaler formulations, budesonide–formoterol fumarate dihydrate and salmeterol xinafoate–fluticasone propionate. The interparticle adhesion may negatively affect suspension stability and decrease aerosolization performance, resulting in a decreased dosage emission. Individual drug particles were mounted on tipless cantilevers and probed against model drug substrates. Force curves were recorded to assess the adhesion between budesonide and formoterol particles and salmeterol and fluticasone revealing that the latter had a stronger tendency to interact and form heteroflocculation that potentially results in a decrease in fine particle fraction and thus reduce the dose to the respiratory tract. The results have important implications for development of new metered dose inhaler formulations.

In an early study, others have investigated drug–enzyme interactions in dihydrofolate reductase (DHFR), a key player in biosynthesis that is a target for various anticancer, antibacterial and anitmalarial therapies [133]. They investigated the rupture forces between substrate-bound DHFR and AFM cantilevers-bound methotrexate-modified agarose beads in dependence of pH and made an attempt to elucidate the kinetic parameters of drug–enzyme complex formation by performing dynamic force spectroscopy.

Sulchek *et al.* [134] notably demonstrated the use of dynamic force spectroscopy to study targeted delivery of therapeutics to cancer cells and provided an accurate measurement of the kinetic off-rates that are important determinants of drug

efficiency. In particular, they studied monovalent and multivalent interactions of AFM tip tethered single-chain variable fragments of Abs that specifically bind to the Mucin1 (MUC1) peptide, which is overexpressed in a reduced glycosylation form in a range of solid tumors. The force spectra with the functionalized AFM tips and isolated MUC1 peptide immobilized on the surface showed the superiority of multivalent binding of targeting Ab fragments, indicating extended and more accurate delivery of drugs and molecular labels to target tissues.

Vincent and coworkers [135] employed SMFS to quantify the electrostatic adsorption of transferrin (Tf) to biocompatible cerium oxide nanoparticles (CNPs) as a potential drug carrier for targeted transport and release of drugs to malignant cells with Tf binding receptors (TfR). As changes in local environmental pH during delivery and cell uptake can alter the surface charges and affect Tf binding to CNP, force spectroscopy was used to determine CNP surface charge-dependent adsorption forces of Tf. Although the specific interaction between Tf on the CNP surface with TfR at the cell surface remained unchanged, the binding force of Tf to the CNP surface could be strongly enhanced through protonation of the CNP surface. This ultimately yielded higher numbers of Tf bound to CNPs as confirmed by FTIR and XPS and increased cellular uptake in human lung cancer cells (A549) and normal embryo lung cells (WI-38).

These selective examples underline the significance of force spectroscopy experiments on isolated ligand–receptor and ligand–nanoparticle systems to determine the binding efficiency of different targeted drug-delivery systems to help establish optimal configurations of targeting agents and to guide design of next-generation drugs and molecular labels.

5.2 Monitoring drug/nanoparticle binding to cells

Quantification of ligand–receptors unbinding force has been shown for a large number of molecules such as proteins, nucleic acids, peptides or viruses, with one binding partner tethered to the cantilevered probe and the isolated receptor adhered to rigid substrates such as mica and Au [18]. However, of more relevance to drug–cell interactions are experiments where the target receptor is presented in a native environment, that is, in the cell membrane. Though in earlier work cells were mostly fixed (e.g., [136-139]), today live-cell measurements in buffer solution or cell medium are routinely conducted. Recognition of the capabilities of live-cell SMFS experiments in pharmacological settings is growing. Interesting applications have been demonstrated for the assessment of binding strength of antibiotic drugs to bacterial cell surface receptors [140], therapeutic agents to fungal pathogens [110], targeting of cancer cells by hormone analogues [141,142] or quantification of forces and chemical rate constants for binding of psychostimulants and antidepressants to serotonin transporters [143]. In addition, force spectroscopy with tip-bound micro- and nanoparticles can be used to measure adhesion forces of bare (hydrophobic or charged) [144-146] and bio-functionalized (e.g., antibody conjugated or lectin coated) particles [114,147,148] to the cell surface in order to evaluate their capacity for cellular uptake and delivery of therapeutic molecules. To further emphasize the considerable potential of SMFS for exploring and improving our basic understanding of the interactions of drugs and nanoparticles with cells in the scope of the development of novel targeted drug-delivery vehicles, we highlight two studies conducted in our own lab in cooperation with collaborating groups.

In a pilot study, Oliveira *et al.* have employed SMFS as a screening tool to optimize the density of targeting moieties on polyethyleneimine nanoparticles complexed with DNA towards improving receptor-specific binding of the nanoparticle-based gene-delivery system to ND7/23 neuronal cells [149]. The researchers correlated the targeting capacity of the functionalized DNA-polymer NPs to the binding event probability between complexes tethered to the AFM tip and their binding to the cell surface. In more detail, 50 kDa tetanus toxin fragments (HC) were used as a targeting moiety that are known to specifically bind to the trisialoganglioside GT1b-receptor found in the membrane of cells in the nervous system. HC fragments as well as the HC fragment conjugated ternary nanoparticles were tethered to the AFM tip via a heterobifunctional PEG-linker and probed against isolated GT1b receptor molecules attached to flat mica and against live ND7/23 cells. Force–distance curves showed specific binding events (**Figure 4A**) that could be clearly distinguished from nonspecific adhesion by the characteristic shape of the PEG-linker stretching. The binding event probabilities for HC fragment and HC fragment functionalized nanoparticles on isolated receptors were found to be 16.2 and 22.0% respectively with a dramatic drop after blocking GT1b with free HC fragments as a specificity proof. Specific interactions with ND7/23 cells had a similar probability of around 16% for both fragment and nanoparticles interaction. Dynamic force spectroscopy was performed by varying the tip retraction velocity. The unbinding forces were plotted against the force loading rate (**Figure 4B**) to determine the width of the interaction potential (x_β) and the kinetic off-rate constant (k_{off}) for the dissociation of the HC fragment–GT1b receptor complex. Finally, nanoparticles with different HC fragment densities were tested showing the highest binding probability for 7.5 µg of HC-PEG per 2 µg of plasmid DNA, yielding an optimal ligand density, whereas surpassing this density resulted in a detrimental effect with the reduction of binding probability.

We very recently employed a similar approach to visualize and quantify binding of folic acid (FA) functionalized carbon nanotubes (CNTs) to FA binding receptors in live T24 urinary bladder carcinoma cells in terms of binding force and binding probability [150]. In this experiment, CNTs were first suspended at the AFM tip apex using PEG-linkers and then functionalized with the targeting ligand FA (**Figure 4C**) and then probed against FA-deprived cells. With this configuration, an average binding probability of around 38% and a high frequency of multiple rupture events were observed (**Figure 4C**). The specificity of this interaction was proven

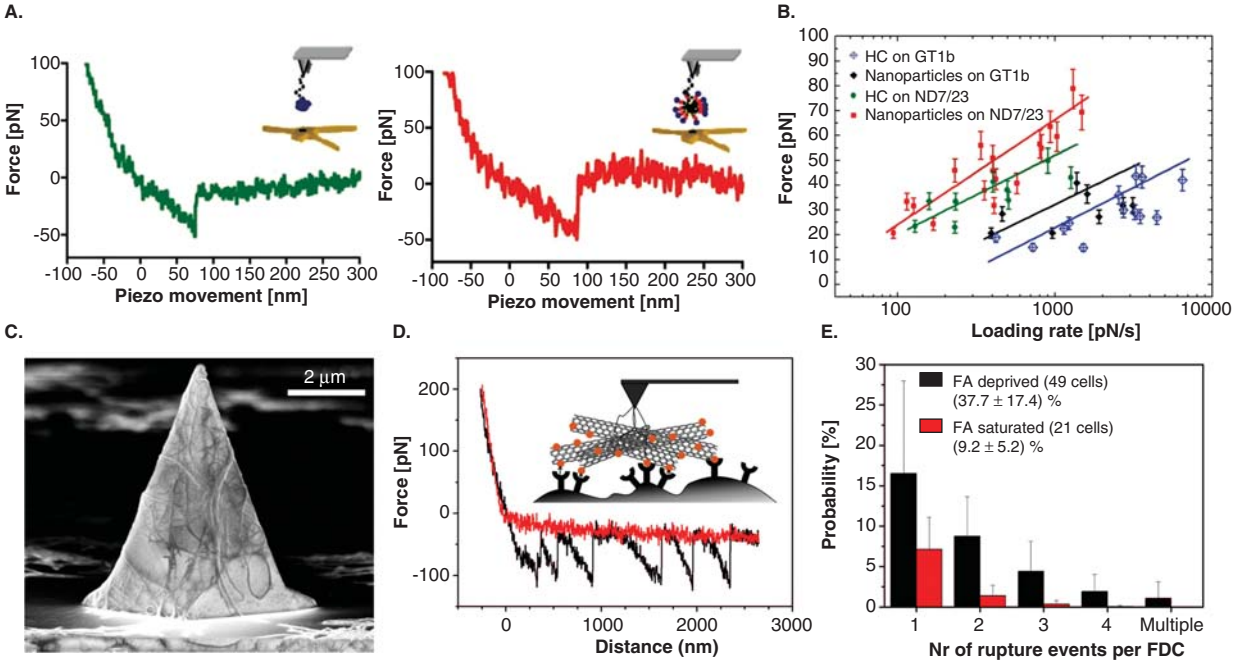

Figure 4. (**A**) Force traces acquired with the AFM in force spectroscopy mode showing single molecular unbinding events between tip-bound tetanus toxin (HC) fragment and neuronal cell (left), and functionalized nanoparticle and neuronal cell (right). (**B**) Loading rate dependence and resulting Bell–Evans fit of the measured unbinding forces considering the interaction between isolated tetanus toxin fragment (HC fragment) and GT1b trisialoganglioside receptor grafted on mica (blue diamonds), ternary nanoparticle and GT1b trisialoganglioside receptor (filled black diamonds), HC fragment and neuronal cells (ND7/23; green circles) and ternary nanoparticle and neuronal cell (red squares). (**C–E**) Binding assay to probe interaction of folic acid (FA) functionalized CNTs with living cancer cells. (C) SEM image of CNT functionalized AFM tip. (**B**) Typical force–distance cycle (FDC) of FA-CNT functionalized tip and folate-deprived T24 cells showing multiple sequential binding events. (**E**) Quantitative comparison of binding probabilities and sequential rupture events for folate-deprived (black columns) and folate-saturated (red columns) T24 cancer cells. A total number of 16,500 FDCs for FA-deprived cells and 13,000 for FA-saturated cells were evaluated using 10 different functionalized AFM tips.

A and B Reprinted with permission from [149].

C–E. Adapted with permission from [150].

AFM: Atomic force microscopy; CNTs: Carbon nanotubes; SEM: Scanning electron microscopy.

though exposure of cells to high concentrations of free FA led to a significant decrease in binding probability (~9%). The receptor-specific targeting and internalization of FA functionalized CNT was further proven using fluorescence microscopy as a complementary technique.

These two examples demonstrate for the first time the use of SMFS for comparative analysis of nanoparticle drug-delivery systems and their interaction with live mammalian cells. They may illustrate AFM's potential as a possible screening tool for the design of targeted nanoparticle systems and drug formulations as a new approach in the growing field of nanomedicine.

6. Conclusion

In this review, we have provided a snapshot of the use of AFM techniques for monitoring drug and nanoparticle delivery with a special focus on imaging and biosensored applications. Our specific aim was to introduce the wealth of experimental possibilities provided by the AFM toolbox to researchers in pharmaceutical and biomedical fields who are new to these techniques. The experiments reviewed here demonstrate the capabilities of AFM imaging to decipher cell responses to drug and nanoparticles exposure, whereas when used as a biosensing and force-measuring tool in SMFS, the interaction between individual drug molecules or whole nano- and microparticles and cell surfaces can be quantified. Exploration of these techniques hold great promise to increase our basic understanding of drug- and drug-carrier interactions with cell membranes and whole cells and their overall role in drug delivery, thereby addressing key questions for the development of new pharmaceuticals.

7. Expert opinion

AFM is becoming a valuable addition to the drug discovery and development toolbox. The highlighted examples in this

review illustrate the considerable potential of imaging applications and force spectroscopy in monitoring the interaction of drugs and (targeted) drug-delivery systems with biological systems, foremost live cells. A few years ago, live-cell experiments required high-level expertise, technologically advanced AFM equipment and were restricted to a few laboratories. Today, commercial (bio-)AFM setups that are mounted on inverted optical microscopes or provided with upright optical configurations are available from several sources. They are equipped environmental chambers and sample stages that offer great flexibility and experimental control. Data processing and analysis has been considerably simplified, as a growing amount of adequate software is more directly integrated with the AFM instrumentation or can be purchased. All these advancements greatly facilitate the access to AFM techniques for scientists who are new to the field. Nevertheless, accurate data acquisition and interpretation still depend strongly on sample preparation, surface chemistry and the quality of the AFM probe and remain the crucial task [151]. These factors can suffer alterations during the experiment, and therefore a basic understanding of the techniques and critical assessment of recorded data are indispensable.

Live-cell imaging in buffer or medium has become a routine task, and temperature control is available for most setups. Nevertheless, they continue to need a great deal of patience and newcomers to AFM will generally need to practice before obtaining good data. Imaging of soft tissues and cells requires the restriction to blunt AFM tips (opening semi-angles 18° or 35°), very low loading forces (< 1 nN) and reduced scan speed (< 1 line/s) to prevent penetration of the cell membrane by the tip. Although dynamic imaging modes (e.g., tapping mode or MAC mode) induce far less shear force compared with contact mode, the viscoelastic response and dynamic nature of the soft cell surface essentially reduce the resolution to ~ 50 – 100 nm. In addition, the AFM tip is easily contaminated by loosely bound molecules (e.g., glycocalyx) or exocytosed material. This might explain why we continue to find publications where cells are not only fixed but moreover dried for imaging to simplify and accelerate the imaging process. Unfortunately, fixation artifacts due to crosslinking of proteins and drying artifacts such as pore formation or deposits due to insufficient washing steps are often enough misinterpreted. Time resolution is a crucial factor that currently limits cell imaging studies with conventional AFM instrumentation. Acquisition of 'high' resolution images takes several minutes, where dynamic processes in biology usually occur at much shorter time-scales. Finally, with regards to cell-uptake studies, it is important to bear in mind that AFM is foremost a surface technique, but the combination with optical setups has significantly widened the observation window.

SMFS and SCFS experiments are relatively straightforward to set up, but here also careful sample and AFM tip preparation and critical data evaluation are of paramount importance [152,153]. First, as the interaction force is generally calculated from the bending of the cantilever, the spring constant has to be known. Although a nominal value is provided by the manufacturer, even the actual spring constant between cantilevers from the same lot may vary tremendously, which makes proper calibration of each cantilever mandatory before use in order to obtain precise force data [154]. Further considerations in force spectroscopy include the separation of specific binding interactions between the molecules of interest from unwanted and nonspecific ones, and their distinction can be challenging even under well-controlled sample conditions. In SMFS, this distinction is facilitated by using, for example, PEG-linkers as described in detail in Section 2.3 and simple standardized tip-functionalization protocols are increasingly available [155]. However, validation of probe functionalization usually happens during the actual experiment to ensure that the tethered ligand is present and correctly oriented so that it can actively bind to its receptor. Also, unequivocal demonstration of binding specificity through, for example, competitive binding that eliminates measurable binding events with soluble agonist, ion-dependent disruption of receptor binding or blocking/cleaving of the tip bound ligand are absolutely necessary. Finally, data interpretation and automation of force-spectroscopy analyses continue to be time-consuming tasks that (partially) require in-house programmed algorithms. Many AFM manufacturers have started to implement analysis algorithms into their data processing software and continue to develop automated routines, which will contribute to making force spectroscopy accessible for pharmacologists, cell biologists and researchers in the biomedical field.

Ligand–receptor interactions on rigid surfaces and on cell membranes were initially acquired 'blindly', that is, force curves were collected at individual, randomly selected sites, with no corresponding image of receptor distribution. The combination of AFM instruments with optical microscopy [156] has greatly eased imaging and force spectroscopy applications on cells with the possibility of independently or simultaneously correlating acquired topographic and fluorescence images [157,158]. The continued development of combined AFM and optical techniques like confocal laser scanning microscopy [159-161] and high-resolution correlative microscopy methods such as total internal reflection fluorescence [162,163] or stimulated emission depletion and stochastic optical reconstruction microscopy [164] will further improve live-cell applications and allow sophisticated experimental settings.

In addition, novel scanning probe methods such as scanning microwave microscopy [165] are important trends that will allow us to probe intracellular structures. Another interesting new development is the combination of AFM imaging with force mapping and spectroscopy [137,158,166-168], which allows the investigator to perform an image scan of the surface and simultaneously acquire adhesion or recognition images, thereby quantifying physical and chemical properties with the same accuracy. Moreover, remarkable advances have been made in developing scanning probe instruments with increased data

acquisition rates, giving access to unprecedented time scales (millisecond resolution) [169]. The ongoing improvements in the field high-speed AFM open up fascinating new perspectives to explore cellular dynamics and monitor drug- and nanoparticle interactions with receptors [170-172]. Increasing parallelism and high-throughput screening in single-molecule force spectroscopy measurements with unattended operation, automatic variation of experimental parameters and automatic data remain a challenge and will be important developments for the future that will certainly have an influence on drug discoveries.

Declaration of interest

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