Biomedical applications of AFM

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Abstract. The atomic force microscopy (AFM) is a technique that is widely used to study the mechanical properties of biological samples in conditions close to natural. In particular, the technique can deliver the information about both the cell stiffness and the unbinding force of individual molecular complexes. Cell stiffness seems to be a global parameter describing the overall changes occurring in the cell structure, particularly in the cellular scaffold called cytoskeleton. The determination of the unbinding forces, acting between a single pair of molecules, brings more specific information about the interaction forces. Both types of AFM measurements can be successfully applied for the detection and quantification of the alterations of mechanical properties in cancerous cells with the special emphasis on the development of a new technique for the cancer detection on a single cell level.

1. Introduction

For many years, scientists have been employing diverse techniques to characterize mechanical properties of cells in their physiological conditions in order to study alterations related to pathological states. In particular, the quantitative description of characteristics for cancerous cells has been of a great interest.

Cancer transformation introduces significant changes in cell structure and behaviour. Alterations in cell growth, morphology, cell-to-cell interaction, and also in cell membrane and organization of the cytoskeleton have been reported [1, 2]. These differences may also induce changes in mechanical properties of the cell, usually leading to higher cell deformability and to distinct adhesive interactions that can be related to the higher invasive capacity of cells. Modified deformability of cells is generally attributed to the altered cytoskeletal organization [3]. Low stiffness of cancer cells may be caused by a partial loss of actin filaments and/or microtubules, and therefore by the lower density of the cellular scaffold [4]. The adhesive interactions involved in all processes occurring either between cells or between a cell and its environment are mostly governed by the binding/unbinding of complementary molecules. Malignant transformation can be also characterized by altered adhesion-dependent responses. The loss or alterations in cell adhesion properties may result in cell
malfunctioning, leading to many pathological states [4]. In addition, many proteins participating in normal biochemical processes are glycosylated, i.e. they have oligosaccharide moieties attached what often leads to changes in their functions. For many tumors, the plasma membrane oligosaccharides linked to proteins or lipids are altered during cancer transformation [5].

There are rather few methods capable to assess cell mechanical properties. Historically, the first technique was the micropipette aspiration [6]. Other researchers have employed the magnetic bead rheology [7], microneedle probes [8], acoustic microscopes [9], and the manipulation of beads attached to cells with optical tweezers [10]. Among these techniques, the atomic force microscopy is able to detect most of malignant changes with a very high resolution, being applied either in imaging mode or as the technique providing information about the mechanical properties of living cells (i.e. their ability to deform and to adhere) in a quantitative manner.

In this paper, the applicability of AFM to studies of cancer transformation, based on the mechanical properties of living cells, is presented. They can describe the cell’s ability to deform and to adhere. The cell deformation (i.e. cell stiffness) is usually quantified by the Young’s modulus while the unbinding force determines the adhesive properties of a cell by characterization of the unbinding forces between a pair of molecules. The quantitative analysis of cell deformability and the expression of adhesion proteins between normal (reference) cells and pathologically changed cells (e.g. cancerous cells), and, more precisely, their characterization (qualitative and quantitative) may have a significant impact on the development of methodological approaches to a detection of cancerous cells.

2. The atomic force microscopy (AFM)

The atomic force microscopy is a technique that bases on the measurements of the cantilever deflection. A sharp probing tip, mounted at the end of a compliant cantilever, moves in a close proximity of the investigated surface. The cantilever senses forces acting between the tip and the sample surface causing its deflection which is usually, recorded by the optical system: the laser beam is focused at the cantilever end and then, after reflection, is detected by a position sensitive photodiode. The position of the beam spot brings information on the cantilever displacement that can be converted into force using Hook’s law [11].

The measurements of the mechanical properties require the AFM working in the so-called force spectroscopy mode where scanning is disabled. In this mode, the cantilever approaches the sample at a given spatial position, contacts it, and when it reaches the maximum deflection value, it is withdrawn backward. The recorded cantilever deflection contains the information about the sample stiffness and also about any adhesive interaction occurring between the probing tip and the investigated surface.

2.1. Cell stiffness measurements

The determination of cell elastic properties is determined on the basis of the force curves representing the dependence between the cantilever deflection (converted into a loading force) and the relative sample position (displacement), usually measured on stiff and compliant surfaces (Figure 1, [12]). When a stiff material (not easily deformable, like a glass coverslip) is investigated, the deflection reflects the position of the sample. This is represented by a straight–sloped line and is usually employed as a reference line needed for the force calibration. For compliant samples like cells, cantilever deflection is much smaller and the resulting force curve has a non–linear character. The subtraction of these two curves leads to
the relation between the loading force and the indentation (a so called force versus indentation curve), which is characteristic for a given material.

\[ F(\delta) = \frac{2}{\pi \cdot \tan(\alpha)} \cdot E' \cdot (\delta')^2 \]  

\[ F(\delta) = \frac{4}{3} \cdot \sqrt{R} \cdot E' \cdot (\delta')^3 \]  

where \( \alpha \) - open angle, \( R \) - radius of curvature of the AFM tip, \( E' \) - reduced Young’s modulus. The reduced Young’s modulus is given by:

\[ \frac{1}{E'} = \left( \frac{1 - \mu_{\text{tip}}^2}{E_{\text{tip}}} + \frac{1 - \mu^2}{E_{\text{sample}}} \right) \]  

For the case of living cells (\( E_{\text{sample}} \ll E_{\text{tip}} \)):

\[ \frac{1}{E'} = \frac{1 - \mu_{\text{tip}}^2}{E_{\text{sample}}} \]  

The Young’s modulus can be a measure of the cell stiffness. Its value is usually evaluated in the frame of the Hertz contact mechanics, by taking into account an infinitely stiff indenter with a selected geometry of the AFM tip (i.e. spherical, paraboloidal, conical or flat–ended) and a flat, deformable substrate [13, 14]. Usually, the AFM probe tip is a four-sided pyramid that can be modeled either by cone or by paraboloid. Thus two formulae are usually applied to describe the relationship between the load force \( F \) and the resulting indentation depth \( \delta \):

– for a conical tip:

\[ F(\delta) = \frac{2}{\pi \cdot \tan(\alpha)} \cdot E' \cdot (\delta')^2 \]  

– and for a paraboloidal tip:

\[ F(\delta) = \frac{4}{3} \cdot \sqrt{R} \cdot E' \cdot (\delta')^3 \]
difficult in determination of the Young’s modulus absolute value. The main sources of errors are as follows:

– the applied physical model. The most popular Hertz model is used under assumption that the cells are purely elastic and isotropic. Cells are characterized by a high level of heterogeneity linked with its internal structures and surface properties.

– the approximation of the AFM tip shape. The four-sided pyramid of the AFM tip is usually approximated by a cone or a paraboloid. The cone matches well to the experimental data for large deformations, but if the measured indentations are small, the paraboloidal approximation can also be successfully applied.

– the assumption should be made concerning the value of the Poisson ratio of cell which is difficult to determine. Very often this value is set to 0.5, since cells can be treated as incompressible material.

– the presence of the stiff substrate below the investigated cells. Its influence leads to dramatic increase of the estimated Young’s modulus value. The strong correlation between the sample thickness and the Young’s modulus value was demonstrated by Domke et al. [15] for gelatin films of different thickness.

– uncertainties of the cantilever spring constant what influences the loading force values.

– the determination of the point of contact between the indenting AFM tip and cell surface what strongly affects the accuracy of the indentation force determination.

All these sources of errors may raise doubts in usefulness of the AFM technique for measurements and analysis of the mechanical properties of living cells. However, first of all, the exact knowledge of the absolute Young’s modulus is not always needed. Very often the relative changes or only observation of the tendency may be sufficient to bring the valuable information on the state of biological material.

2.2. Unbinding force determination

The measurements of the unbinding force between a single pair of molecules require one molecule to be attached to the AFM tip [16, 17]. Then, the modified probe is brought into contact with the cell surface in search of complementary molecules to those immobilized on the AFM tip. The unbinding event (i.e. the moment when the AFM tip detaches from the surface) is recorded during withdrawing of the tip from the cell surface (Figure 2).

![Figure 2](image_url). The unbinding force determination (only retracting part is shown of the exemplary curve). All quantities needed for the unbinding force determination are marked (after [17]).
The value of the force $F$ needed to separate two interacting molecules (defined as an unbinding force) is defined as a difference between the force $F_b$, corresponding to the free cantilever position (when the interacting forces are negligible), and the maximum value of the force $F_a$:

$$F = |F_a - F_b|$$

However, it should be pointed out that the measured single value of the force (delivered from a single force curve) may contain information about two components: i) discrete, short–range component, dominating within the binding site and related to the interaction strength of the single molecular pair and ii) the one originating from long–range, distance–depended forces, dominating outside the binding site. Moreover, the unbinding force measured by AFM depends on the logarithm of loading rate [18]. Therefore, if the results obtained for several samples are planned to be compared, all measurements should be performed at the same loading rate value.

3. Mechanical properties of cancerous cells studied by AFM

3.1. Cell ability to deform

The cell ability to deform is maintained by its internal structure – cytoskeleton – a filamentous network composed of three types of elements i.e. actin filaments, microtubules, and intermediate filaments. The contribution of these elements to the AFM–determined mechanical properties was discussed in several studies where the importance of both the structural proteins of cytoskeleton and cytoskeleton associated proteins was demonstrated [2, 19]. The results have shown that for small indentations (up to 500–1000 nm) the actin filaments are mostly responsible for the mechanical properties of cells, as the disruption of microtubules showed no effect on the mechanical properties of cells measured by AFM.

It has been reported that cells in vitro have the Young’s modulus values in the range of 1–100 kPa [20–22], which encompasses different types of investigated cells, including vascular smooth muscle cells, fibroblasts, bladder cells, red blood cells, platelets, and epithelial cells. Since different cell types are measured, the large modulus variation is fully justified. Despite the lack of absolute value of the Young’s modulus, the obtained relative change of the elastic modulus is sufficient to describe the alterations in cancerous cells. One of the first measurements on a cancer cells was performed in 1993. The human lung carcinoma cells were measured and the obtained Young’s modulus value varied from 13 kPa to 150 kPa [23]. Later on, in 1998, some studies reported that the vinculin–deficient F9 mouse embryonic carcinoma cells had slightly lower Young’s modulus (2.5 ± 1.5 kPa) than the wild-type cells (3.8 ± 1.1 kPa). The authors attributed this change to the altered actin cytoskeletal organization [24], indicating an important role of vinculin as an integral part of the cytoskeletal network. Lekka at al. reported in 1999 the comparative studies of the mechanics of non-malignant and malignant cells [12]. The Young’s modulus of three human cancerous bladder cell lines was one order of magnitude lower than that for the reference cells. These measurements were confirmed in 2005 by Guck et al. [25] and by Park et al. [26]. In the former paper, the authors showed that the cell deformability is sensitive enough to monitor changes during the progression of mouse fibroblasts and human breast epithelial cells from normal to cancerous cells. The latter showed a much wider distribution of the Young’s
modulus of normal fibroblasts than the malignantly transformed cells.

Cell stiffness determined via AFM is a very local feature, showing discrepancy in the Young’s modulus not only if measured on a single cell (Figure 3), but also when determined for a population of cells (Figure 4).

![Figure 3. The Young’s modulus distribution obtained on a single cell (bladder T24 cancerous cells). 20 curves were recorded within a square 4 µm x 4 µm around cell center.](image)

![Figure 4. Histogram of the Young’s modulus calculated for 15 cells separately. On every sample 20-30 curves were taken at different positions on the cell surface.](image)

The experimental errors increase also together with the large degree of sample (cell) heterogeneity. However, despite that, the comparison between different cell types is possible, especially for cancer cells, where large alterations of cell cytoskeleton are expected.

3.2. Cell ability to adhere

So far, the AFM technique has been mostly applied to study the interaction of different molecular complexes for isolated molecules in buffer conditions [27, 28]. However, some attempts have been also focused on studies of receptors present on the living cell surface. Such measurements require that the force to uproot the receptor from the cell membrane is higher than the force necessary to rupture the molecular bonds [29].

The unbinding force measured by AFM is not a fundamental property of a single ligand-receptor complex but it depends on how fast the external force applied to a single bond changes in time (i.e. on loading rate). Studies of the unbinding force as a function of loading rate bring the information on the properties of the energy landscape of the interacting molecules. Such studies, called dynamic force spectroscopy, were applied to investigate the single-molecule interactions on a living cell surface. The most common integrin receptor is the \( \alpha_5\beta_1 \) integrin, which interacts with a fibronectin (FN). An important intrinsic property of the \( \alpha_5\beta_1-\text{FN} \) interaction is the dynamic response of the complex to a pulling force. The AFM measurements carried out for this interaction revealed two distinct regimes during unbinding: a fast loading rate and a slow one, characterizing the inner and outer activation barriers of the complex, respectively. The use of genetically changed fibronectin showed that both inner and outer activation barriers were suppressed by the mutation [30]. In other studies, the adhesion mediated by another integrin (\( \alpha_4\beta_1 \)) has been investigated. The mechanical strength of the interaction allows resisting the large shear forces imposed by the bloodstream [31]. The employed single-molecule dynamic force spectroscopy showed that the dissociation of the \( \alpha_4\beta_1 \) from its ligand complex involves overcoming of at least two energy barriers: a steep
inner barrier and a more elevated outer barrier. The inner barrier grants the complex the tensile strength to withstand large pulling forces (> 50 pN). The outer barrier of the complex was stabilized by integrin activation. Together, these findings can provide a molecular explanation for the functionally relevant kinetic properties of the studied interaction involving α4β1 integrin. Recently, Taubenberger et al. characterized early steps of integrin-mediated cell adhesion to a collagen type I by using single-cell force spectroscopy [32]. In agreement with the role of α2β1 integrin as a collagen type I receptor, the α2β1-expressing Chinese hamster ovary (CHO)-A2 cells spread rapidly on the matrix, whereas α2β1-negative cells adhered poorly. Probing CHO-A2 cell detachment forces over a contact time range of 60 s revealed a nonlinear adhesion response. During the first 60 s, cell adhesion increased slowly, and forces associated with the smallest rupture events were consistent with the breakage of individual integrin–collagen bonds. Above 60 s, a fraction of cells rapidly switched into an activated adhesion state marked by increased up to 10-fold detachment forces. Elevated overall cell adhesion coincided with a rise of the smallest rupture forces above the value required to break a single integrin–collagen bond, suggesting a change from single to cooperative receptor binding. Transition into the activated adhesion mode and the increase of the smallest rupture forces were both blocked by inhibitors of actomyosin contractility.

The cell ability to adhere is mainly governed by the binding/unbinding phenomena between single molecules (i.e. single molecular complexes). The measurement of the unbinding force can provide direct information about the expression of cell surface molecules only at given experimental conditions. This expression can be quantified by two factors. The first parameter is the unbinding force of a single pair of molecules. Its larger value means the more difficult rupture of the molecular complex indicating the more stable complex formed. When a certain ligand type, recognizing the specific structural fragment of its receptor, is immobilized on the AFM probe, variations of the force value indicate the alteration in the binding site structure of the receptor. The other parameter, the unbinding probability, corresponds to the number of molecules that are present on a surface of living cells and can be related to the density of surface receptors.

Several studies have been focused on differences between normal and cancerous cells, where the AFM technique has been shown to be complementary to other biochemical methods [22, 23]. Using AFM, the characterization of the expression of cell surface molecules in different cancerous cells can be used to show either the antigenic identity of receptors, or their dissimilarities, or alterations of the binding site within the same receptor type [33, 34].

As a first example demonstrating the potential of AFM usage in the characterization of the cell surface molecule expression, the studies on the prostate specific antigen (PSA) are presented. This antigen is nowadays widely studied in search of new types of cancer markers that allow better detection of the prostate cancer. In normal prostate, PSA is generally present in soluble form in cytoplasm. During cancer progression, cells begin to express more membranous form of PSA (i.e. prostate specific membrane antigen, PSMA). Investigations of certain growth factors affecting the PSMA expression showed that one of them, estradiol, was able to up regulate the expression of this protein [33]. Parallel to other techniques applied, AFM was chosen as a method giving an additional evidence of the restoration of the expression of membrane form of PSMA in PC-3 prostate cancer cell line that lost the ability to express this protein (Table 1). The presence of PSMA antigen was investigated on the surface of three types of prostate cell lines: LNCaP (androgen-dependent), PC-3 and Du 145 (androgen-independent) with the use of monoclonal antibody. The calculated unbinding force of the PSMA–antibody complexes present on LNCaP, PC-3 or Du 145 was in the range of
58–65 pN regardless of the estradiol treatment.

Table 1. The unbinding force between PSMA and its antibody measured directly on a cell surface of cancer prostate cells in a culture medium containing foetal calf serum (FCS) without or with estradiol (data taken from [33]).

| Cell type | Unbinding force [pN] | Unbinding probability [%] |
|-----------|----------------------|----------------------------|
| Measurement condition | Only FCS | FCS + estradiol | Only FCS | FCS + estradiol |
| LNCaP | 63 ± 27 | 58 ± 25 | 6.2 | 7.5 |
| PC-3 | 61 ± 23 | 65 ± 16 | 2.9 | 6.5 |
| Du 145 | 65 ± 27 | 60 ± 14 | 4.9 | 2.7 |

Taking into account the experimental errors, these results suggest the antigenic identity or a very close similarity of the membrane form of expressed PSMA in all studied cell lines. The calculated unbinding probability (related to the number of PSMA antigens present on cell surface) showed constant number of PSMA on the surface for LNCaP prostate cells, almost two-fold decrease in Du 145, and two-fold increase in PC-3 prostate cells.

The feasibility of AFM to cancer research was also demonstrated for the oligosaccharides expression on a surface of living bladder cancer cells studied with the use of lectins as probes [34]. Sialic acid type oligosaccharides were probed directly on a surface of living cell using AFM with the probing tip functionalized with lectin from Sambucus Nigra (SNA).

![Figure 5](image_url)

**Figure 5.** The unbinding force (left) and the unbinding probability (right) determined in search of the quantification of the expression of sialic acid-type oligosaccharides present on the surface of bladder cells: cancerous T24 and reference ones HCV29 (data from [34]).

The obtained results showed the differences in oligosaccharide’s expression in cancerous cells compared with the reference cells. The smaller unbinding force observed for the same ligand type probed on a surface of cancer cells indicated the alteration of its structure leading to forming non-stable complexes that can be easily disrupted during cancer metastasis. The unbinding probability attributed to the number of formed ligand–receptor bonds, showed the
higher content of sialic acid residues in cancer cells (5.3 % versus 8.4 % for HCV29 and T24 cells, respectively) being a consequence of an increased sialylation of cancer cells [35].

The presented above examples indicate that both parameters (the unbinding force and the unbinding probability) can be used to trace cancer-induced changes of a given pair of molecules, bringing information about changes in a number of cell surface molecules or about alterations occurring within the binding site. One should also point out that the adhesion of cells studied using standard biochemical/biological assays cannot be directly translated to the unbinding force values. This “classical” adhesion is a combination of the unbinding force and the probability.

4. Summary

It is obvious that novel techniques are in the limelight if they are able to bring more precise, local information about cancerous changes as early as possible. The atomic force microscopy is one of such candidates since it has many advantages that make it a valuable tool in biology. The main feature is the possibility to measure biological objects directly in their natural environment, such as buffer solutions or culture media.

The cell elasticity determined via AFM is a very local feature, showing large discrepancy in the Young’s modulus measured on a population of cells as well as on a single cell. In general, malignant cells respond either more elastically (softer) or less viscously to the applied stress. This is because metastatic cells must be squeezed to go through the surrounding tissue matrix when they make their way into the circulatory systems where they are directed to establish distant settlements. These findings suggest that the cell stiffness can be used as a marker and also as a diagnostic parameter for the underlying disease. Therefore, the measurements of Young’s modulus of cells can in future help to determine the range of cytoskeleton changes and allow quantifying them. The cancerous alterations linked with cell stiffness seem to be relatively straightforward and non-specific. They are induced in the whole volume of cells, and therefore are easier to be measured by different techniques.

The cancerous transformation changes also the adhesive properties, therefore, a natural direction for the studies is the determination of changes in cell’s adhesive interactions through measurements of unbinding forces between particular molecules that are well-established (as potential) markers of the disease. The expression of different types of molecules can be studied using several other methods. The most popular are the immunodetection of blotted proteins (i.e. Western blot technique) and fluorescence based methods (fluorescence microscope or flow cytometry). In the former method, the presence of a given interaction can be detected and used for estimation of the molecular mass of the formed complexes. However, these experiments deliver only qualitative information related to bond strength. Furthermore, the cell membrane is usually blocked in order to prevent non-specific antibody binding. This step is not needed in the AFM measurements since the specific interaction is characterized by a single, discrete value of the unbinding force. The fluorescence-based methods require fluorescently labelled molecules. Again, such labelling is not needed in AFM, where if there are no specific requirements, the native protein is immobilized using a rather simple protocol. Applying fluorescence, the detection of bound complexes is relatively easy. However, the quantitative information about their number (or density), and about the strength of interaction is indirect. These parameters can be estimated only by measuring fluorescence intensity. Therefore, the AFM measurements of the unbinding forces are of great interest for many biologists since, in contrast to standard biochemical or cell biology methods,
this technique can be used not only for the detection of molecules on the cell surface but also it gives additional information about the physical properties of the studied interaction.

5. References

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