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Scale-independent roughness value of cell membranes studied by means of AFM technique

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ABSTRACT

The roughness of cell membrane is a very interesting indicator of cell's health state. Atomic Force Microscopy allows us to investigate the roughness of cell membrane in great detail, but the obtained roughness value is scale-dependent, i.e. it strongly depends on measurement parameters, as scanning area and step size. The scale-dependence of the roughness value can be reduced by means of data filtration techniques, that are not standardized at nanometric scale, especially as far as biological data are concerned. In this work, a new method, based on the changes of values of some roughness parameter (root mean square roughness and skewness) as a function of filtration frequencies, has been implemented to optimize data filtering procedure in the calculation of cell membrane roughness. In this way, a root mean square roughness value independent of cell shape, membrane micro-irregularities and measurement parameters can be obtained. Moreover, different filtration frequencies selected with this method allow us to discriminate different surface regimes (nominal form, waviness and roughness) belonging to the raw cell profile, each one related to different features of the cell surface.

1. Introduction

The roughness of plasma membrane is an important cytological parameter, because it is involved in several cellular mechanisms as motility, adhesion, intracellular contact, etc. [1–3] and consequently, it is a sensitive indicator of the cell's health state. Indeed, it has been used to monitor the damages caused to the erythrocytes by artificial organs [4], the action of antimicrobial peptide PGLa on bacteria [5], the changes induced in cardiomyocytes by aldosterone [6] and the red blood cell aging [7].

The use of the membrane roughness as cytological parameter is related to the capability to get high resolution topography of cell's surface. This can be mainly obtained by the Atomic Force Microscopy (AFM) technique, which provides images of cellular surface with a lateral resolution of a few nanometers and a vertical sensitivity of 1 Å without damaging the cellular structure [8–11].

Despite of this, the use of roughness values to characterize cell membrane is not widespread, because of the large variability of roughness value calculated from the height data of cell profiles. In fact, it is well-known that the roughness value is dependent on scanning parameters [12–15]. In particular, it strongly increases with the scanning area, i.e. it is 'scale-dependent'. Indeed, the resolution of the measurement (defined by scan step and probe size), as well as the scan length, might cause the inclusion or exclusion of some surface features. So, the value of roughness parameter in cytological field is rarely used because of the ambiguity about which surface features it is related to, of its poor reproducibility (for the variability of the cell structure) and of its scale-dependence.

In surface metrology, such problems have been overcome by standardizing as ISO norms the parameters, procedures and instrumentations of roughness measurement [16–18]. In particular, these norms point out a filtration procedure of the height data in order to cut-off the data related to long wavelength (low frequency) sampling and to obtain a profile containing only high frequency data, from which it's possible to calculate the roughness parameters. This means that, using the same cut-off frequency for the filtration procedure, the calculated roughness would be the same at all scan lengths (if the same resolution has been preserved). Unfortunately, the standards defined by ISO norms concern the milli- and micro-scale roughness and no indication exists for the nano-roughness domain. Even if some efforts have been made in the analysis of roughness for inorganic surfaces to adapt the standard methods to the nanoscale regimes [19], no application of this procedure is known in the cytological field yet. In this work, we investigate the possibility to extend the standard method for the calculation of roughness parameters of inorganic surfaces to cellular surfaces, although the variability of structure, size and surface features according to the type of cell prevent a real standardization of membrane roughness measurements. For these reasons, a new procedure for the selection of scanning and filtering parameters is proposed and applied to two different kinds of cell. The obtained results demonstrate that not only the proposed method strongly reduces the dependence of roughness value on the scale length, but it is also useful to characterize different-size features of the cell surface.

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2. Roughness computation theory

In this section, the main items about one-dimensional roughness theory are reported: general definition, computation with and without filtration methods and scale-dependence issues.

2.1. Roughness parameters

The roughness of a real surface is evaluated from the deviations of the two-dimensional measured profile (z values measured by means of the AFM technique on the surface) with respect to an ideal surface. Such deviations can be classified in three groups (which hereinafter will be named 'regimes'), depending on the value of the irregularity steps [20], as shown in Fig. 1, where the total profile (Fig. 1a) includes the contributions of:

- *Nominal form*: corresponding to macroscopic deviations from the mean line profile (nominal shape), cleaned out of the irregularities and characterized by a big sampling step P_f (Fig. 1b);
- *Waviness*: irregularities with large amplitude and step P_w (Fig. 1c),
- *Roughness*: irregularities with small amplitude and step P_r (Fig. 1d).

Several parameters have been defined to characterize the irregularities, all described by the ISO norm 4287 [16]. The most interesting one is the Root Mean Square Roughness R_{RMS}, as it is very sensitive to isolated deviations from a regular profile. It is calculated from the following relation:

$$R_{RMS} = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\left|z^2{}_i\right|} \tag{1}$$

where n is the number of data points and z_i is the height deviation of i-th point from a mean line, defined so that arithmetic sum of all z_i is equal to zero.

Another useful roughness parameter is the Skewness (R_{sk}), a statistical parameter that measures the asymmetry of the Amplitude Distribution Function, that is the probability density of the profile heights. The skewness R_{sk} is defined according to the following equation:

$$R_{sk} = \frac{1}{(R_{RMS})^3}\frac{1}{n}\sum_{i=1}^{n} z_i{}^3. \tag{2}$$

The skewness characterizes very well surfaces with different structures, which could even have the same arithmetic roughness values [21]. In particular, a negative R_{sk} value means that a larger number of valleys with respect to peaks are present on the surface profile, while a positive R_{sk} value means that the presence of peaks is prevailing [20].

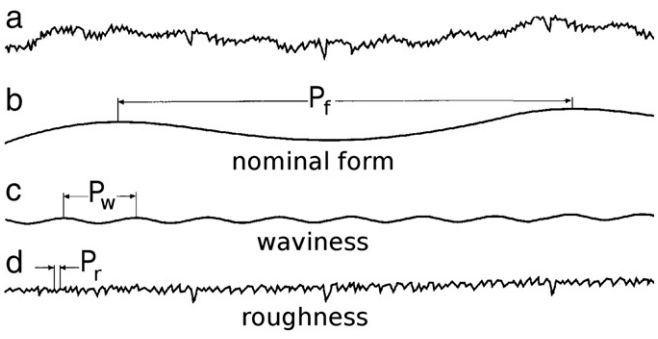

Fig. 1. An example of a graph describing a generic line profile (a) consisting of the overlapping of (b) the nominal form, (c) the waviness and (d) the roughness. In b), c) and d) the sampling step is indicated as P_f, P_o and P_r, respectively.

2.2. Digital filtering theory applied to roughness computation

The calculation of the roughness parameters from the height data measured by means of the AFM technique (raw data) provides values containing information of all kinds of irregularities discussed above. A proper application of digital filtering helps to separate the features related to the nominal form (low frequency information) from those of the waviness and roughness (medium and high frequency information) respectively. To operate a proper data filtration, it is particularly important to choose a suitable cut-off frequency f_c.

The general filtering procedure used in surface metrology is based on the use of Discrete Fourier Transform (DFT), which is applied to the M data points measured along a scan line of length L (with a scan step $\Delta x = L/M$). The transfer function $H(\omega)$, which contains all the signal frequency components, is expressed in terms of the normalized frequency ω_p (with $p = 0,\ldots M-1$; $\omega_p = p/(\Delta x\, M)$) in the following way:

$$H\left(\omega_p\right) = \sum_{k=0}^{M-1} h(k)\ \exp\ \left(-j2\pi\left[\frac{p}{M}k\right]\right). \tag{3}$$

The transfer function is designed to form high-pass, low-pass, band-pass and band-stop filters with appropriate cut-off frequencies, just depending on the desired frequency components.

The function $h(k)$, which is the inverse DFT of $H(\omega)$, is a filter impulse response function.

$$h(k) = \sum_{p=0}^{M-1} H\left(\omega_p\right)\exp\left(j2\pi\left[\frac{p}{M}k\right]\right) \tag{4}$$

From the convolution of the impulse response with the original data matrix $z(x)$, is possible to obtain the final filtered profile $f_{iltered}(x)$, from which the roughness parameters can be calculated.

$$^{z}filtered(x_i) = z(x_i) \times h(x_i) = \sum_{k=\left(-\frac{M}{2}+1\right)}^{\frac{M}{2}-1} h(k)z\left(x_{i+k}\right) \tag{5}$$

with $i = 0,\ldots,M-1$ [19,20].

A critical point for evaluating the roughness parameters is a proper choice of the scanning parameters, as scanning length and sampling step, in order to measure the desired features.

The scanning parameters are also important in the filtering procedure since, in spatial frequency components, the extent of spectral range of the Fourier space is defined by Δx and L. In fact, the low and high frequency limits of the spectral range are [15] $\omega_l = 1/M\Delta x$ and $\omega_h = m^{-1}/2\Delta x$ (Nyquist limit), respectively, where m is the frequency index varying between 0 and M/2.

The low frequency limit, ω_l, is determined by the length of the profile $L = M\Delta x$ and it plays a fundamental role in the problem of the scale-dependence. In fact, the longer the scanning length, the more the features included in the length profile, the larger the computed R_{RMS} value. For each analysed profile, there will be a length value that will include all the bigger surface features: for length values larger than this one, the R_{RMS} will remain quite constant. This is just the result obtained when R_{RMS} values of raw unfiltered data are calculated [13,14]: in this case the R_{RMS} includes contribution from all the three surface regimes, among which the larger ones (that is waviness and nominal shape) dominate.

On the other hand, the high frequency limit, ω_h, is related to scan step value Δx, which depends on the instrumental resolution. In fact, it is quite obvious that the AFM microscope has to be calibrated to resolve the scan step size. So, if the curve radius of the tip is larger than Δx, the tip's geometry acts as a low pass filter by cutting out the high spatial frequencies of the surface morphology [15].

3. Materials and methods

3.1. Cells

Two kinds of cells have been investigated: murin microglial cells (BV2) and keratinocytes from a normal human primary cell line (HUKE). The BV2 and HUKE cells were provided by ATCC (United Kingdom) and Zooprophylactic Institute of Brescia (Italy), respectively. They were separately grown in RPMI 1640 and Epilife medium, respectively, supplemented with a proper Medium Supplement, incubated at 37 °C and 5% CO_2. The samples for AFM measurements consisted of cells seeded on poly(lysine)-coated glass coverslips, washed three times in phosphate-buffered saline (PBS) and fixed in paraformaldehyde 3.7%. The samples were stored in petri dishes filled with PBS solution until AFM images were performed. Before such measurements, cells were rinsed twice in deionised water, in order to remove residual PBS from the surface of the cells. AFM measurements of BV2 cells were carried out on both untreated cells and cells exposed to an aqueous solution of a Deltamethrin-based commercial pesticide, prepared at a cytotoxic concentration ($5 \cdot 10^{-6}$ M of Deltamethrin), as assessed by MTT method.

3.2. Atomic Force Microscopy

A Perception (Assing S.p.A., Italy) Atomic Force Microscope was used to record AFM images. The measurements were performed in air, with the microscope working in the weak repulsive regime of contact mode. Aluminum coated Si_3N_4 cantilevers (model MSCT from Veeco) with a spring constant of 0.01 N/m and Silicon tips with a nominal apical radius of 10 nm were used.

The raw AFM data of a cell profile have been filtered with a mathematical procedure implemented in the free software Gwyddion [22]. Such a software computes several standard roughness parameters at different high-pass cut-off frequencies, which can be selected inside a range of frequencies (normalized to the Nyquist one), from 0 (no-filter) to 0.3. In this specific case, in spatial terms, the use of Nyquist value means that the data are automatically high-pass filtered for wavelengths greater than two times the scan step, in order to avoid measurement uncertainty (due, for example, to instrumental noise).

4. Results and discussion

BV2 cells have been analysed to build up the procedure for the computation of plasma membrane roughness described by R_{RMS} parameter. Such cells are relatively small (10–30 μm in diameter), they appear club-shaped, as shown in Fig. 2, and their surface results very smooth, as shown in the inset of Fig. 2. Ten of BV2 cells have been analysed by randomly measuring several areas over each cell surface.

Firstly, the well-known scale-dependence of R_{RMS} has been tested. In fact, seven topographical images have been acquired for each cell at increasing scan lengths (from 1 to 12 μm) and at constant scan points (256×256), so that resolution decreases with increasing scan length, causing the loss of some detailed features of the surface (aliasing). The value of R_{RMS} has been calculated for each image on the same line profile (accurately included in all the images). The obtained results for a typical BV2 cell are shown in Fig. 3: it is clearly visible the increase of R_{RMS} with increasing scan length, due to the contribution of large features (low frequency information). Similar trends have been observed in all the investigated cells, in agreement with what reported in the literature [13,14,19,23].

4.1. Choice of scanning parameters

Before approaching any filtration procedure, a proper choice of scanning parameters has to be done. In fact, the choice of the scan length and scan step values should be related to the lateral size of the interested features. The scan area should be chosen larger enough to include a representative sample of features, and the scan step should be chosen little enough to resolve each single feature.

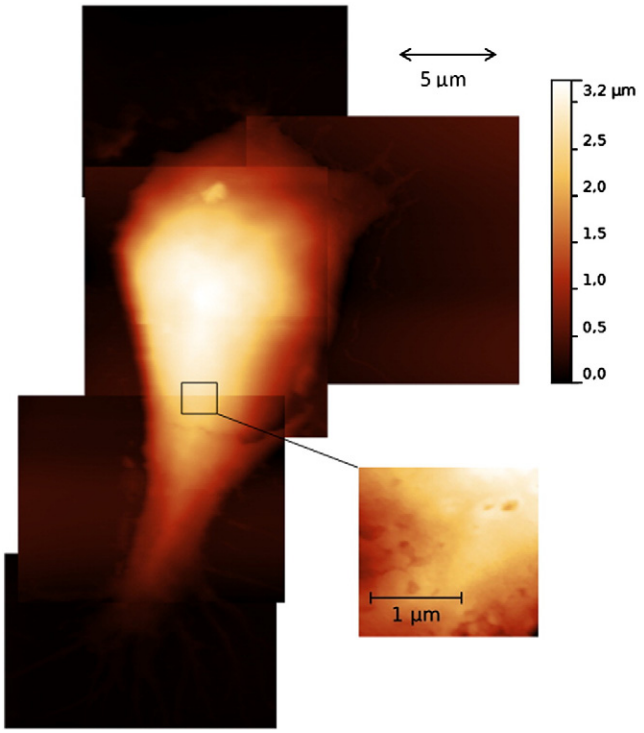

Fig. 2. AFM topography image of a microglial cell; the inset shows a particular of the cell membrane with evident micro-irregularities. Scale bars of the pictures are reported.

The importance of such scanning parameters can be pointed out by considering what happens when the same filtration operations are applied to a line profile measured with different scan length and scan step. In Fig. 4, two profiles scanned with different lengths and resolutions (dashed lines) and filtered with the same cut-off frequency value (continuous lines), are shown. In particular, Fig. 4a shows a profile 12 μm long, scanned with 256 points (scan step $\Delta x = 46.9$ nm) and Fig. 4b shows a profile 2 μm long which is also scanned with 256 points (scan step of 7.8 nm). A filtration procedure has been applied to both profiles, using an arbitrarily chosen value of cut-off normalized frequency $f_c = 0.04$. In the first case all features sampled with steps λ longer than 2345 nm ($\lambda = 2 \cdot \Delta x / f_c$) have been removed (i.e. cell profile shape). On the contrary, in the second case, using the same filtering value, features larger than 390 nm have been removed (i.e. the contribution to the profile of the micro-irregularities or waviness). Therefore, R_{RMS}

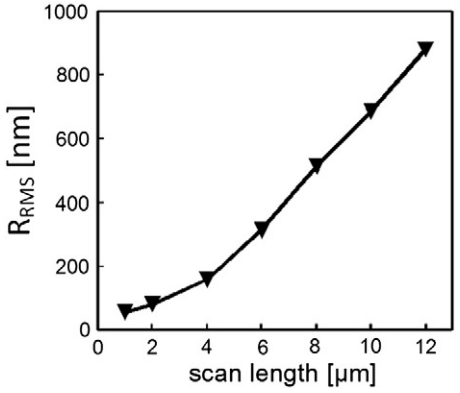

Fig. 3. R_{RMS} variation as a function of scan lengths for a typical microglial cell. The values have been calculated on the same line profile scanned seven times at increasing scan lengths.

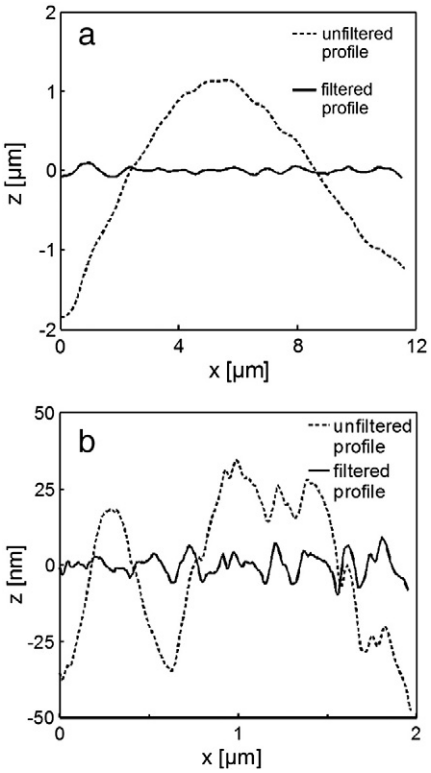

Fig. 4. a) An unfiltered (dashed line) 12 µm profile scanned on a microglial cell and a filtered one (continuous line) with a cut-off normalized frequency of 0.04; b) an unfiltered (dashed line) 2 µm profile scanned on the same cell line as in a) and a filtered one (continuous line) with the same cut-off frequency value.

parameters computed on the two filtered AFM profile have different meanings.

In the first case (Fig. 4a), the R_{RMS} parameter calculated on filtered profile has a value of 36 nm, instead of 881 nm calculated for raw data. This parameter characterizes the micro-irregularities of the cell profile (superimposed to the membrane roughness information) and it can eventually be used to monitor their variation in different cell conditions.

For the profile of Fig. 4b, the R_{RMS} value calculated for filtered profile characterizes the plasma membrane roughness: its value is of 3.5 nm. On the other hand, the R_{RMS} value calculated for raw data is 22.7 nm and it refers to micro-irregularities with a superimposed contribution of nominal form (related to cell shape).

Some quantitative indications about the choice of sampling parameters for roughness computation of inorganic surfaces are reported in ISO norms [17]. They are taken from empirical observations and stated that a good 'evaluation length' (the length of profile used to compute roughness parameter) should be long approximately 5 times the 'sampling length' (the 'length of assessment' over which the surface roughness can be considered representative [17]). This latter parameter represents the 'meter cut-off length' and, for inorganic surfaces, it is quantitatively defined in several ways [20]. Variability and complexity of the cells require a flexible method to compute the 'meter cut-off length' to be used in the filtering procedure. This problem will be handled, in frequency terms, in the next section.

4.2. Choice of the cut-off frequencies

In the above example, an arbitrary cut-off frequency has been used. On the contrary, a reliable method to choose the cut-off frequency should be investigated in order to get a standard procedure of roughness calculation for biological samples. To this aim, several AFM images of BV2 cells have been recorded, with a scanning area of 2 µm × 2 µm, scan step of 7.8 nm and by using a probe with a nominal curvature radius of 10 nm. As the lateral resolution is limited by the probe size, it isn't worth decreasing the scan step furtherly, as it would result in redundancy in consecutive points, with an overall decreasing effect on roughness value (data not shown). Afterwards, a line profile of 2 µm length has been arbitrarily chosen inside the scanned area of each investigated cell. For each profile, the raw AFM data have been filtered many times at various cut-off frequencies f_c, from 0 (no-filter applied) to 0.30, with a step of 0.01. For every filtering process, the R_{RMS} and R_{sk} values have been evaluated.

The results obtained for a typical microglial cell are reported in Fig. 5, where a comparison of the trends of the R_{RMS} and R_{sk} parameters as a function of cut-off frequencies f_c is shown. In particular, the R_{sk} values have been compared even with respect to the zero line. Fig. 5 shows that it's possible to select some cut-off frequency values (corresponding to the vertical lines) at which the R_{RMS} curve presents slope variations and the R_{sk} curve has cross-over points with the zero-line (dashed horizontal line). In other cases (not shown), the R_{sk} curve presented a maximum point near the zero line (and not a cross-over as in Fig. 5) in correspondence of the slope change of R_{RMS} curve. Such abrupt changes of R_{RMS} and R_{sk} curves correspond in the cell profile to the filtration of the information related to a certain surface wavelength regime. So the first cut-off value in Fig. 5 (0.04), can be used to filter the information relative to the cell shape, and the second one (0.07) to filter the cell shape and the micro-irregularities information. On the other hand, the largest cut-off frequency shown in Fig. 5 (the 0.16 value) has not been considered, since it is related to features too near the resolution limit of the measurement. The graph in Fig. 5 is a typical one among those obtained from ten different microglial cells. The lowest cut-off frequency, among those selected with the previous method, has been found between 0.02 and 0.04 for seven cells, indicating a weak or strong influence, respectively, of cell slope on the 2 µm profile (randomly scanned, both over a plane and a sloped region). Another value of the cut-off frequency has been found in the range 0.06–0.08 for nine cells. So, choosing the 0.07 value as cut-off frequency, a profile filtered from nominal form and micro-irregularities features and containing only membrane roughness information can be reconstructed: the roughness parameters can be computed for it.

An example of calculation of roughness parameters related to different surface regimes is shown in Fig. 6, where the cell profile indicated by the continuous line in the picture a) (2 µm long) has been filtered two times at the cut-off frequency values of 0.04 and 0.07. In Fig. 6b the original profile (continuous line) and the profile subtracted to the raw data

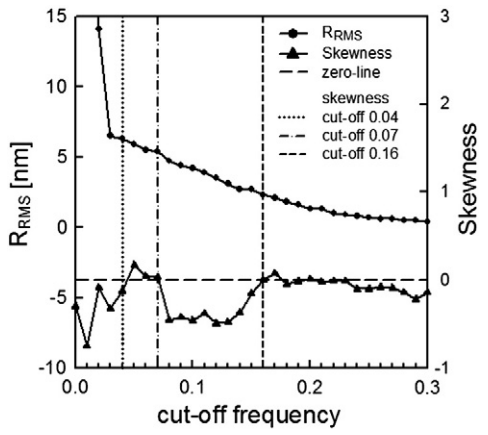

Fig. 5. Filtered R_{RMS} (circles) and R_{sk} (triangles) values calculated for the same line profile of a BV2 cell as a function of normalized cut-off frequency values. Vertical lines indicate the cut-off values at which the two curves have simultaneously slope variations (R_{RMS}) and cross-over of zero line (R_{Sk}).

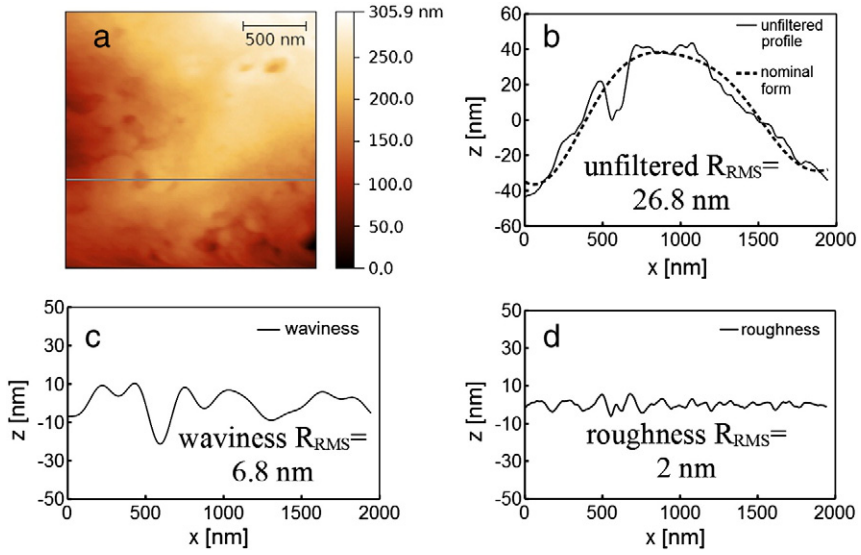

Fig. 6. a) AFM image of a microglial cell, for which filtering procedure at cut-off frequency values of 0.04 and 0.07 has been carried out. b) The unfiltered profile (raw data) and the cell shape removed in the first filtration (cut-off value of 0.04) are illustrated. c) The filtered profile obtained with the first filtration process is shown. d) The filtered profile obtained filtering the raw data with the cut-off value of 0.07 is presented. In each case the R_{RMS} value has been calculated and reported for comparison.

(dashed profile) during the first filtration at 0.04 are plotted. The filtered profile obtained with this filtration procedure is presented in Fig. 6c, containing the information related to the micro-irregularities (waviness) and to the roughness of the cell membrane. In Fig. 6d, it is shown the filtered profile obtained with the cut-off frequency of 0.07, containing only the information related to the membrane roughness. The obtained R_{RMS} values were 26.8 nm for the unfiltered profile, 6.8 nm for waviness and 2 nm for the roughness profile. Such values show that cell shape and micro-irregularities dominate the unfiltered roughness parameter.

The above procedure represents a quite easy and reasonably method to select the proper cut-off frequency value for filtering the raw data.

After such selection of the different surface regimes, a R_{RMS} value representative of the membrane of microglial cells can be estimated. Using the scanning and filtering parameters previously chosen, the R_{RMS} value has been calculated as the average of about 200 R_{RMS} values computed for ten microglial cells (scanning for each cell a number of 15–20 profiles at least in three different cell areas). The R_{RMS} value obtained is 1.90 ± 0.09 nm, whereas the unfiltered R_{RMS} value is 16.37 ± 0.71 nm.

The most important result obtained with the proposed method is shown in Fig. 7, where the R_{RMS} values estimated by filtering the data obtained from AFM measurements of a typical BV2 cell with different scan sizes are shown. The R_{RMS} value results almost independent on the scan size. Even if the scale-dependence is not completely removable from roughness measurements, the proposed filtration method reduces such variability of more than one order of magnitude. In fact, in the unfiltered case the R_{RMS} value is 56.5 nm at 1 μm scan length and 881 nm at 12 μm scan length. On the other hand, the R_{RMS} value filtered with the cut-off frequency of 0.07, although varies from 1.9 nm to 25 nm, rapidly tends to a "plateau" value at about 4 μm scan length. The slight R_{RMS} increasing from 1 to 4 μm is due to the change of resolution (it decreases with increasing scan length) and also to residual scale-dependence. However, the obtained drastic decrease of the scan length dependence is sufficient to indicate the new filtered roughness value as 'scale-independent'.

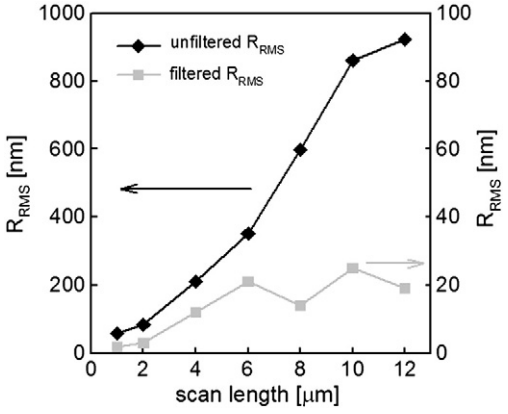

Fig. 7. Estimated R_{RMS} variation as a function of scan lengths. The diamonds corresponds to R_{RMS} values computed on unfiltered profiles, the squares correspond to R_{RMS} value computed on filtered profiles. In the unfiltered case the roughness scale-dependence is evident and it nearly disappears in the filtered case.

4.3. Cell characterization by means of scale-independent roughness parameter

In the following, two examples of the use of scale-independent roughness parameter in cell characterization will be introduced. In particular, firstly an investigation about assessment of cell damages after exposure to a toxic chemical will be presented. Then, the possibility to characterize two different cellular features at the same time will be explored.

4.3.1. Assessment of cell membrane damage induced by exposure to a toxic chemical

The BV2 cells have also been exposed to a commercial pesticide compound (as described in 'Materials and methods'), in order to evaluate the changes of the cell membrane features, due to the action of the chemical, by monitoring the membrane roughness value. In Fig. 8 an AFM image of an exposed cell (Fig. 8a) and a 2×2 μm^2 detail of the same cell (Fig. 8b) are shown. It is evident that the chemical induces damages on the cell structure, resulting in cell profile irregularities. Therefore, the calculation of the roughness parameter on an unfiltered cell profile corresponds to sample structure irregularities and consequently, in this case the damages of the cellular structure will be characterized. On the contrary, the employ of the scale-independent roughness parameter, obtained by computing the R_{RMS} value on a filtered profile, allows us to evaluate the effects of the chemical only on the plasmatic membrane. In order to evaluate the membrane roughness, ten different glial cells have been measured,

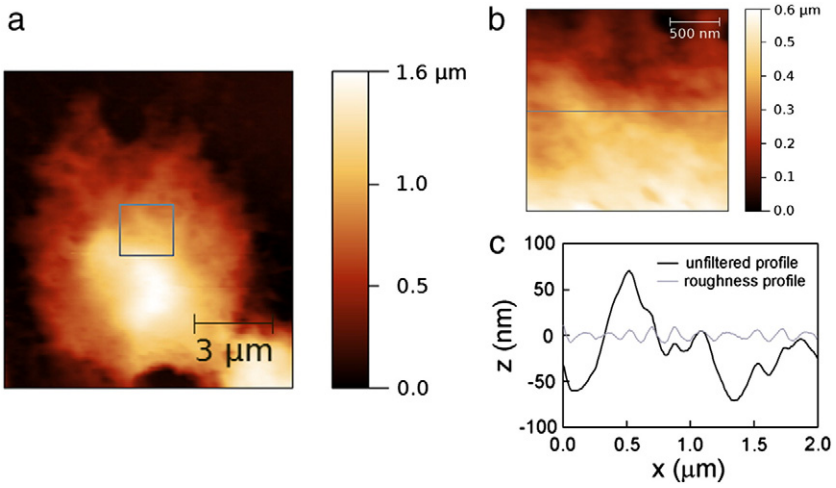

Fig. 8. AFM image of a BV2 cell exposed to a pesticide compound containing Deltamethrin at the cytotoxic concentration ($5 \cdot 10^{-6}$ M) (a). The image in the inset (b) corresponds to the squared area in (a). In c) the profiles taken on the line drawn in b) (black line) and the same profile filtered at the cut-off frequency 0.07 (gray line) are shown.

obtaining 100 profiles 2 µm long (scanned with 256 points). The profiles have been filtered at the same cut-off frequency value used for the untreated cells (0.07). In Fig. 8c typical profiles, before (dark line) and after (gray line) filtration procedure, are shown. The R_{RMS} value results 5.08 ± 0.23 nm. This value, if compared with 1.90 ± 0.09 nm obtained for the untreated cells, reveals an increase of membrane roughness after exposure. This result suggests a modification of the membrane ultrastructure, according to previous works [24–27].

4.3.2. Use of filtered roughness to monitor different surface features simultaneously

During the filtration procedure, a part of data (related to nominal form and waviness profile) are filtered out in order to get a profile containing only the information about membrane roughness. However, such data, particularly the waviness ones, can be useful in the characterization of cell micrometric features as apoptotic structures [28], microvilli [29] and surface depressions caused by cytoskeletal alterations [30]. The waviness R_{RMS} (that is the root mean square roughness value computed on the waviness profile) can be an interesting parameter when it can be unambiguously related to a specific feature of cell surface. On the contrary, if more than one surface feature with similar lateral size (comparable with the waviness wavelength cut-off) is present on the cell surface, no specific meaning can be ascribed to the waviness R_{RMS}. So, using the method proposed in this work, it is possible to extract from the same profile (provided that the same scanning parameters are able to resolve both types of features at the same time) two kinds of information: one related to the membrane ultrastructure and the other one related to the micrometric surface features, without any restriction in the selection of the area to be scanned. This can be done by filtering the original profile twice at two different cut-off frequencies, both selected with the proposed method, and computing on the 'roughness profile' and on the 'waviness profile' two different R_{RMS} values.

To test this possibility, several HUKE cells have been measured by the AFM technique: in Fig. 9 a typical cell is shown. The topography images show that the cells are covered by microvilli, as it is evident in the inset of Fig. 9. The microvilli are microscopic cellular membrane protrusions that increase the surface area of cells and are involved in a wide variety of cellular functions, including absorption, secretion, cellular adhesion, and mechanotransduction. Therefore, the monitoring of the density of microvilli results interesting to evaluate the functionalities of the cell. The application of the exposed method to HUKE cells yields the results shown in Fig. 10, where the results about R_{RMS} and R_{Sk} parameters for a typical cell among ten cells tested are reported. In all the cases two frequency ranges have been identified, from 0.01 to 0.04 (with 0.03 as the more frequent value) and from 0.06 to 0.10 (with 0.09 as the more frequent value). Then, we selected the frequency values of 0.03 to cut-off the information related to cell shape (in order to evaluate the microvilli density) and 0.09 to cut-off the information related to cell and microvilli shape (in order to evaluate the membrane roughness). The selection of the surface information made by means of the filtration procedure is shown in Fig. 11. The profile drawn in Fig. 11a (3D visualization of the 2×2 µm^2 image in the inset of Fig. 9) has been filtered two times at 0.03 (Fig. 11b gray line) and at 0.09 (Fig. 11b dark line) cut-off frequencies. The roughness profile contains the information related to roughness of the plasmatic membrane (covering also the microvilli surface) and the waviness profile clearly reveals the microvilli outlines. From the latter profile the microvilli density can be approximately evaluated by means of the waviness R_{RMS} value). In Fig. 12 the scale-dependence of unfiltered (circles), waviness (squares) and roughness

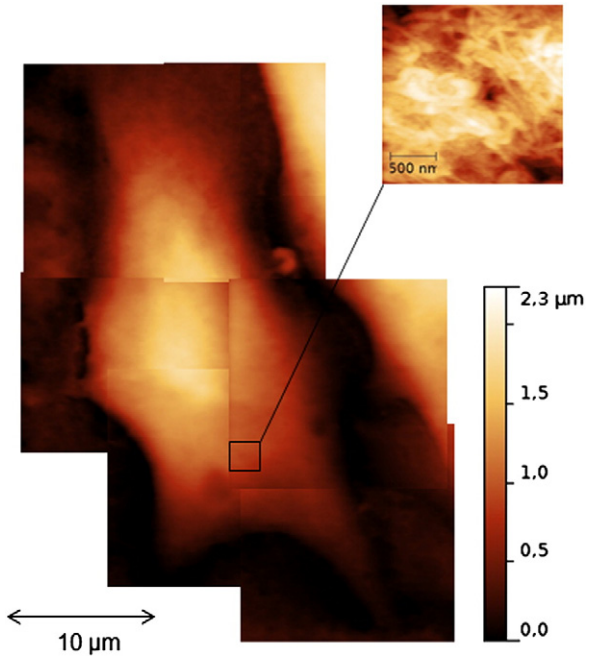

Fig. 9. AFM image of a keratinocyte cell; in the inset a cell area where the presence of microvilli is shown. The scale bar of the inset is 500 nm.

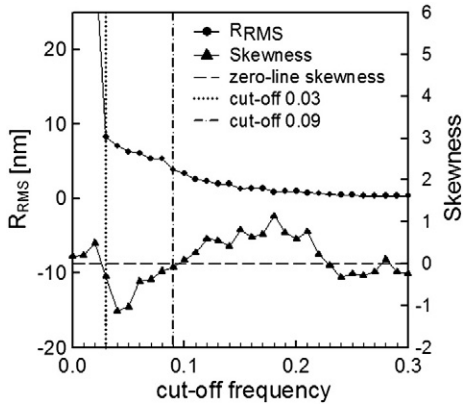

Fig. 10. Filtered R_{RMS} and R_{sk} values calculated for the same line profile of a HUKE cell as a function of normalized cut-off frequency values. Vertical lines indicate the cut-off values at which the two curves have simultaneously slope variations (R_{RMS}) and cross-over of zero line (R_{Sk}).

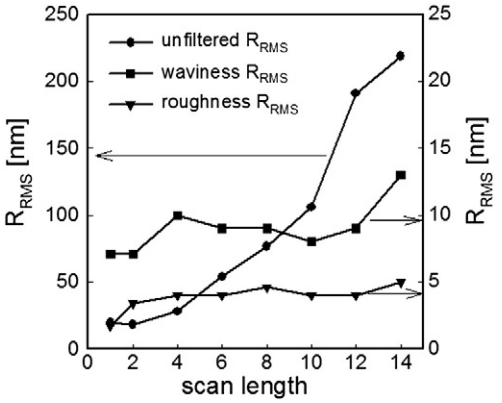

Fig. 12. Estimated R_{RMS} values as a function of scan lengths for unfiltered profile (circles), waviness profile (squares) and roughness profile (triangles). In the unfiltered case the roughness scale-dependence is evident whereas it nearly disappears in the filtered case.

(triangles) R_{RMS} has been shown, demonstrating that the filtered R_{RMS} values result quite scale-independent. In particular, the roughness R_{RMS} is quite constant between 2 μm and 12 μm, while the waviness R_{RMS} is quite constant between 4 μm and 12 μm. In fact, the 1 μm and 2 μm profiles are too short to appreciate micrometric surface features, whereas the 14 μm profile has a scan step too large to sample nanometric and micrometric surface features.

A total number of 140 profiles have been analysed by scanning 6 different cells (2 μm × 2 μm scan area). By using the selected filtering parameters, the R_{RMS} values obtained to characterize the presence of microvilli and the membrane roughness are 8.31 ± 0.2 nm and 2.44 ± 0.06 nm respectively (whereas the unfiltered R_{RMS} value was 25.34 ± 0.74 nm).

Another interesting result is the trend of the relative standard deviation (also known as Coefficient of Variation CV), which is a normalized measure of the dispersion of the data distribution [31]. It has been calculated that the CV relative to the unfiltered data is larger with respect to that of the filtered ones (0.35 in the unfiltered case and 0.28 and 0.29 for the two filtered set of values). The decrease of the data dispersion is indicative of the process of information selection made by the filtering procedure and it confirms the success of the method proposed in achieving specific parameters instead of general and overlapping information obtained from raw data.

5. Conclusions

In this work, some efforts have been done to fit typical metrological procedures to the nano-biological field, particularly concerning the choice of scanning and filtering parameters for the calculation of cell membrane roughness. After a proper choice of scanning parameters, a new method has been tested to choose the cut-off frequencies to be used in the filtration process. In this way, a roughness value almost scale-independent and related to the real membrane roughness has been obtained. The proposed method has been tested in two experiments. The first one was carried out in order to assess cellular damages induced by exposure to a toxic chemical: in this case the R_{RMS} value has been confirmed as a discriminating parameter of the plasmatic membrane health state. In the second experiment, the possibility to characterize two different cell surface features at the same time has been successfully explored. In fact, by applying the proposed method to a cell membrane covered of microvilli and operating two different filter procedures, two roughness values have been obtained characterizing the microvilli density and the membrane roughness, respectively.

The membrane roughness is a parameter full of interest for a lot of cytological studies and clinical applications. In fact, it can characterize quantitatively both the micrometric-size membrane features (apoptotic changes [28], microvilli density [29], and cytoskeletal alterations [30]), as well as the nanometer-size ones (membrane components [24–27], and extracellular polymeric substances [32]). In our opinion, the scale-independent roughness parameter could be useful in both cases as, by properly selecting the information present on the cell surface, it has been demonstrated to be an unambiguous and reliable cellular parameter.

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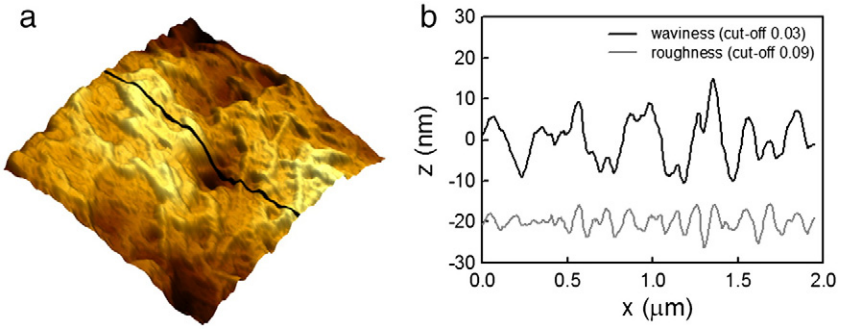

Fig. 11. In a) a 3D image of the inset of fig. 9 is shown. The line profile in a) has been filtered at two different cut-off frequency values. The resulting filtered profiles are shown in b), corresponding to the waviness profile (black line) and the roughness profile (gray line).

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