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Effect of Trypsin Concentration on Living SMCC–7721 Cells Studied by Atomic Force Microscopy

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Abstract

Trypsin is playing an important role in the processes of cancer proliferation, invasion, and metastasis which require the precise information of morphology and mechanical properties on the nanoscale for the related research. In this work, living human hepatoma (SMCC–7721) cells were treated with different concentrations of trypsin solution. The morphology and mechanical properties of the cells were measured via atomic force microscope (AFM). Statistical analyses of measurement data
indicated that with the increase of trypsin concentration, the average cell height and the surface roughness were both increased, but the cell viability, the cell surface adhesion and the elasticity modulus were decreased significantly. The force required to puncture the cells was also gradually reduced. It indicates that trypsin not only hydrolyzes the proteins between the cell and the substrate but also the membrane proteins. The results offer valuable clues for the cancerous process study, pathological analysis, and trypsin inhibitor drug development. And this work provides an effective way for overcoming the cell membrane in drug injection for cell–targeted therapy.

**KEYWORDS:** Trypsin, Living cell imaging, Cell mechanics, Cancer mechanics

1 | INTRODUCTION

Trypsin can degrade the basal membrane and extracellular matrix, also activate various matrix metalloproteinases and matrix serine proteases, and hence contribute to cancerous proliferation, invasion, metastasis, and angiogenesis.\(^1\)\(^{-}\)\(^4\) During the trypsin involved cancer progressions, the physical properties of cells such as stiffness, adhesion, roughness and morphology change significantly. They offer pathological information for interactions between the trypsin and microenvironments. In the last decades, the roles of trypsin were usually studied by activation or inhibition of trypsin expression\(^5\)\(^{-}\)\(^7\) in the pathological analysis of cancers.\(^8\)\(^,\)\(^9\) The methods can clearly prove the positive correlation between the trypsin and cancer development. However, they cannot directly observe the mechanical properties of cancer cells and subcellular structures which is an important dimension for the pathophysiology of trypsin in cancer development.

AFM provides a powerful toolkit for real–time studying the mechanical properties of living cells with piconewton and sub–nanometre resolution,\(^10\)\(^,\)\(^11\) hence becomes popular on cancerous nanomechanics studies in recent years. For example, AFM can obtain nanomechanical fingerprints of cancer cells for cancer diagnosis.\(^12\)\(^,\)\(^13\) Mechanical clues are revealed by AFM showing that the cancer cells are usually softer than the normal cells,\(^14\) and the strong adhesion between cancer cells reflecting the high malignancy.\(^15\) Recent studies have shown the direct evidence that tumor cells soften during the confined migration and support cell softening as a mechano–adaptive mechanism during invasion.\(^16\) The positive correlation between trypsin and cancer progression (as above mentioned) has been confirmed by pathological analysis methods. However, the nanomechanical mechanism of cancer progression, like a piece of the puzzle, is still missing.

Cell detection based on micro–nano manipulation can accurately reflect the physiological state and process of living cells, providing an intuitive and effective tool for the study of cancer development and cell–targeted therapy.\(^17\)\(^{-}\)\(^19\) Meister et al. combined AFM with nanofluids and
developed a fluidic force microscopy (FluidFM). It had a micro channel integrated in the probe cantilever, used for the local liquid distribution and stimulation of a single living cell. By increasing the contact force between the tip and the cell, the tip penetrated the cell membrane for drug injection.\textsuperscript{20, 21} Gene or drug delivery is an emerging cell–targeted therapy method, which is flexible, effective and applicable to a variety of diseases. Overcoming various physiological barriers so that the drugs or genes can smoothly reach the nucleus of target cell from the contact point has become the key to solving the problem of low drug delivery efficiency.

In this work, the effect of trypsin concentration on living SMCC–7721 cells was studied. The cells were exposed by varying concentrations of trypsin solution in the same dosing time. The relationship between the alterations of cell characteristics and the concentrations of trypsin was examined by AFM. The investigation indicated that via adjusting the concentration of trypsin on the cell, the mechanical properties of the cell could be adjusted. By controlling the indentation force and the depth based on the micro–nano manipulation system, it is possible to directly transfer the gene or drugs into the cell nucleus, which is of great significance for bioengineering and cell manipulation.

2 | METHODS

2.1 | Cell culture

RPMI–1640 with 10% fetal bovine serum (FBS) was chosen to be the culture medium. A flask containing cells which had reached the full confluence level, was treated with 0.25% trypsin (2.5 mg/ml) for the detachment of cells. The detached cells were plated on glass coverslips (18 mm×18 mm) in culture dishes (1×10\textsuperscript{4} cells/culture dish) and incubated 48 h for the trypsin treatment.

2.2 | Preparation of trypsin treatment solution

The trypsin powder (1:250, Gibco Amresco, from porcine pancreas) was dissolved in the phosphate buffered saline (PBS) at the concentration of 4.0 mg/ml, then filter-sterilized with a 0.22 \(\mu\) m filter. After that, the solution was stored at 4 \(^\circ\) C. Before the test, it was diluted with PBS to make the different concentrations of 1.5 mg/ml, 2.0 mg/ml, 2.5 mg/ml, 3.0 mg/ml and 3.5 mg/ml. The optimal operating temperature of the trypsin was 37 \(^\circ\) C. Thus, the treatment solution was heated to 37 \(^\circ\) C to make the trypsin most active which was necessary to the experiment.

2.3 | Sample preparation

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After 48 h of incubation, the culture medium of SMCC-7721 cells was replaced with PBS to rinse the cells and remove the unbound cells and the cell metabolites. The SMCC-7721 cells were immersed in the 1 ml trypsin treatment solution for 2 min, and then removed the liquid with a pipette. The trypsin-treated cells were gently rinsed 3 times with PBS for at least 20 s each time to ensure that the trypsin attached to the cell surface had already been washed away. After that, 2 ml of RPMI-1640 with 10% FBS was carefully added for AFM measurements. The cells treated with the trypsin solution at different concentrations were set as the six test groups, and the cells without the trypsin treatment were set as the control group.

2.4 | Cell fluorescence staining

Alexa Fluor 488® Phalloidin is a high-affinity filamentous actin (F-actin) probe (phalloidin), conjugated to bright, photostable, green-fluorescent Alexa Fluor 488 dye. Phalloidin can specifically bind to F-actin in eukaryotic cells, and display the distribution of the actin skeleton in cells. 4',6-diamidino-2-phenylindole (DAPI) is a blue-fluorescent DNA stain, which can penetrate the cell membrane and bind strongly to DNA. They were used as fluorescent staining reagents in this work. 1 ml of 4% methanol–free formaldehyde was added to the cells. After 15 min of cell fixation, the fixed cells were rinsed twice with PBS for 5 min each time. Alexa Fluor 488® Phalloidin solution (300 assays, Cell Signaling Technology) was added in the dark room (500 μl/culture dish). After incubating for 15 min at room temperature (25 °C), PBS was used to rinse the cells once for 5 min. DAPI (1 μg/ml, Cell Signaling Technology) was added to the cells for 5 min (1 ml/culture dish). Then rinsed with PBS for 5 min. In the cell fluorescent stained images, the cell membrane was green and the nuclei were blue.

2.5 | MTT assay

Suspended SMCC-7721 cells in 200 μl of RPMI-1640 with 10% FBS were cultured in 96-well plates (1.0 × 10⁴ cells/well). 10 same treated wells were prepared for each group. Removing the culture media after 24 h incubation, and 30 μl of trypsin solution at different concentrations were added to each corresponding experimental group well. The solution was discarded after 2 min, and then added 200 μl of new culture medium to each well. 20 μl of MTT solution (5 mg/ml) was added in the cells. After 4 h incubation, the solution was removed carefully and blue formazan crystals appeared in the living cells. After adding dimethyl sulfoxide (DMSO, 150 μl/well) with slow shaking for 10 min, the crystals dissolved. Optical density (O.D) was obtained via a microplate reader at the wavelength of 498 nm. In order to better illustrate the effect of trypsin on cell viability, the culture medium was added to the trypsin-treated cells, and the MTT assay was performed on them after further 24 h cell culture.
2.6 | AFM detection

The QI mode AFM (JPK, Germany) was selected for the examination of cell properties, which included the cell morphology, cell average surface roughness, cell average surface adhesion, and cell average elasticity modulus. It was also applied to the cell indentation experiment.

The working principle of AFM-based nanoindentation experiment is shown in Figure 1. The atoms on the tip surface and the sample surface will be attracted when they are far apart, and will be repelled when they are too close.\(^22\) In a liquid environment, the capillary forces are absent, and the ions in the liquid can shield the long-range electrostatic forces.\(^23\) As the probe gradually approaches to the cell surface, it enters the range of van der Waals attraction, which makes the cantilever deflect downward (Figure 1a). When the probe fully contacts with the cell, the cantilever deformation is proportional to the applied force and bends upward (Figure 1b). When the probe deformation reaches the maximum, the probe starts to withdraw. As the probe continues to be far away from the cell surface, the tip will not detach from the cell surface when it reaches the original contact point, due to the cell surface adhesion. The probe continues to move upward, and the cantilever becomes bending downwardly (Figure 1c). They will not separate until the elastic force overcomes the adhesion force between the tip and the cell surface. Finally, the cantilever returns to the free state (Figure 1d).\(^24,25\)

The vertical displacement of the probe \(h\), the depth of the cell indentation \(\delta\), and the cantilever deflection \(z\) correspond to the following relationships:\(^{36}\)

\[
h = z + \delta \tag{1}
\]

For a quadrangular pyramid probe, the indentation force \(F\) can be expressed as:\(^{37}\)

\[
F = \frac{1}{\sqrt{2}} \times \frac{E}{1-\nu^2} \times \tan \alpha \times \delta^2 \tag{2}
\]

where \(\nu\) is the Poisson's ratio of the cell,\(^{38}\) \(\alpha\) is the pyramidal angle of the probe, \(E\) is the elastic modulus of the cell. The motion trajectory of the probe in the QI mode is shown in Figure 2c.

In this work, the probe DNP–10 was used to examine the cell properties (tip shape: quadrangular pyramidal; spring constant: 0.06 N/m; tip radius: 20 nm; tip height: 5 \(\mu\)m). After the calibration, the spring constant was 0.059 N/m. The AFM was working at room temperature, and the imaging scan frequency was 0.4 Hz with the setpoint of 2 nN. The cells of the experimental groups and the control group were all from the same cell culture flask, and the samples of each group were prepared at the same time. The AFM measurements (morphological and mechanical parameters) of each group

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were all completed within the same day. The whole experiment was repeated six times and completed in two months. JPK data processing software (JPK Instrument, Germany) was used for the image and the force data processing. OriginPro8.0 software (OriginLab, USA) was used for graphing and data statistical analysis. The results were represented as the mean and standard deviation (±).

3 | RESULTS AND DISCUSSION

3.1 | Influence of different concentrations of trypsin on cell morphology

In order to better observe the effects of different concentrations of trypsin on the morphology of SMCC-7721, the overlapped fluorescent images of the experimental and control cells are shown in Figures 3b, 4d-f and 4j-l. Figure 3a shows the morphological images of SMCC-7721 cells, which have been cultured for 48 h. Figures 3c–d show the effects of the trypsin solution at the concentration of 2.5 mg/ml on the morphology of living SMCC-7721 cells after 48 h culture. The comparisons of the two images before and after dosing show that the edge of the cell lamellipodium can be obviously observed in Figure 3c, and the shape of the cells before dosing is similar to an ellipse. After the trypsin solution (2.5 mg/ml) treatment for 2 min as shown in Figure 3d, the cell morphology contracts significantly, and the lamellipodium attached on the substrate is damaged. The shape of the cells changes into rotundity.

AFM morphological images and fluorescent stained images of the experimental cells, treated with different concentrations of trypsin, are shown in Figure 4. The distribution of F-actin in the experimental cells is dependent on the concentration of trypsin. The higher the concentration of trypsin, the more visibly the f-actin distribution shrinks. The reason for the shape varying was that as the protein between the cell and the substrate was hydrolyzed by trypsin, the cell ability to adhere to the substrate decreased. When the cell was subjected to the pressure from all directions by the interior of liquid, the cell shape changed towards to round. The degree of morphological variation was obviously related to the trypsin concentration.

Figure 5a shows the data analysis of the average cell height. After 2 min exposing in the trypsin solution, the average cell height of the six test groups is increased. The minimum value appears in the control group (5.7±1.5 μm). An obvious rising trend in the average height is observed for the trypsin treated cells with the concentration from 1.5 to 2.5 mg/ml (6.3±1.4 μm for 1.5 mg/ml, 7.1±2.1 μm for 2.0 mg/ml, and 8.2±2.3 μm for 2.5 mg/ml). A rapid increase appears in the concentration of 4.0 mg/ml (10.8±1.7 μm), but there is no obvious variation tendency from 3.0 mg/ml (8.7±1.3 μm) to 3.5 mg/ml (9.0±2.0 μm).
In this work, the minor axis and the major axis of the cell are regarded as the length and width of the cell, respectively (Figure 3a). Most of the SMCC–7721 cells in the experiment are polygonal, showing an approximately elliptical shape with an average cell length of $23.0 \pm 3.0 \, \mu m$ and an average cell width of $32.9 \pm 2.6 \, \mu m$. Compared with the control group, the trypsin–treated cells show a decrease trend in the average length and width in Figure 5b. From the statistics of the average cell length, a clear downward trend can be observed from the control group to the 2.0 mg/ml treated cells ($19.0 \pm 2.7 \, \mu m$ for 1.5 mg/ml and $16.0 \pm 2.1 \, \mu m$ for 2.0 mg/ml). When the trypsin concentration is higher than 2.0 mg/ml, the average cell length of the experimental groups tend to be stable, and they are not significantly different from each other ($16.2 \pm 1.5 \, \mu m$ for 2.5 mg/ml, $16.9 \pm 2.7 \, \mu m$ for 3.0 mg/ml, $14.9 \pm 1.2 \, \mu m$ for 3.5 mg/ml and $14.9 \pm 2.3 \, \mu m$ for 4.0 mg/ml). Compared with the control group in average cell width statistics, 1.5 to 2.5 mg/ml trypsin–treated cells show a significant downward trend with the increase of trypsin concentration ($26.0 \pm 3.3 \, \mu m$ for 1.5 mg/ml, $23.6 \pm 4.7 \, \mu m$ for 2.0 mg/ml and $19.5 \pm 3.3 \, \mu m$ for 2.5 mg/ml). When the concentration of trypsin is higher than 2.5 mg/ml, the changes of the average cell width tend to be stable ($20.5 \pm 5.1 \, \mu m$ for 3.0 mg/ml, $18.5 \pm 2.6 \, \mu m$ for 3.5 mg/ml and $17.7 \pm 2.3 \, \mu m$ for 4.0 mg/ml).

Figure 5c shows the average aspect ratio (length: width) of the cells. After the two experimental groups treated with 1.5 mg/ml and 2.0 mg/ml trypsin, the average cell aspect ratio was closer to the control group by the contrast of the rest. It was indicated that the two groups of cells were as similarly elliptical as the control ones ($0.73 \pm 0.12$ for 1.5 mg/ml, $0.70 \pm 0.16$ for 2.0 mg/ml and $0.70 \pm 0.09$ for the control group). The average aspect ratio of the cells treated with trypsin at the concentration of 2.5 mg/ml to 4.0 mg/ml is closer to the value one, which illustrates that the cell morphology changes from ellipsoid to rotundity ($0.84 \pm 0.15$ for 2.5 mg/ml, $0.86 \pm 0.24$ for 3.0 mg/ml, $0.81 \pm 0.10$ for 3.5 mg/ml and $0.85 \pm 0.19$ for 4.0 mg/ml). We speculate that the cytoskeleton contracts due to the internal pressure of cytoplasm, which gives rise to the increase of average cell height, and makes the cell morphology close to rotundity.

Previous studies have shown that the cell surface roughness is related to the cell viability. Select the area ($8 \, \mu m \times 8 \, \mu m$) where the nucleus is located as the test one. RMS roughness is obtained by JPK data processing software. The data analysis of the average surface RMS roughness of each group of cells is given in Figure 5d. $370 \pm 55 \, nm$ is the control group value. The increase trend at the concentration higher than 2.5 mg/ml ($523 \pm 50 \, nm$ for 2.5 mg/ml, $646 \pm 81 \, nm$ for 3.0 mg/ml) is significant, but the increase tendency is slightly at the concentration ranging from 1.5 to 2.5 mg/ml ($444 \pm 52 \, nm$ for 1.5 mg/ml, $499 \pm 51 \, nm$ for 2.0 mg/ml). There is no obvious change that
can be seen between the high concentrations 3.5 and 4.0 mg/ml (764±42 nm for 3.5 mg/ml, 773±74 nm for 4.0 mg/ml).

3.2 | Influence of different concentrations of trypsin on cell mechanical properties

Cell adhesion is crucial for cell transfer, shape maintenance and mechanical signal generation. It is also the basis of the physiological and pathological processes such as wound healing, recombination of neurons and cancer metastasis. Trypsin can hydrolyze proteins on the cell surface, which in turn affects cell surface adhesion. We selected 8 μm×8 μm on the cell surface for the data analysis in the experiment. As shown in Figure 6a, the average cell surface adhesion of experimental groups shows different degrees of declines compared with the control group (2.21±0.20 nN). The higher the trypsin concentration, the more obvious the cell adhesion declined in the experimental groups (2.05±0.10 nN for 1.5 mg/ml, 1.85±0.15 nN for 2.0 mg/ml, 1.91±0.08 nN for 2.5 mg/ml, 1.76±0.16 nN for 3.0 mg/ml, 1.67±0.07 nN for 3.5 mg/ml, and 1.65±0.16 nN for 4.0 mg/ml). This phenomenon supports the feasibility of regulating cell surface adhesion via adjusting the concentration of trypsin acting on cells.

Elasticity measurement is essential for studying the biophysical properties of cells and the effects of drugs on cancer cells. The measurement area was 10 μm×10 μm, where the cell nucleus was located. Figure 6b shows the average elasticity modulus of the experimental and control cells. The cells after trypsin treatment show an overall downward trend as compared with the control cells (2.85±0.43 KPa). The experimental group cells treated with the trypsin concentration less than 2.5 mg/ml, the average elastic modulus of each group is not much different from each other (2.50±0.42 KPa for 1.5 mg/ml, 2.41±0.44 KPa for 2.0 mg/ml, and 2.66±0.39 KPa for 2.5 mg/ml). The experimental cells treated with trypsin at the concentration more than 2.5 mg/ml have more significant changes in elastic modulus than those of the control cells (2.17±0.40 KPa for 3.0 mg/ml, 1.77±0.60 KPa for 3.5 mg/ml and 1.93±0.68 KPa for 4.0 mg/ml). It can be inferred from the statistics that trypsin can reduce the elastic modulus of the cell, and the degree of the change is more pronounced as the trypsin concentration is increased.

It can be seen from the above experimental results that trypsin acts on SMCC–7721 cells, which causes not only the changes in cell morphology, but also the changes the elastic modulus of cells. That is to say, the action of trypsin on SMCC–7721 cells can cause the change in cell stiffness. In order to verify this viewpoint, nano-indentation tests were performed on experimental and control cells. The force–distance curve measured by AFM reflects the quantitative tip–sample force, which can be used to quantitatively measure the elastic modulus, adhesion and stiffness of the sample. The force–distance curves of the tests are shown in Figure 7. P points in the figure represent the
probe tip successfully piercing into the cell surface. P0 is the puncture point of the control group cells, and P1–P6 represent the puncture points of the six experimental group cells. As the trypsin concentration increases, the slope of the force curve gradually decreases, which means that the cell stiffness also decreases. The decline in the position of P point means that the force requires for the probe to penetrate the cell is gradually reduced. The changing trends of the cell elastic modulus and the cell puncture force are consistent with the related results of Liu et al.36

As shown in Figure 8, the penetration force and depth of trypsin–treated cells show an overall downward trend (1.84±0.28 nN and 1.64±0.26 μm for 1.5 mg/ml, and 1.51±0.15 nN and 1.89±0.18 μm for 2.0 mg/ml), as compared with those of the control cells (2.16±0.29 nN and 2.21±0.32 μm). In the experimental groups, with the concentration between 2.5 mg/ml and 3.5 mg/ml, the average penetration depth and force show a gradual downward trend, and no significant reduction is obtained in this range (0.98±0.19 nN and 1.23±0.15 μm for 2.5 mg/ml, 0.91±0.14 nN and 1.18±0.18 μm for 3.0 mg/ml, 0.67±0.14 nN and 1.16±0.16 μm for 3.5 mg/ml). The average penetration force and depth of the 4.0 mg/ml experimental group change the most significantly (0.25±0.10 nN and 0.74±0.24 μm for 4.0 mg/ml). The statistical results reveal that the trypsin in different concentrations can regulate the cell stiffness and the ease of penetrating cells.

To assess the effect of the trypsin on cell viability, MTT assay was applied to detect cell viability. In the first part of the test, the experimental and the control cells were cultured under the same conditions for 24 h. Then, the cells in the experimental groups were exposed to different concentrations of trypsin for 2 min, and MTT solution was added for the cell viability detection. In the second part of the test, the trypsin–treated experimental cells and control cells were sent to the incubator for further incubation. After 24 h, MTT assay was performed. The experimental results are shown in Figure 9. The cell viability of the control group is regarded as 100%. In the experimental groups in test 1, the cell viability of the cells treated with 1.5 mg/ml trypsin reaches 92.6%±4.5% of the control group. While the other experimental groups are below 90%. The cell viability of the experimental groups with the concentration ranging from 2.0 mg/ml to 3.0 mg/ml is close to each other (88.7%±2.5% for 2.0 mg/ml, 85.2%±3.2% for 2.5 mg/ml, 86.1%±2.9% for 3.0 mg/ml), but the values of 3.5 mg/ml and 4.0 mg/ml decrease obviously (82.8%±3.5% for 3.5 mg/ml and 80.3%±3.4% for 4.0 mg/ml). The results show that trypsin can affect cell viability. Under the same dosing time, the higher the trypsin concentration, the more obvious the cell viability declines. Trypsin can hydrolyze the proteins on the cell surface and cleave the peptide bonds on the carboxyl side of lysine and arginine in the polypeptide chain.37 Thus, the membrane integrity could be damaged by increasing trypsin amount and treatment time.
After the cells cultured for further 24 h in test 2, the cell viability of the experimental groups increases significantly. The cell viability of the experimental groups at 4.0 mg/ml reaches 89.9%±1.7% of the control group, and the cell viability of the rest experimental groups reach more than 90% of the control group (94.7%±1.6% for 1.5 mg/ml, 92.4%±0.8% for 2.0 mg/ml, 90.4%±1.6% for 2.5 mg/ml, 93.3%±1.3% for 3.0 mg/ml, and 93.6%±1.3% for 3.5 mg/ml). It can be inferred that, in this work, high concentration of the trypsin can affect cell viability more obviously. However, after 24 h cell culture, the cell viability will be restored, the adverse effects of trypsin on the cells will be alleviated.

4 | CONCLUSION

A better understanding of the effects of trypsin solution at different concentrations on living SMCC-7721 cells were obtained from the investigations via AFM. Combining with the results of cell viability tests, the trypsin solution at the concentration higher than 2.5 mg/ml, had a negative influence on cell viability. With the increase of trypsin concentration, the protein hydrolysis was enhanced, and caused more serious damage of cell surface. However, as the further culture time reached 24 h, the negative effect on cell viability was weakened. There was no obvious toxic effect on cells. As the trypsin concentration increased, the adhesion force on the cell surface was significantly reduced. The shape of the cell changed into rotundity from ellipsoid. In the meantime, the average cell height increased as a result of the cytoskeleton contraction. The average roughness of the cell surface also increased due to the proteolysis. The increases in height and roughness of the cells treated with the trypsin solution at the concentration more than 2.5 mg/ml were outstanding, while the rest were gentle. The average elasticity modulus of SMCC-7721 cells decreased with the increasing of trypsin concentration. The corresponding cell stiffness was reduced, and the force required to puncture the cell was also reduced. The application of appropriate concentration of trypsin can be used to regulate cell mechanical properties without causing cell death, which provides powerful support for cell targeted therapy, and has great research potential in cell biology, biomedicine, and drug delivery.

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CONFLICTS OF INTEREST

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REFERENCES


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FIGURE CAPTIONS

FIGURE 1 Schematic diagrams of the indentation process of single cell by a quadrangular pyramid probe under the physiological medium.

FIGURE 2 (a), (b) Schematic diagrams of the relationship between the probe vertical displacement $h$, cantilever deflection $z$ and sample indentation depth $\delta$. (c) Direction of the probe in the QI mode.
FIGURE 3 (a) AFM morphological image of SMCC–7721 cells in the QI mode with the scanning range: 60 μm × 60 μm (pixels: 256×256). (b) The fluorescent stained image at 40× magnification of SMCC–7721 cells. (c), (d) Comparison of the morphology of the same SMCC–7721 cells before and after the trypsin treatment.
FIGURE 4 AFM morphological images in the QI mode and cell fluorescent stained images at 40× magnification of SMCC-7721 cells treated with different concentrations of trypsin for 2 min (scanning range: 50 μm×50 μm; pixels: 256×256).
FIGURE 5 The variations in morphology of the cells treated with different concentrations of trypsin for 2 min. (a) Average cell height. (b) Average cell length and width. (c) Average cell aspect ratio. (d) Average cell surface RMS roughness. The differences between the experimental groups and the control group were considered statistically significant at *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (a, b, d). The results are represented as the mean and standard deviation ($\pm$).
**FIGURE 6** (a) Average cell surface adhesion. (b) Cell surface average elastic modulus. Statistical differences compared to the control group were considered at *$P < 0.05$*, **$P < 0.01$** and ***$P < 0.001$***. The results are given as the mean and standard deviation (±).

**FIGURE 7** Force-displacement curves of SMCC–7721 cells and the cells after 2 min exposing to the trypsin at different concentrations.
FIGURE 8 Average penetration depth and force of control and experimental cells. The differences between the experimental groups and the control group were considered statistically significant at *P < 0.05, **P < 0.01 and ***P < 0.001. The results are given as the mean and standard deviation (±).
FIGURE 9 MTT assay of the SMCC-7721 cells. The results are expressed as the mean and standard deviation (±).