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THE BIOMECHANICS OF DRUG-TREATED LEUKAEMIA CELLS INVESTIGATED USING OPTICAL TWEEZERS

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The Leukaemia is a very common cancer worldwide, and different drugs have been applied to treat leukaemia. However, the influence of the drugs on the biomechanical properties of leukaemia cells, which are related the risk of leukostasis, is still unknown. Moreover, accurate measurement of biomechanical properties of leukaemia cells is still a challenging task because of their no-adherent nature and high sensitivity to the surrounding physiological conditions. In this study, a protocol to measure the biomechanical properties of leukaemia cells by performing indentation tests using optical tweezers is proposed. The biomechanical properties of normal leukaemia cells and cells treated with various cancer drugs, including phorbol 12-myristate 13-acetate (PMA), all-trans retinoic acid (ATRa), Cytoxan (CTX) and Dexamethasone (DEX), were measured. The adhesion between the cells and certain proteins existing in the extracellular matrix, i.e. fibronetin and collagen I, was also characterized with the help of a static adhesion assay. It was found that after treatment by ATRa, CTX and DEX, the cells became softer, and the adhesion between the cells and the proteins became weaker. PMA treatment caused no change in the stiffness of the HL60 cells, but increased the stiffness of the K562 cells, and increased the cell-protein adhesion of both K562 cells and HL60 cells.

Keywords: optical tweezers, indentation, Leukaemia cells, cell mechanics

1. Introduction

The biomechanical properties of cells are directly determined by their protein structures and thus closely related to their biochemistry and genetics. Therefore, physiological changes caused by various diseases in the human body may result in alteration of the cells' biomechanical properties[1]. For example, the chondrocytes suffering from osteoarthritis are generally less stiff than normal chondrocytes[2], and it was also reported that cancer cells can deform more easily than normal cells[1]. To study the biomechanics of cells may therefore be helpful in revealing quantitative information about the progression of certain diseases[1, 3].

Leukaemia is a cancer of the blood or bone marrow due to an abnormal proliferation of blood cells, usually white blood cells. It can be classified into two groups: acute and chronic leukaemia[4]. Acute leukaemia is caused by the rapid increase of immature blood cells, the malignant cells rapidly progress and accumulate in the human body, split over into bloodstream and then spread out to other organs. Chronic leukaemia is due to the excessive build-up of relatively mature, but still abnormal, white blood cells. Studies have been done to investigate the cell mechanics of leukaemia[4-7]. Tsai et. al. employed micropipette aspiration to investigate the deformability of HL60 cells at different cell cycles and found that the HL60 cells at G1 cycle is less stiff than in G2/M cycles[8]. Rosebluth et al used atomic force microscopy (AFM) nanoindentation to measure the biomechanical properties of HL60, Juket cells etc., and found that their biomechanical properties are related to the risk of leukostasis, a complication of leukaemia, and a major reason to cause death, due to the heavy aggregation of leukaemia cells inside the blood vessels[9]. Lam et. al investigated the stiffness of leukaemia cells, and found that their stiffness increased more than 100 times after treated with DEX. They therefore suggested that the DEX will have the chance to increase the risk of leukostasis[7]. It was also reported that the adhesion between the leukaemia cells and their extracellular matrix plays an important role in leukostasis, especially for acute myeloid leukaemia[10-12], in which the escape of leukaemia cells from bone marrows is determined by the adhesion between the bone marrow fibroblasts and the cells[13, 14].

Accurate measuring the biomechanical properties of leukaemia cells is challenging, due to the difficulties in simulating the in vivo conditions of the cells and their non-adherent nature. The results for the single cell mechanics of leukaemia reported so far suffer from large scattering and are debatable[9, 15]. The previously used techniques of micropipette and AFM nanoindentation both have their short comings. In AFM nanoindentation, an AFM tip is used as a probe to indent on the cells. Based on the mechanical response recorded during the indentation, the mechanical properties of the cells can be extracted[1, 9], [16], but it is well known that the properties of the cells are closely related to the physiological conditions, e.g. the pH value, the temperature and even the atmosphere[9], and it is difficult to build up an experimental setup that can completely simulate the physiological conditions of the human body on an AFM. In a micro-pipette system, normally the cells are aspired by the pipette and their deformation is recorded and analyzed to evaluate deformability. However, the shapes of cells are usually irregular, and this limits the accuracy of the measurement because precise knowledge of the cells' shape is required in the calculation.

Optical tweezers are a novel technique for manipulating small objects. In this technique a focused laser beam is utilized to provide an attractive or repulsive force to physically hold and move micro-particles[17, 18]. Because of its feasibility to perform test on biological samples in physiological conditions, it has been adopted by the biological community for cell manipulation, isolation, etc[19-21]. Meanwhile, the feasibility of using the optical tweezers to study single cell mechanics has been demonstrated recently[3, 22-24]: the most pronounced study was to use optical tweezers to perform tensile tests on red blood cells suffering from malaria to investigate their deformability[3, 22]. Comparing with AFM nanoindentation, optical tweezers do not need direct physical contact with the "probe" to apply a force on them, and so the tests can be performed in a completely isolated container, thus enabling physiological conditions very close to those in the human body to be easily achieved. In the previous study by Suresh et. al[3], tensile tests were performed with the help of optical tweezers, but in this method, the maximum applied force is actually determined by the interaction force between the micro beads and cells, which is usually in tens of piconewtons. Such small forces may not be able to generate significant deformation in cells stiffer than red blood cells.

In this work, a new protocol to evaluate the deformability of cells by performing indentation tests using optical tweezers is developed. Currently, different drugs have been developed to treat leukaemia, but their influence on the mechanical properties of leukaemia cells, which should be closely related to the risk of leukostasis, has not yet been fully understood. For this reason, we applied the optical tweezers indentation protocol to measure the biomechanical properties of leukaemia cells, in order to study the influence of different drugs on the biomechanical properties of the treated cells. Apart from the mechanical testing, the cell-fibronetin and cell-

collagen I adhesion was also studied with the purpose to better evaluate the relationship between the drug-treatment and the risk of leukostasis.

2. Experimental details

2.1. *Cell culture*

In this study, the HL60 and K562 cells were used as models for acute and chronic leukaemia respectively. The incubator used is a Bionex@ type. The cells were cultured in RPMI 1640 containing 10% fetal bovine serum, and the atmosphere inside the incubator was humidified and maintained in 5% CO_2 at 37 ^oC.

2.2. *Optical tweezers*

The optical tweezers used in this study are a MMI Cellmanipulator System supplied by MMI Company in Switzerland. This machine is specially designed by MMI to enable laser trapping to be performed with a maximum laser power of 8W (Nd:YAG laser, wavelength 1064 nm). The laser focus was adjusted following the standard calibration procedure suggested by the supplier before the experiments. A microscope stage incubator (Okolab Company, Italy) was integrated into the optical tweezers system which enables control of the experimental conditions, such as the atmosphere and temperature.

2.3. *Laser power-force calibration*

The relationship between the applied laser power and the generated maximum laser trapping force must be carefully calibrated before the mechanical testing. A calibration method integrated into the optical tweezers system, which involves estimating the maximum viscous drag force exerted by the fluid when the trapped particle just breaks loose from the trap during oscillation by it, was employed in this study[25]. Typically a calibration procedure is as follows: first, the laser is focused and used to trap a microsphere at a given laser power. Then the trapped microsphere is vibrated periodically by rocking the laser beam. The frequency and amplitude of the vibration are increased gradually until the laser cannot hold the microsphere. The critical frequency ω and amplitude A_c are then used in the follow equation,

$$F_{\max}(x,t) = 6\pi^2 \eta a A_c \omega , \quad (1)$$

to calculate the maximum laser trapping force[25]. Here η□□ is the dynamic viscosity of the solution and *a* is the radius of the microsphere. Fig. 1 shows the calibrated plot of laser power vs. maximum trap force, which was achieved by vibrating microspheres in the culture medium mentioned above. Clearly, the maximum trapping force increases linearly with increasing laser power, in agreement with previously reported result[25].

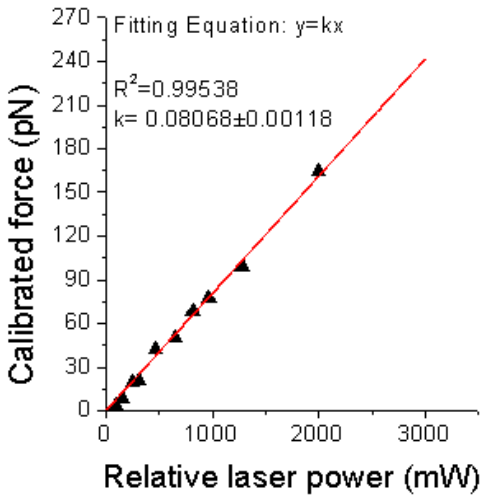

Fig. 1. The relationship between the laser power and maximum trap force calibrated by the viscous drag method.

2.4. *Cancer drugs testing*

The leukaemia cells were divided into 5 groups. One of them was used as control, and the other four were treated by different common drugs for leukaemia, including the two differentiation therapy drugs, namely, phorbol 12-myristate 13-acetate (PMA) with a concentration of 10 μg/L and all-trans retinoic acid (ATRa) with a concentration of 1 μM/L, as well as two chemo-therapy drugs, namely, Cytoxan (CTX) with a concentration of 0.1 μM/L and Dexamethasone (DEX) with a concentration of 20 μg/L. These drugs were applied to the 4 groups for 24 hours before mechanical testing.

2.5. *Cell structure observation*

Scanning electron microscopy (SEM) and fluorescence microscopy were used to study the morphology and

the structure of the cells. The SEM used is a Hitachi S4800 FEG SEM and the fluorescence microscope used is a Nikon Ti-E inverted microscope integrated into the optical tweezers system.

2.6. *Static adhesion assay*

96 well microplates (BD Falcon MicroTEST^{TM} 96) were pre-coated with 4 μg/ml fibronetin (sigma, from human plasma) and collagen I (sigma, from rat tail) respectively overnight at 4 ^{o}C, then washed twice with PBS buffer, blocked by 1% BSA in PBS buffer at 37^{o}C for 1 hour and then washed again. K562 and HL60 cells were cultured with the drugs in 2 hours and 24 hours. The cell density was counted by haemocytometer to be 2.5× 10^{6} cells/mL. 100 mL drug treated and untreated cells were added into each well and incubated at 37^{o}C for 30 minutes. The cells inside the wells were gently washed with PBS twice, fixed with 2.5% glutaraldehyde and incubated at room temperature for 10 minutes, and then they were washed again and stained with 0.5% crystal violet in 20% methanol for 10 minutes. The stained cells were washed with water gently and immersed with 10% acetic acid on an orbital shaker (Heidolph Vibramax 100) for 5 minutes. Finally the cells inside the microplates were measured by a Bio-Tek instruments EL808 microplate reader at wavelength 550 nm.

2.7. *Optical tweezers indentation*

Indentation tests were performed on the cells with the help of the calibrated optical tweezers mentioned above. Before doing the indentation, the cells were transported from the large Bionex$^{@}$ incubator to the microscope incubator integrated into the optical tweezers system. All the indentation tests were performed in this isolated microscope incubator thereafter. The temperature as well as the atmosphere during the mechanical testing could therefore be well controlled. The culture medium inside the microscope incubator was RPMI 1640 containing 10% fetal bovine serum, which served to maintain the cells' normal physiological behaviour. The bottom of the microscope incubator, which actually is a transparent glass slip, was coated with fibronetins by culturing with PBS containing 0.1 μg/mL fibronetins for 24 hours at 37^{o}C, and so on it the leukaemia cells could be attached firmly. The microspheres used in this study were supplied by Polysciences, Inc. They were made of polystyrene and had diameters in the range 2.49±0.02 microns. The typical indentation procedure is as follow: first, the microscope's focal plane was adjusted to overlap with the largest circular section of the cell under study. Then, a microsphere was laser-trapped with a given laser power and it was then brought to contact with the cell. The microsphere was then indented into the cell slowly by moving the motor-driven sample stage toward the trapped microsphere at a velocity of around 1 μm/s. The critical indentation depth as well as the corresponding laser power at which the microsphere broke loose from the trap was then recorded. In this protocol, since the indentation depth is larger than the radius of the microsphere, the microsphere-cell contact radius should always be equal to the radius *a* of the microsphere. The contact stiffness, *S*, is therefore

$$S = P_{\max} / h_{\max} \tag{2}$$

where $h_{\max}$ is the indentation depth at which the laser trap was lost, and can be directly measured based on the recorded microscope images; $P_{\max}$ is the corresponding applied force, and can be calculated from the laser power based on the calibration results shown in Fig. 1. The elastic moduli *E* of the cells, can therefore be obtained by the equation

$$E = E_r(1-\nu^2) = \frac{S}{2a}(1-\nu^2) \tag{3}$$

where *a* is the contact radius which is equal to the radius of microsphere used, and v is the Possion's ratio which is assumed to be 0.5.

Mechanical testing was performed on the drug-treated cells 24 hours after the drug were applied to the cells. For each cell group, tests were performed on 12 randomly selected cells. Experiments were also performed on an individual group of K562 cells at different time points to investigate the biomechanical properties of the cells during their life cycles.

3. Results and discussion

Fig. 2 and 3 show the SEM images of K562 and HL60 cells before and after the drug treatment respectively. For the CTX and DEX group, no significant change in morphology was observed, because the major function of these two types of drug is to directly kill the leukaemia cells. However, for the leukaemia cells treated with PMA and ATRa, their morphology changed significantly. For example, some K562 cells treated with PMA became elongated (Fig.2e) and some HL60 cells treated with PMA expanded and stuck to the substrate (Fig. 3e). This might be due to a change of adhesion and cytoskeleton, making the cell more adhesive and therefore easier to attach on a substrate. This should also be due to the nature of the drugs applied: the PMA and ATRa are differentiation therapy drugs and will lead to the differentiation of leukaemia cells.

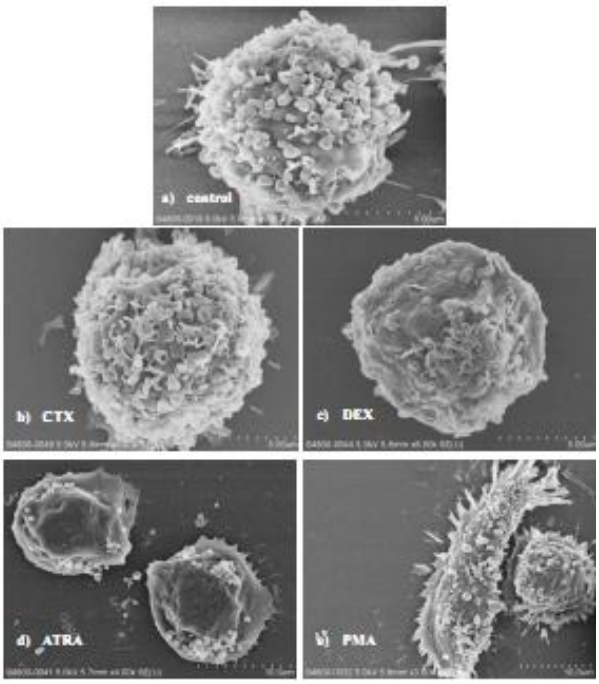

Fig. 2 SEM images of K562 cells. (a) shows the normal K562 cells; (b) - (e) show the K562 cells after the treatment of CTX, DEX, ATRa and PMA respectively.

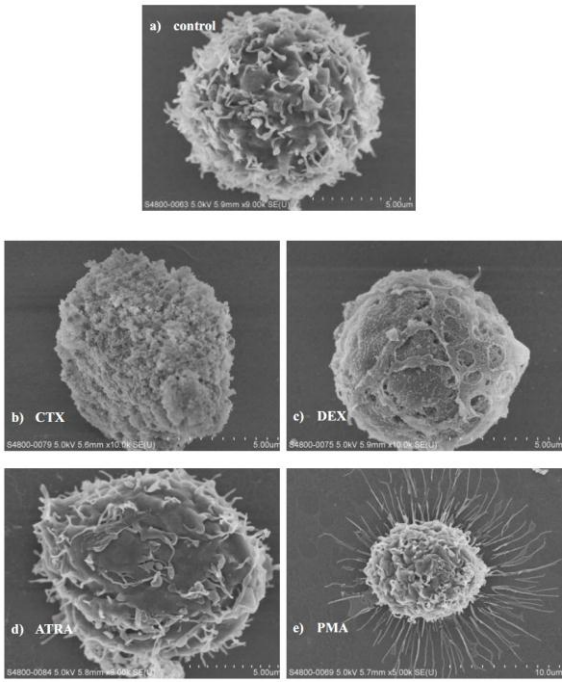

Fig. 3 SEM images of HL60 cells. (a) shows the normal HL60 cells; (b) - (e) show the Hl60 after the treatment of CTX, DEX, ATRa and PMA respectively.

Fig. 4 and 5 show the HL60 and K562 cells stained with Hoechst 33342 (Sigma), which is commonly used to make DNA visible under the fluorescence microscope. It can be seen that the nuclei in the CTX and DEX treated cells showed a condensed shape with part of the DNA fragments spread in the cytoplasm, indicating the feature of apoptosis, while the control group and the leukemic cells treated with PMA showed homogeneous nuclei and not DNA in the cytoplasm. The cells were also stained with phalloidin to observe the distribution of F-actin under the fluorescence microscope. It was found that the cells treated with CTX and DEX became less condense than the control group. Both the SEM and the fluorescence microscope study indicate that the drugs affect the cells efficiently 24 hours after the treatment.

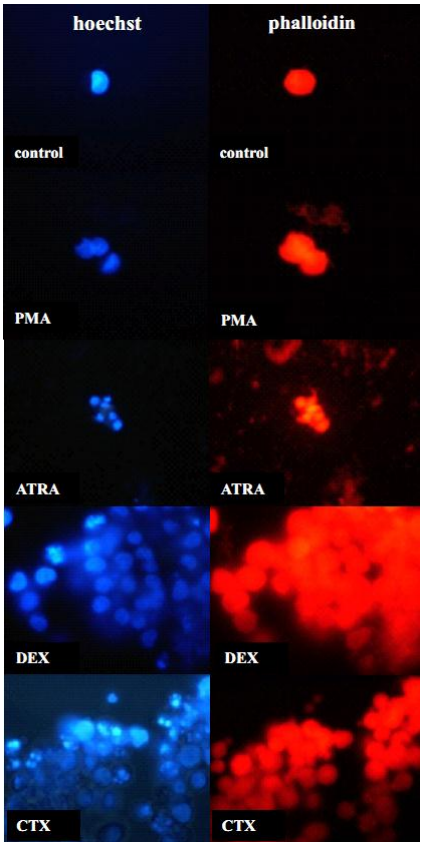

Fig. 4. Fluorescence microscope images of normal K562 cells and drug-treated K562 cells. On the left side are cells stained with hoechst to observe the nucleus and on the right side are cells stained with phalloidin to observe the distribution of F-actin.

The cell-protein adhesion was characterized by a static adhesion assay, and the results are shown in Fig. 6. Here we classify the cells into three groups: normal cells, the cells exposed to drugs for 2 hours and 24 hours respectively. Results from the negative control group, i.e. adhesion between the leukaemia cells with drug-treatment and BSA, are also reported. It can be seen that K562 cells showed stronger binding to the proteins comparing with HL60 in all the results reported here, and this may also suggest that the

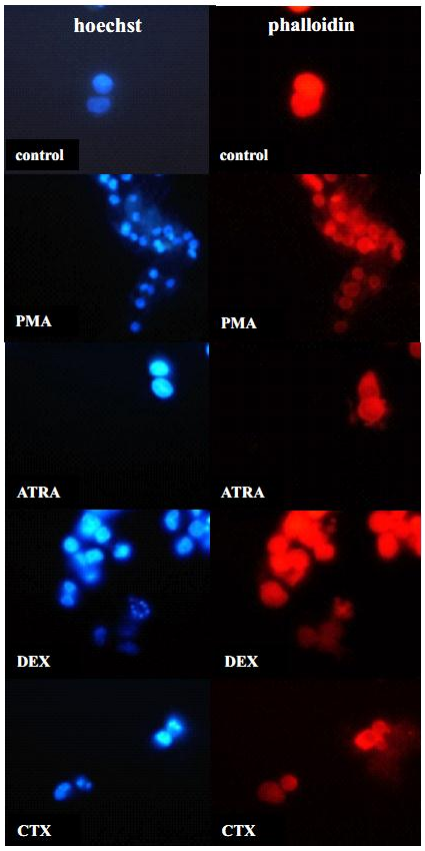

Fig. 5. Fluorescence microscope images of normal HL60 cells and drug-treated HL60 cells. On the left side are cells stained with hoechst to observe the nucleus and on the right side are cells stained with phalloidin ato observe the distribution of F-actin.

chronic leukemic cell K562 is a more mature cell type than the acute leukemic cells HL60. The HL60 cells, as less adhesive leukemic cells, should therefore be easier to flee away from the bone marrow extracellular matrix. In the CTX and DEX drug treatment groups, the adhesion of both K562 and HL60 cells decreased rapidly, from 2 hours treatment to 24 hours treatment, indicating that CTX and DEX have a reverse effect on the cell-protein adhesion, and it is suspected that this may be caused by the cell apoptosis[26]. Cells treated with PMA show different behaviour – the cell-fibronetin and cell-collagen adhesion increases for both HL60 and K562 cells. This may also indicate that PMA may be helpful in slowing down the releasing process of leukaemia cells into the blood stream, by increasing the adhesion between the acute leukemic cells and the bone marrow extracellular matrix.

Fig. 7 shows the optical microscope images taken during the indentation procedure and fig. 7-d shows the image just before the loss of the laser trap. The measured elastic moduli of normal K562 and HL60 cells and the cells treated with different drugs are

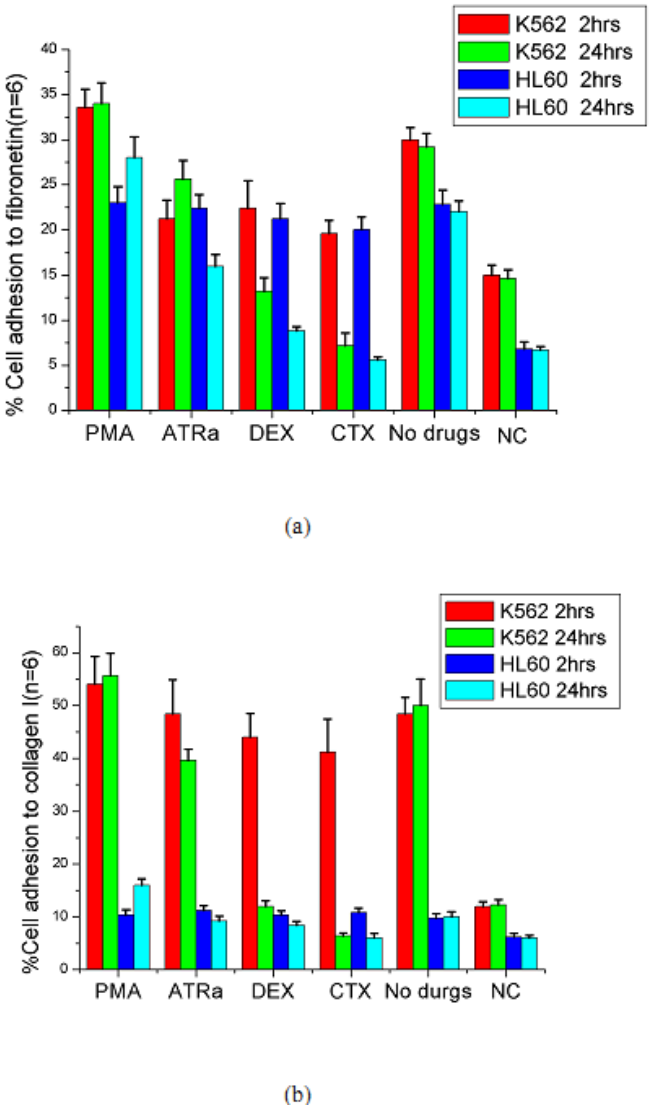

Fig. 6. Adhesion between the leukaemia cells and the extracellular matrix proteins measured with static adhesion assay (n=6). (a) and (b) show the cells-fibronetin adhesion and cell-collagen I adhesion respectively. The adhesion between protein and cells treated with different drugs at time points of 2 hours and 24 hours are shown. The 'no drugs' groups show the adhesion of proteins and leukaemia cells without drug-treatment, and the "NC" group is the negative control of leukaemia cells without drug-treatment tested in BSA- blocked well.

listed in Table 1. It can be seen that the normal HL60 cells show higher elastic moduli than K562 cells, i.e. the HL60 cells are less deformable than the K562 cells. It was reported that the risk of leukostasis of chronic leukaemia patients is smaller those suffering acute leukaemia[9, 27], and so the elastic moduli measured here for normal leukaemia cells agree with this finding, if leukostasis is linked to increased stiffness. The elastic moduli of normal HL60 cells was measured as

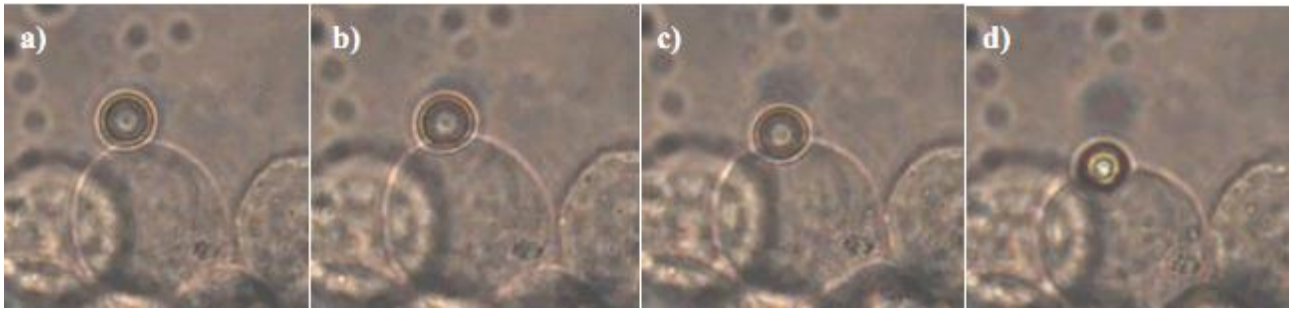

Fig. 7. Microsphere images taken during an indentation test performed by optical tweezers.

213.1±21.4 Pa, which is significantly lower than the 855±670 Pa measured with AFM nanoindentation previously[9], but the results reported previously have large scattering presumably because a larger sample size of 60 was used, and the elastic moduli measured here are still in the range of the previously report ones, albeit with much less scattering. It therefore seems that the present measurement protocol is more reliable.

Measured Elastic Modulus of Leukemia Cells

	Control	PMA	ATRa	DEX	CTX
K562	162.8±19.2	183.5±15.6	145.6±25.4	116.3±30.8	113.6±31.9
HL60	213.1±21.4	211.6±29.8	174.0±24.2	145.6±36.3	138.6±32.8

Table 1. The measured elastic modulus of leukemia cells with and without drug treatment (n=12, Mean±SD, Pa)..

It was found that almost all the drugs will significantly influence the biomechanical properties of the leukaemia cells. For the cells treated with CTX and DEX, which are chemical drugs to kill the leukaemia cells, the foggy images of nucleus as well as the loss of cytoskeleton structure shown in Fig. 4 and 5 indicate the apoptosis of the cells, and so it is not surprising that the measured elastic moduli of both K562 and HL60 after drug treatment are smaller than those measured from normal leukaemia cells. The PMA and ATRa will induce the leukaemia cells to differentiate, and the condensed nuclei of the cells treated with these two drugs indicate that the cells are still in a healthy state. However, the cells' structure were actually affected by the drugs, for instance, the nuclei became smaller for the ATRa treated HL60 cells. Our measurement shows that all the cells treated with ATRa became less stiff, but cells treated with PMA either exhibited no significant change in stiffness (HL60), or actually an increase in stiffness (K562). The increase of stiffness of K562 cells after PMA treatment might be related to the change of their shape and cytoskeleton after the drug treatment (c.f. Fig. 2-e).

Noticeable scattering of the elastic moduli measured from each group of cells was found. Since it was reported that the leukaemia cells in different life cycle phase will have different mechanical properties, a control experiment was conducted using K562 cells. In this experiment, first the cell synchronization was done by culturing the cells in RPMI 1640 without fetal bovine serum at the same atmosphere conditions for 24 hours[9], and then the synchronized cells were cultured in the microscope incubator with the normal culture medium for another 24 hours. Their mechanical properties were measured every 2 hours when culture with normal culture media. The measured elastic moduli are shown in Fig. 8.

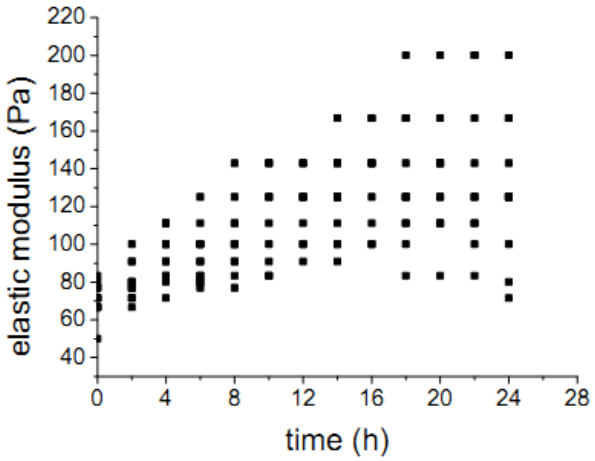

Fig. 8 Elastic modulus of normal K562 cells measured at different time points within 24 hours after cell synchronization.

Interestingly, we found that scattering of the measured elastic moduli increases with the time, and the lower limit of the measured results is more or less

the same during the 24h period while the upper limit increases rapidly with time. The cell doubling time for a K562 cell to shift from the G1-M phase is around 24 hours[28], and the speed to shift the cell life cycle will be different between individual cells. It is therefore believed that with the increase of time, the originally synchronized cells inside the incubator evolved into different life cycle phases, and this causes the increasing scatter. It was reported previously that the HL60 cells will become stiffer following the life cycle G1->S->G2/M8, and the results shown in Fig. 8 indicate that the K562 cells also become stiffer when as they enter later phases in their life cycle, just as HL60 cells. The drug-treated cells should actually be in different life cycle phases when they were mechanically tested, because the tests were performed 24 hrs after the drug treatment. It is therefore believed that the scattering found in the elastic moduli measured from the drug-treated cells should be partially caused by differences in the different cells' life cycles.

In our experiment, the microsphere used was around 2.5 μm in diameter, and the size of the cells was in the range from 10 μm-20 μm in diameter. Therefore, the microsphere can only deform the cell partially and the results measured should be local properties. Actually, the local elastic moduli of HL60 measured in this study agree with those measured with AFM indentation in previous study[9]. Our results still have quite significant scatter, although this is smaller than those reported previously with AFM nanoindentation[9]. The scatter may be due to the inhomogeneous nature of the cells. The changes in mechanical properties of HL60 cells treated by DEX were reported previously by Lam et al.[7], and the DEX cells' stiffness was found to increase by more than 100 times after exposure to DEX for 6 hours. This is totally different from the stiffness of DEX treated HL60 cells measured in our work. This discrepancy may be due to the differences of the measurement techniques. In Lam et al.'s work, AFM nanoindentation tips were used, and therefore the stiffness of cells measured should represent the surface properties of the cells. However, in our experiment, we deformed the cells with microspheres with a diameter of around 2.5 μm, and so the elastic modulus measured should closely relate to the cytoskeleton structure of the cells or the size of cell's nucleus.

The long-term laser operation will influence the cell's growth and division, as reported by Ayano et al. in 2006[29]. However, in our study, the laser was not directly shot on the cells, and the time of the contact between the laser trapped bead and the cell was very short at typically less than 1 min. Therefore, it is believed that the influence of laser in this study is negligible.

It should be admitted that the current protocol may not be very accurate, since the cells' deformation during indentation should actually be viscoelastic, but in our protocol, viscoelasticity is not considered[30, 31]. The current experimental technique can also be further improved. For example, if a photodiode bead detection system is installed in the optical tweezers, the movement of the bead and the force applied onto the cells can be accurately monitored, and the recorded force-displacement curve can therefore be analyzed by certain viscoelasticity correction protocol to extract the elastic modulus accurately.

4. Conclusions

In this study, a new protocol was proposed to measure the mechanical properties of leukaemia cells under well controlled physiological conditions by performing indentation test using optical tweezers. The proposed method was employed to measure the elastic moduli of the normal HL60 and K562 cells, and their counterparts treated with four different drugs PMA, ATRa, CTX and DEX. The measured elastic moduli of K562 and HL60 cells are 162.8±19.2 Pa and 213.1±21.4 Pa respectively. These measured moduli agree with the previously reported relationship between the deformability of leukaemia cells and the risk of leukostasis, and also agree with previously measured elastic moduli of HL60 cells by AFM nanoindentation. It was found that the ATRa, CTX and DEX drugs all decreased significantly the stiffness of the leukemia cells, and so they possibly can decrease the risk of leukostasis. PMA treatment, however, resulted in no change of stiffness of HL60 cells, or actually increased the stiffness of K562 cells. A static adhesion assay was employed to investigate the adhesion between the cells and proteins existing in their extracellular matrix. It was found that cells treated with ATRa, CTX and DEX showed weaker

cell-protein interaction, while those treated with PMA showed stronger cell-protein interaction, than the cells without drug treatment.

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