Detection of cancerous cervical cells using physical adhesion of fluorescent silica particles and centripetal force

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Here we describe a non-traditional method to identify cancerous human cervical epithelial cells in a culture dish based on physical adhesion between silica beads and cells. It is a simple optical fluorescence-based technique which detects the relative difference in the amount of fluorescent silica beads physically adherent to surfaces of cancerous and normal cervical cells. The method utilizes the centripetal force gradient that occurs in a rotating culture dish. Due to the variation in the balance between adhesion and centripetal forces, cancerous and normal cells demonstrate clearly distinctive distributions of the fluorescent particles adherent to the cell surface over the culture dish. The method demonstrates higher adhesion of silica particles to normal cells compared to cancerous cells. The difference in adhesion was initially observed by atomic force microscopy (AFM). The AFM data were used to design the parameters of the rotational dish experiment. The optical method that we describe is much faster and technically simpler than AFM. This work provides proof of the concept that physical interactions can be used to accurately discriminate normal and cancer cells.

1. Introduction

Cervical cancer is the second leading cause of cancer death in women worldwide. Early detection of this cancer can substantially decrease fatality of this disease. The objective of screening for cervical cancer is to prevent persistent human papillomavirus (HPV) infection and to avoid death by detecting and treating high-grade squamous intraepithelial lesions, which are precursor lesions for invasive cancer. A simple and effective screening method is of prime and utmost importance especially in many developing countries where cervical cancer rates are particularly high.

The Papanicolaou (Pap) smear and liquid-based cytology tests have proven to be the most successful methods of cervical cancer detection over the years. The biggest advantage of these tests is that the cells are collected by scraping them off the cervix and analyzing in vitro without the need for biopsy. Although the Pap test is the most widely used cancer screening method in the world and its impact in the incidence of cervical cancer is well known from a historical perspective, recent reports suggest that the sensitivity of Pap smear is 50–60%, with the relative proportion of sampling to screening errors being 2 : 1. Each year in the United States alone approximately 3.5 million Pap smears are classified equivocal, out of which 75% of women do not have cancer (similar accuracy is found for the liquid cytology test*). DNA tests (detection of HPV, human papillomavirus infection) are rather accurate, but identify only a risk of cancer (the fact of HPV infection) but not cancer. Thus, there is an obvious need for more accurate methods of detection of cervical cancer, methods which could work with cells collected with cell cytology tests without tissue biopsy to be analyzed in vitro. In addition, a recent study showed that women consider accuracy, comfort, and time as critical issues of any modification of the test procedure.*

Here we describe an approach that can result in a more accurate method of detection of cancer cells. The approach is based on detection of the difference in physical adhesion of non-functionalized (bare) silica beads to the surfaces of cancer and normal cervical epithelial cell. The work described is a proof of the concept aimed to show that physical interactions can be used to discriminate normal and cancer cells.

One other motivation of the presented work comes from the hope that physical sciences could bring new insights and provide new ways to attack cancer, which may fall outside of the traditional channels. The reason for this is as follows. A biological cell is an example of an extremely complex biochemical machine. While each individual chemical reaction can be understood by using modern biochemical methods, the work of cell as a unit is still far from clear. Modern cell biology is based on the hypothesis that the cell can be understood by analyzing individual biochemical reactions (“bottom-up” approach). However, there are many instances of complex systems where this approach...
is either ineffective or even not possible. An example is Statistical Physics, in which the behaviour of a system (for example, gas) cannot be derived from the behaviour of individual components (gas molecules). Thus, it is rational to consider a cell as one single object rather than a “bag” containing a collection of biochemical reactions (“top-down” approach). This approach shows definite success in some biomedical research in which cells are treated as a sort of “black box” responding to treatment with various chemicals. One of the physical approaches is to treat the cell as just a complex material with unknown properties.

Recently there have been many activities in studying mechanical properties of cells. In particular, it was shown that atomic force microscopy (AFM) can effectively be used to identify the difference in surface physical properties of cancerous and normal epithelial cervical cells. In that approach, the cell was treated as an elastic material covered with a “brush” comprised of microvilli, microridges, and various polysaccharides of the glococaly. It was shown that these surface brush layers are significantly different for cancerous and normal cells. This made it plausible to expect the difference in adhesion properties of these two types of cells. It was shown that adhesion of silica particles to cells depends on the time of contact, and can be statistically different for cancerous and normal cells. For a short period of time, cancerous cells showed higher adhesion to silica particles than normal cells. This regime was used to develop a method to identify malignant cells, which was based on an optical detection of the observed difference by utilizing ultrabright fluorescent silica particles. Unattached silica beads were removed by gentle washing. The total number of fluorescent particles was found by measuring fluorescent intensity of the particles. The difference was found to be significantly different at \( p > 0.05 \).

Here we report the use of another adhesion behaviour of silica particles to the cells. After increasing the time of contact with the silica particle, normal cells develop higher adhesion compared to cancerous cells. Physically, this regime is more robust compared to the previous one because of the weak dependence of the adhesion on time. However, the adhesion force becomes excessively high, and consequently, the silica particles cannot be removed with simple washing used in ref. 18. We resolve this issue in the present work by introducing centripetal force due to rotation of a culture dish, which contains cells with the attached fluorescent silica particles. We used modelling to predict the range of parameters (particle sizes and speed of rotation) required to discriminate normal and cancerous cells. Due to the different balance between adhesion and centripetal forces, cancerous and normal cells demonstrate clearly distinctive gradients of the fluorescent particles as a function of radius of rotation. Due to their ultrabright fluorescence of the used silica beads, the particles can be detected using rather simple experimental equipment consisting of an UV light source and a consumer level digital camera.

2. Experimental

2.1. Materials

Cetyltrimethylammonium chloride (CTACl, 25 wt% aqueous solution) and tetraethyorthosilicate (TEOS, 99.999%) precursors were obtained from Aldrich. Hydrochloric acid (HCl) (37.6 wt% aqueous solution, Baker, Inc.) and Rhodamine 6G (R6G) perchlorate dye (Sigma-Aldridge, Inc.) were used. All chemicals were used as received.

2.2. Cell culture

Primary cultures of human cervical epithelial cells were prepared from biopsies of cervical tissue collected from three cancer patients (from primary cervical carcinomas) and three individuals (from normal cervical tissue). The cells were isolated by a two-stage enzymatic digestion as described. Cells were maintained in keratinocyte serum free medium (Invitrogen, Carlsbad, CA). All human tissue was obtained from the Cooperative Human Tissue Network. Informed consent was obtained from patients according to their published guidelines (http://chtnci.nih.gov/phspolicies.html). Normal (healthy) cervical cells were used at 20 to 40 population doublings (PD), when they were actively growing, and carcinoma cell lines were used at 90 to 120 PD. The higher number of PDs of cancer cells was used to avoid possible confusion between cancer and normal cells (any normal cells present in the cancer culture dish would die out before that number of PDs). All cells were plated in 60 mm culture dishes. Epithelial cells adhered tightly to the bottom of the tissue culture dishes. The dishes were used for the experiments when cells were 80 to 100% confluent.

2.3. Fluorescent silica beads

Ultrabright fluorescent silica particles with encapsulated organic dyes were synthesized according to the procedure described in the literature. Briefly, it is a straightforward one-step synthesis. The surfactant, acid, dye, formamide, and distilled water (Corning, AG-1b, 1 M \( \text{cm} \)) were stirred in a polypropylene bottle at room temperature for 2 hours, after which TEOS was added and the solution stirred for ca. 5 min. The solution was then kept under quiescent conditions for 3 days. The molar ratio of \( \text{H}_2\text{O} : \text{HCl} : \text{formamide} : \text{CTACl} : \text{R6G} : \text{TEOS} = 100 : 7.8 : 9.5 : 0.11 : 0.01 : 0.13 \). The materials so formed were washed by centrifugation to avoid damaging of the surface, washed with copious amounts of water, and mixed with HBSS solution for the further use on the cell culture.

Comparing fluorescence of these particles with the brightest micron-size particles assembled with quantum dots, one can find that the used here particles (scaled to the same size) are up to two orders of magnitude brighter. This makes the particles the brightest tags presently available.

2.4. Detection of adhesion of fluorescent silica particles to cells in rotating culture dish

For the detection of affinity of fluorescent silica beads to either cancerous or normal cells, we studied the removal of silica beads from cells by the action of centripetal force. We subject the cell culture dish to rotational motion with respect to its centre to measure how centripetal force influences particle–cell affinity. The concentration of the particle’s dispersion was maintained at approximately 20 gm \( \text{L}^{-1} \). The cells cultured in 60 mm culture dishes were washed twice with HBSS solution, and then exposed to 0.5 ml of the colloidal dispersion premixed at 3 ml of \( 1 \times \text{HBSS} \) solution for 2 min. A hole of about 5–10 mm in diameter was
made in the centre of the lid of the cell culture dish. The dishes were then sealed using parafilm and mounted on a spin coater. The dish was consequently filled completely with more HBSS solution through the opening at the centre of the dish lid. The excess HBSS solution should be transferred gently so as not to displace many particles from the centre to the surrounding areas of the dish. The dishes were then spun with an acceleration time of 3 minute from 0 to 1000 rpm. The parafilm seal was intentionally broken by puncturing small holes with a pin. While rotating, the fluorescent silica particles can escape through the holes made in the parafilm. This allows removing excess particles from the dish avoiding accumulation of the particles near the edges. It also eliminates the need to wash excess particles from the dish after the rotation because the majority are discarded through the holes in the parafilm. The dish was then left to dry in ambient air at room temperature.

2.5. Determination of the gradient of particle distribution as a function of radius

To image fluorescent light coming from the silica particles, the culture dishes were illuminated with 375 nm UV light. Each dish was then imaged using a consumer Canon 5D SLR camera. The images were processed using Image metrology’s SPIP software version 5.0.6. Fluorescent intensity profile as a function of radius was found using profiling option of the software. The collected profiles were averaged over the places where the particle distribution was quite isotropic (to avoid any artifacts due to inhomogeneous cell distribution over the culture dish surface). The particle distributions at the centre as well as the edges of the dish were neglected due to the preparation artifacts. The radial gradient of fluorescent intensity was found by linear fitting of the averaged curves for the radii interval between ~5 and 25 mm.

3. Results and discussion

The study of adhesion of silica particles to normal and cancerous cells can be done directly with the help of AFM. As shown, the cell surface is covered with a brushy structure of microvilli and glycocalyx. When in contact with such brushes, silica particles develop different contact area with the brush over the time due to the Brownian motion. silica Surface is rich in hydroxyl groups, which interact with polysaccharides of the glycocalyx layer coating the plasma membrane. Moreover, silica interacts with the lipids of the membrane itself. As a result, the adhesion increases with time. Because the cell brush is different for cancer and normal cells, the time dependent adhesion is different.

3.1. Modelling

The force required to detach silica particles in the horizontal direction by means of rotation or centripetal force is obviously correlated with the adhesion. Therefore, we will use the information obtained with the AFM technique to find the set of parameters for the rotation and particle’s sizes which allows us to see a clear difference between cancerous and normal cells when rotating the culture dish.

The condition to detach a silica particle from the cell surface is described by the balance of torques at a particular lateral shift of the particle required for its detachment $\Delta x$:

$$6\pi W_A R_p^2 \theta = m\omega^2 R_p \theta = \Delta x R_p,$$

where $R_p$ is the radius of silica particle, $R_c$ is the radius-coordinate of the rotating dish, $\omega$ is the angular speed of rotation, $m$ is the particle mass, and $W_A$ is the energy of adhesion between two flat surfaces per unit area.

$W_A$ can be found by using Derjaguin approximation:

$$W_A = F_{adh}/(\pi R_m),$$

where $R_m$ is the harmonic mean of the radii of the contact of silica particle with cell, $F_{adh}$ is the adhesion force.

To find the relation between the adhesion and rotational condition to detach the silica particle from cell surface, we use Johnson–Kendall–Roberts theory combined with classical formula for centrifugal forces. As a result, one obtains the minimum adhesion required to keep the particle attached to cells:

$$F_{adh} = 2/3 (\rho_p - \rho_{buffer}) V_p \rho_p^2 R_p R_p/\Delta x,$$

where $\rho_p$ is the mass density of the fluorescent silica particle (1650 kg m$^{-3}$), $\rho_{buffer}$ is the mass density of HBSS buffer (997 kg m$^{-3}$), and $V_p$ is the particle volume.

All parameters are easily defined in the above formula, except the detachment distance $\Delta x$. This distance can be estimated from the previous study of adhesion of micron-size polymer and silica particles to various surfaces in air. For the values of adhesion force recorded on cervical cells, this distance could be estimated as 5–30 nm. This estimation is done based on the data collected in air.

It is obviously easier to remove particles when immersed in liquid. To take into account other possible unknown parameters, we will consider $\Delta x$ of the interval 1–50 nm in our further calculations. Fig. 1 shows the adhesion of silica beads to normal and cancerous cells as measured with AFM$^{17}$ as well as the minimum adhesion force required to keep the particles attached to the cell surface as described by eqn (3). In these calculations, the radius-coordinate of the rotating dish $R_c$ was taken 10 mm, and the rotation speed $\omega = 1000$ rpm. When the adhesion is larger than the minimum adhesion force, the particle remains attached to the cell surface. As we can see from Fig. 1, the size of the particles that can be detached from cancer but will stay on normal cells is to be from 3 to 20 $\mu$m in diameter. Particles with larger diameters will be detached from both types of cells, while the beads with smaller diameters will stay adherent to both kinds of cells.

Based on the above calculations, one can conservatively conclude that working with fluorescent silica particles of ~5 to 15 micron in diameter makes it possible to detect the difference in the adhesion of particles to cancerous and normal cells. The time of contact is expected to be longer than the AFM contact time shown in Fig. 1. This is because the silica particle attached to the AFM probe pushed cells with much higher force compared to the force of gravitational precipitation, which acts in the culture dish. Based on the data of ref. 17, this time has to be more than two minutes. The experimental method developed here is based on these estimations.
3.2. Observation of gradients of distributions of adherent fluorescent silica particles for culture dishes with cancerous and normal cells

An example of fluorescent images of culture dishes with adhered fluorescent silica particles to cancerous and normal cells is shown in Fig. 2. Note a residual fluorescence of plastic material of culture dishes. Being a constant, this fluorescence does not influence the sought gradient.

One can see a dark artefact in the middle of the culture dishes, which occurs due to the hydrodynamic shear force when adding buffer (HBSS solution added to the dishes when they are mounted on the spin coater). We also ignore distribution of the particles near the edge of the dish due to a complex hydrodynamic behaviour of liquid at that location. One can also see a round bright artefact in Fig. 2b typically associated with multilayered cell growth, which is hard to prevent when cells are nearly confluent.

Averaged gradients of distributions of adherent fluorescent silica particles calculated for various cell cultures are shown in Fig. 3. The results are grouped for normal (left panel) and cancerous (right panel) cells. One can clearly see an unambiguous difference between the gradients of cancerous and normal cells. It is clear that we are dealing with the behaviour highlighted in grey shown in Fig. 1. The adhesion to normal cells is stronger than the minimum adhesion force predicted by eqn (3). Therefore, fluorescent particles stay attached to normal cells. At the same time, the adhesion force to cancerous cells is smaller than the minimum adhesion, eqn (3). As a result, fluorescent beads are being removed from cancerous cells located in the culture dish at the radii greater than ~10 mm. Presumably the removal is not instantaneous, but is accelerating closer to the dish edges. This results in the observed negative gradient (decrease) of the number of silica particles attached to cancerous cells with the increase of $R_c$.

It is useful to analyse the consistency of our model with the observed experimental data. Using eqn (3), one can find the ratio of the adhesion force at the radius where silica particles started to detach from cancerous cells (~5 to 10 mm) and the minimum adhesion force at the largest radius (~25 mm) where the particles still stay on normal cells. Fig. 4 shows this ratio as a dashed line, which was calculated with the used experimental parameters.

Note that this ratio depends only on the radius-position of particles and does not depend on the particle size $R_p$ and detaching distance $\Delta x$. Fig. 4 also shows the ratios between the actual adhesion forces of silica particles to cancerous and normal cells found in the AFM experiments (shown in Fig. 1).

To compare these ratios, one should note that the true ratio of the adhesion forces found from the rotational experiments using eqn (3) should be less than shown by the dashed line because we used only the minimum adhesion force for normal cells. Thus, one can see that we are indeed working at the region where the adhesion of silica particles to normal cells is higher than to cancerous ones. Secondly, the ratio observed in the rotational experiments is

![Fig. 1](image1.png) Solid line curves: the adhesion force between a silica ball (attached to AFM probe) and cell surface as a function of contact time of the probe and surface for normal (curve 1) and cancer (curve 2) cells. Dashed lines: minimal adhesion force calculated from eqn (3) as a function of $\Delta x$ distance and particle radius.

![Fig. 2](image2.png) Fluorescent images of culture dishes with (a) normal and (b) cancerous cells, and examples of gradient profiles for (c) normal and (d) cancerous dishes.

![Fig. 3](image3.png) Gradients of distributions of adherent fluorescent silica particles calculated for various cell cultures. The error bars correspond to the error in the linear curve fit.
experiments is quite close to the ratio expected from the AFM experiments.

It is worth noting that the adhesions shown in Fig. 1 and 4 for the shortest time of contact were used previously\textsuperscript{17} to detect cancer cells by means of fluorescent silica particles. Here we use the other part of the ratio between the adhesions that lie below the dashed line of Fig. 4.

4. Conclusions

We described a non-traditional method to identify cervical cancerous cells \textit{in vitro}. The method is based on the difference in physical (nonspecific in biological sense) adhesion of silica particles to cancerous and normal cells. Using the AFM data and modelling, we suggested and verified a simple method to visualize this higher adhesion of silica particles to normal cells using a rotating culture dish with cells and adherent fluorescent silica particles. Due to the different balance between adhesion and centripetal forces, cancerous and normal cells demonstrate clearly distinctive distributions of the particles. This method is much simpler and faster compared to AFM. The differences are easily detectable by using even consumer type of digital cameras.

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