



ORIGINAL ARTICLE

Capture of cervical exfoliative cells on a glass slide coated by 3-glycidyloxypropyl trimethoxysilane and poly-L-lysine

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Abstract A new modification method for glass slides was developed and applied to make ThinPrep Pap smears, in order to increase the adhesion ability of cervical exfoliative cells. 3-glycidyloxypropyl trimethoxysilane (GOPS) was coated on the glass slides firstly on the slides, then poly-L-lysine (PLL) was covalently modified onto the above epoxy-terminated slides to form GOPS-PLL double decorated slides. The modified slides were characterized using X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM). The cell adhesion ability effect was tested and compared with traditional PLL coated slides by fixing the cervical exfoliative cells on the double adorned slides. The control test was conducted by the bare glass slides unmodified. The cell morphology of cervical exfoliative cells adhered on different slides was observed under the microscope after Papanicolaou staining. The number of cervical exfoliative cells on the unmodified slides, PLL coated slides and GOPS-PLL coated slides was 1030 ± 300 , 3283 ± 226 and 4119 ± 280 ($n=12$), respectively. The data among the three different modification methods showed significant differences (one-way analysis of variance, ANOVA test, $P < 0.05$). The cell capturing effect of the GOPS-PLL slide was the best among the three different modified slides. In addition, the GOPS-PLL slide could enhance the uniformity of the adhered cells and be widely applied to the ThinPrep system for cervical carcinoma screening to increase the accuracy rate of diagnosis.

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1. Introduction

The ThinPrep Papanicolaou-stained (Pap) smear has been one of the precancerous diagnosis methods of high-risk cervical carcinoma and has been widely used to screen the cervical cancer currently since it was developed in the 1990s [1–3]. ThinPrep liquid-based cytology has become a preferred method, of which samples were fixed in time, and blood, mucus and unstructured pieces were eliminated from the specimen. The key to prevention and treatment of cervical carcinoma is the effective cervical

screening at the early stage [4]. The diagnosis results based on the identification of morphological changes within cells, and all grades of cervical lesion cells were sorted according to The Bethesda System (TBS). The accuracy of the diagnosis results is largely dependent on the high quality specimen being collected during the procedure of examination. In a minority of instances, the obtained cell samples were inadequate or easily aggregation leading to high false negative results. For the ThinPrep Pap smear method, microscope glass slides are always used as cell adhesive substrates to make cervical smears. However, the low adhesive effect and easy agglomeration of cervical exfoliative cells on bare slides might lead to a high false negative rate of diagnosis. Therefore, it is important to modify the slides to adhere enough cells and to prevent the cells from dropping off the slides during the complicated operations of Pap staining.

Glass slide is one of the most commonly used substrates for adhering cells which was completely transparent in a broad range of wavelengths employed. The modification of the glass substrates has been done in many reports by introduction of different terminal groups to adhere protein and cells [5–9]. Silane coupling reagent is a kind of classical crosslinkers which has the ability to bind covalently to other molecules and even to biological molecules [10,11] which have amino, carboxyl or sulfhydryl groups [12–14]. Poly-L-lysine (PLL) is an important water-soluble and cationic polypeptide composed of naturally L-lysine—including amine groups on the side chains. It shows great potential in biological application on cell culture as the coating materials via electrostatic interactions [15]. The traditional modified PLL coated slides were used widely [16,17], but they did not work well in making smears because the cells on them could easily agglomerate. In addition, changing the surface morphology and surface roughness of the substrates can also increase the adsorption of protein, and then affect the adhesion behavior of different cells [18–20]. The increased surface roughness could enlarge the cells adhesive area to enhance the cell adhesion ability. Furthermore, the effects of surface charge may mediate the adsorption ability of differential protein to the different substrates [21,22], and the appropriate properties of hydrophilic or hydrophobic of the substrate can contribute to the ability of the cell adhesion and growth [23].

In this work, we employed 3-glycidoxypropyl trimethoxysilane (GOPS) which is one of the silanes coupling reagents and PLL to make double decorated slides as the cell adhesive substrate. The adhesion ability of cells was tested and compared to the traditional PLL coated slides by preparation of the cervical smears. Statistical differences between each group of cells on the different modified slides were compared and analyzed.

2. Experimental

2.1. Reagents and chemicals

3-glycidoxypropyl trimethoxysilane (GOPS, 97%) and poly-L-lysine (PLL, $M_w=70,000\text{--}150,000$) were obtained from Sigma-Aldrich Company (MO, USA). Papanicolaou stain and neutral balsam (60%) were bought from Shanghai Hualan Chemical Technology Company Limited (Shanghai, China). Hydrogen peroxide (H_2O_2 , 30%), concentrated sulfuric acid (H_2SO_4) and acetone were obtained from Beijing Chemical

Reagent Company (Beijing, China). All the reagents were of analytical grade and used as received without treatment under further purity. Deionized water was used during all the experiments.

2.2. Apparatus

Characterization of slides was performed by Atomic force microscopy (AFM, SPM-9600 Shimadzu, Japan) and X-ray photoelectron spectroscopy (XPS, PHI Quantera, Ulvac-PHI, Japan). The cell morphology of cervical exfoliative cells was observed by the microscope (Leica DMI 4000 B, Wetzlar, Germany) equipped with a CCD camera (Leica DFC 300 FX, Wetzlar, Germany).

2.3. Procedure

2.3.1. Substrate preparation

The glass microscope slides ($25\text{ mm} \times 75\text{ mm} \times 1\text{ mm}$) used as cell adhesive substrates were immersed in freshly prepared hot Piranha solution (30% H_2O_2 /concentrated H_2SO_4 , 1:3) for 1 h under slight boiling state. (*Warning: Piranha solution should be handled with extreme caution since it can react violently with organic matter*) Then the substrates were washed with copious amount of deionized water after cooling down to the room temperature, and dried under a stream of N_2 prior to performing the monolayer formation reaction on them. The cleaned slides were stored in deionized water, and used within a week.

2.3.2. Preparation of the PLL monolayer

The glass substrates were immersed immediately after cleaning into a water solution of PLL (0.1%) for 1 h. Then the slides were transferred to a fresh chamber filled with deionized water, and plunged up and down for three or more times to rinse the extra reagent. Finally, these slides were dried under a stream of N_2 before use and stored in a closed slide box to prevent them from being polluted by the air dusts.

2.3.3. Preparation of the GOPS-PLL surface

The glass slides coated with GOPS and PLL were prepared according to the procedures of references [24,25]. In brief, the cleaned glass substrates were immersed into a toluene solution of GOPS (1 mM) for 30 min to form a monolayer on the glass surface with epoxy functional groups. The reacted glass substrates were removed from the reaction vessel and rinsed thoroughly with toluene and ethanol successively to remove any excess silanes. These substrates were then dried under N_2 stream, and immersed immediately in a water solution of PLL (0.1%) for 1 h in order to covalently couple with PLL. Finally, all these double decorated substrates were dried under a stream of N_2 after being rinsed with deionized water and stored in a closed slide box to prevent the air pollution.

2.3.4. Characterization of different slides

The different glass slides were characterized by XPS and AFM. Tapping mode AFM images were conducted and the surface roughness of different slides was analyzed. XPS was performed using monochromatic Al $K\alpha$ radiation at 1486.7 eV in a PHI-Quantera SXM system. Survey spectra (0–1200 eV) were recorded at a 280 eV pass energy with an energy step of

1.0 eV. The slides used in AFM and XPS tests were prepared by cutting 1 cm × 1 cm from PLL coated slide and GOPS-PLL coated slide, respectively. And the untreated slide was used in all the control experiments.

2.3.5. Preparation of smears and Pap staining

The collected cervical exfoliative cells were stored in the vials containing 20 mL of preservation solution. 5 mL of specimen was transferred to a centrifuge tube and centrifuged at 1000 rpm for 5 min. About 3 mL of supernatant was sucked out, and the rest was mixed to form cell suspension. 1 mL of the well-mixed cell suspension was randomly added to the glass slides and stayed for 15 min. The rest of the liquid on the slides was dumped and air dried to make ThinPrep Pap smears. And the steps of Papanicolaou staining were conducted according to the operating instructions of the products. The morphology of cells on different ThinPrep Pap smears was observed under the microscope.

2.3.6. Statistical analysis

Quantitative data of the adhered cells on each slide was presented as the mean ± standard deviation ($n=12$). Statistical analysis was performed using the software of SPSS 17.0, and P values of 0.05 using one-way analysis of variance (ANOVA) test were considered to be a statistically significant difference.

3. Results and discussion

3.1. GOPS-PLL-slide modification

Functionalization of glass surfaces was usually employed by the trialkoxysilane reagents to modify the glass substrates. These functionalized surfaces were applied in the areas of chemistry and biochemistry. Scheme 1 briefly outlined the processes used to prepare the GOPS-PLL coated slides. The silane reagent is usually used for the surface functionalization. The epoxysilanes reacted directly with hydroxyl groups on the glass slide without hydrolysis of the silane reagent to the trisilanol followed by surface reaction and crosslinking [26]. The epoxy groups terminal glass slides were formed. The immobilization of PLL onto the epoxy-terminated surfaces was based on the reaction between the PLL and the head-end

reactive groups of epoxy silanes. The cervical exfoliative cells were fixed on the double decorated slides, and the cell morphology was observed under the microscope after the Pap staining.

3.2. Characterization of different prepared slides

The surface of the slides was examined by XPS and the change of the surface composition after each reaction was easily observed. The survey XPS spectra of the clean glass substrates with hydroxyl groups are shown in Fig. 1d, in which the main peaks are due to Si (2p and 2s), though C 1s and O 1s are observed. The peak of C 1s was possibly due to the presence of organic contaminations [27]. The XPS spectrum of the GOPS modified substrate showed obvious increase in the peak intensity of C 1s (Fig. 1c). When PLL was immobilized onto the above epoxy functionalized substrate, the C 1s signal increased and the N 1s peak at 400.8 eV appeared, indicating the successful binding of the PLL molecules on the epoxy-terminated slide (Fig. 1b). The intensity of the N 1s peak in the

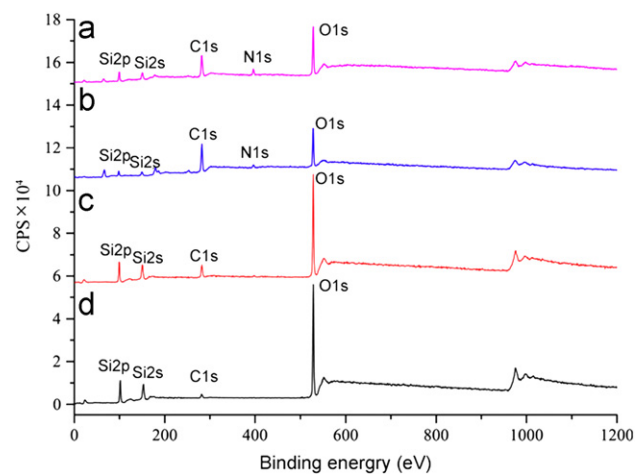
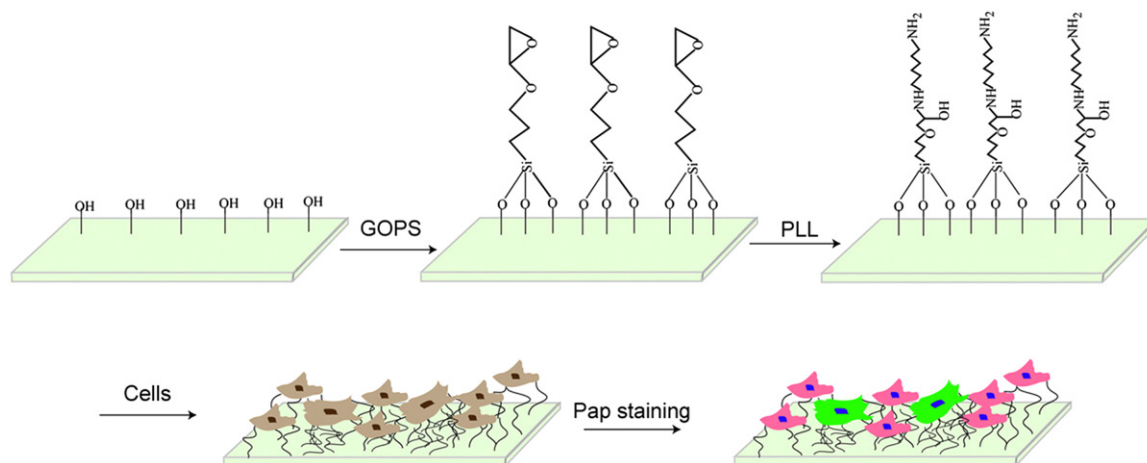


Figure 1 X-Ray photoelectron spectra of different substrates. (a), (b), (c) and (d) indicated the activated silicon substrate with hydroxyl surface, the GOPS-substrate with an epoxide terminated adlayer, the substrate with GOPS-PLL moieties attached, and the PLL coated slide with amino group, respectively.



Scheme 1 Preparation procedure of GOPS-PLL coated slide.

survey XPS spectra of the PLL slides was increased (Fig. 1a) than that of the GOPS-PLL slides. These signals evidenced that the substrates were successfully modified with GOPS and PLL, respectively.

The microroughness of the monolayers on different slides surfaces was imaged by AFM operated in the tapping mode under ambient conditions. Fig. 2 gives topographic views of untreated slide, PLL coated slide and GOPS-PLL coated slide. The surface roughness of untreated slide was small on which cells were adhered with low efficiency (Fig. 2A). Meanwhile, as the slides were modified with PLL and GOPS-PLL respectively, the surface roughness increased accordingly, which enlarged cells adhesive area. After being grafted with PLL and GOPS-PLL, the roughness of the substrates increased and the number of adhesive cells increased.

3.3. Preparation of cervical smears

The preparation of Pap smears was conducted according to the procedures described in the above section. But before the procedures of Pap staining, the cervical smears should be treated by wet fixation and air-dried methods. Pap staining is regarded as the ringleader of stain for assessment of chromatin pattern in cervical smears with a prerequisite of immediate

fixation in 95% ethanol. This wet fixation was conducted to avoid the cell concentration and the effects on nuclear and cytoplasmic staining caused by the air-drying [28]. However, the existed red blood cells and not clear background of the smears could affect the cytologic diagnosis [28–30]. The air-dried method particularly modified by rehydration step and fixed in an aqueous solution immediately before the staining procedure, provides the advantages of rapid cell sample preparation and better morphology. There was no difficulty in reaching cytologic diagnosis with rehydrated smears which is identical or superior to wet fixed smears especially for blood stained smears, while the routine use of the method might result in better smears [28]. In this experiment, we also considered the two different methods between wet fixation and air-dried-rehydrated method of the Pap smears on the GOPS-PLL coated slides. And the microscope photographs are shown in Fig. 3. The air-dried-rehydrated smears with the color of stain are not technicolor as shown in Fig. 3A. In Fig. 3B, the red blood cell background is not seen, possibly because of the function of the cell preservation solution in the ThinPrep system. However, the fragments of the cells appeared in which the cell preservation solution was not acted. The results could be more distinct in squamous cell carcinoma of the cervical cancer patient, while the samples in this text were normal cells. In the following steps, the cervical smears were conducted by the air-dried-rehydrated method.

3.4. Cell adhesion assay on different substrates

The enhancing adhesion effect of the cervical exfoliative cells on GOPS-PLL coated slide was examined. As a control, the cervical exfoliative cells were also fixed on the bare slides and traditional PLL coated slides. The cell morphology was visualized after fixing and staining the adherent cells with Papanicolaou stain. And another control test was conducted on the epoxide terminated glass slides. Cells on the bare slides and the epoxide terminated slides do not adhere well to the hydroxyl and the epoxy groups (Fig. 4A and B). These two control tests of the two different methods showed no significant differences. However, the PLL coated slides and GOPS-PLL coated slides provided strong cell adhesion (Fig. 4B and C). Adhesion to these modified slides was increased because of the higher film surface roughness indicated. The surface roughness of the substrates has been shown to play an important role on cell adhesion [18–20], and cell adhesion strength enhanced is associated with increased

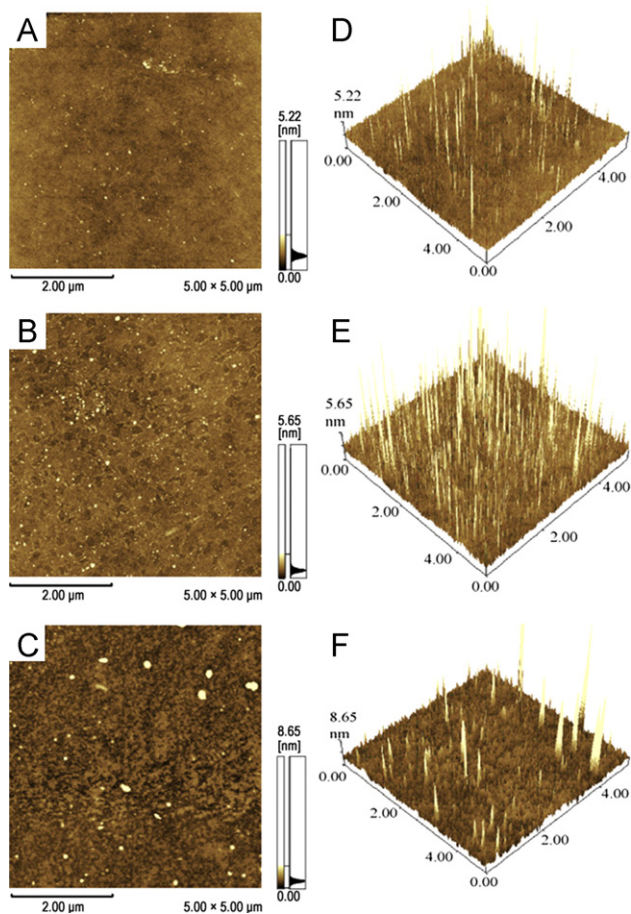


Figure 2 AFM topographic images of slides. (A, B) Indicated the slides with and without PLL treatment, respectively; (C) described the AFM image of GOPS-PLL double coated slide; (D), (E) and (F) were the 3-D AFM images corresponding to (A), (B) and (C), respectively.

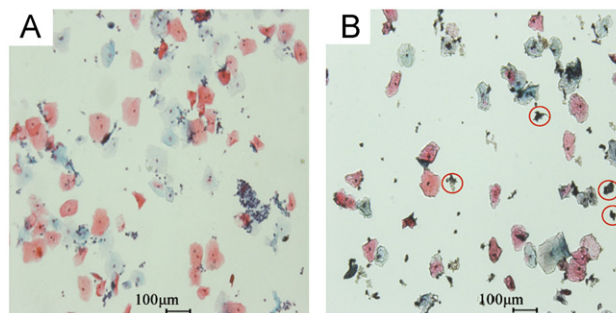


Figure 3 Photomicrographs of cervical exfoliative cells by different preparation methods of smears on the GOPS-PLL coated slides. (A, B) Indicated air-dried-rehydrated unmodified smear and wet fixation smear, respectively (Pap, original magnification $\times 100$).

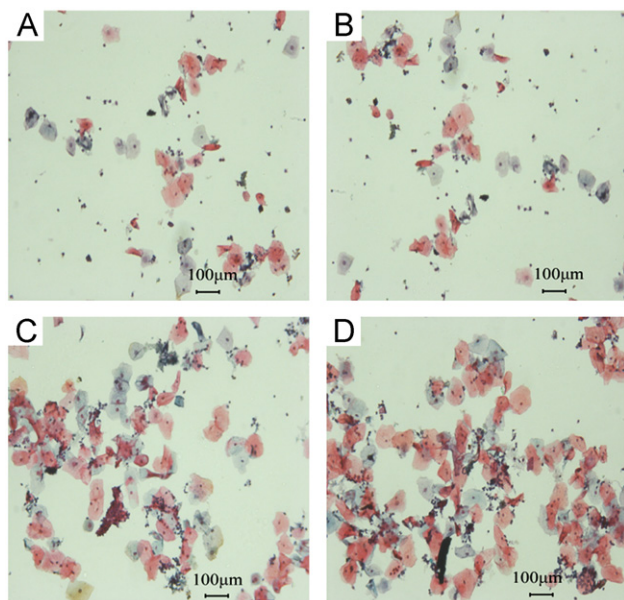


Figure 4 Photomicrographs of cervical exfoliative cells on different glass slides. (A), (B), (C) and (D) indicated unmodified slide, epoxide terminated slides, PLL coated slide and GOPS-PLL coated slide, respectively (Pap, original magnification $\times 100$).

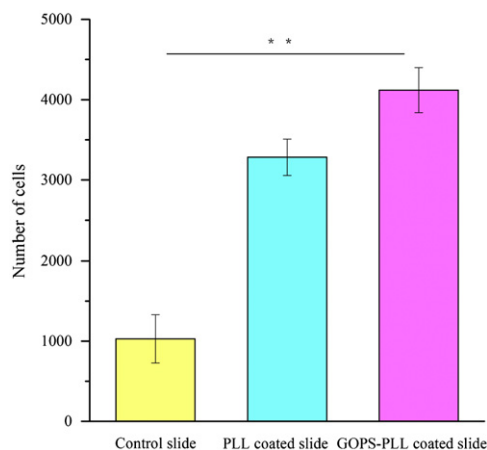


Figure 5 Data of cervical exfoliative cells on different coated glass slides ($n=12$, error bars indicate standard deviations). ** data indicated significant differences (ANOVA test: $P < 0.05$). Number of cervical exfoliative cells on the unmodified slide, PLL coated slide and GOPS-PLL coated slide was 1030 ± 300 , 3283 ± 226 and 4119 ± 280 , respectively.

surface roughness [19]. However, the cell morphology was not significantly affected by the modification methods of the slides, the color of the cells showed a little different. And the cells on the GOPS-PLL coated slide were more uniform than those on the PLL coated slide, facilitating the cytological observation and number counting.

3.5. Analysis of exfoliative cells on different substrates

Quantitative data of adhesive cells on different slides are shown in Fig. 5. The number of the cervical exfoliative cells on the unmodified slides, PLL coated slides and GOPS-PLL

coated slides was 1030 ± 300 , 3283 ± 226 and 4119 ± 280 ($n=12$), respectively. Significant differences in cell adhesion were found among the three different modification methods (ANOVA test, $P < 0.05$). The number of adhered cells on GOPS-PLL coated slides was the largest among three different kinds of glass slides.

4. Conclusion

This work designed a new modification method of slide used in the ThinPrep system, and the Pap smears were produced by fixing the exfoliative cells on the GOPS-PLL coated slides. This GOPS-PLL coated slide could enhance the cell adhesion effect and improve the uniformity of adhered cells. In addition, it would be widely applied to the ThinPrep system for cervical cancers screening to increase the accuracy rate of diagnosis.

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