Construction of a Biomimetic Zwitterionic Interface for Monitoring Cell Proliferation and Apoptosis

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A new zwitterionic monolayer film of sulfobetaine was constructed by grafting novelly designed N,N-dimethyl (β-hydroxyethoxyethyl) ammonium propanesulfonate (DHAPS) to hydroxyl groups of glass in the presence of hexamethylene diisocyanate (HDI) as a coupling agent and dibutyltin dilaurate (DBTDL) as a catalyst. Experiments of blood adhesion proved that the zwitterionic film possessed excellent hydrophilicity and very good biocompatibility and provided an appropriate biomimetic interface for adhesion and proliferation of cells. Thus, the monitoring of the cell proliferation and apoptotic processes on the zwitterionic surface during an incubation process was achieved, using different techniques, such as electrochemical impedance spectroscopy, scanning electron microscopy, flow cytometric assay, and Trypan blue staining. K562 leukemia cells, as a model, cultured in vitro on the zwitterionic surface kept their viability for 5 days and remained healthy and undifferentiated, indicating that the zwitterionic surface did not have a deleterious effect on cell growth in normal conditions. Thus, this man-made interface would be applicable to the growth of cells and the study of biomaterial—cell interaction and has potential applications in medicine and cytobiology.

Introduction

In vitro, cells generally exhibit restricted growth requirements with a finite proliferation. The extracellular microenvironment plays a key role in controlling cellular behavior.1,2 To adapt the need for medical and cytobiological studies, cells are usually attached to a biocompatible support.3 The properties of the support surface, such as roughness,4 hydrophobicity,5 surface chemistry,6 surface topography,7 surface positive/negative charges,8 and specific protein or cell—surface interactions,9 can affect cell activity and adhesion to biomaterials.10 Much research has currently focused on the design of biomaterials for cell adhesion.11,12

To improve cell attachment to man-made polymer materials, the surface treatment of these materials has been performed. For example, strongly oxidizing reagents and sodium hydroxide treatments have been used to improve cell attachment to polystyrene,13 smooth muscle cell attachment to poly(glycolic acid),14 and facilitate fibroblast attachment to poly(hydroxyalkanoates),15 respectively. The surface grafting with neutral functional groups such as hydroxyl groups can improve the cellular matrix for cell spreading. Up to now, several amine-based biocompatible polymers16 such as polyethylenimine, poly(propyleneimine, poly(pyrrrol, and poly(phenylene-diamine) have been demonstrated to be very effective for cell adhesion and proliferation owing to the positive charge in the cell culture medium. More recently, the protein—

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12 (8) Shelton, R. M.; Rasmussen, A. C.; Davies, J. E. Protein adsorption at the surface between charged polymer substrata and migrating osteoblasts. Biomaterials 1992, 13, 897–904.
nanoparticle conjugate modified surface has been designed to keep cells in vitro for 5 days. This indicates that surface modification is an important step for proper anchorage of cells on artificial surfaces and that the study on the interaction of living cells with artificial surfaces is interesting for both scientific and medical reasons.

Although some techniques such as spectrophotometric methods, fluorescence microscopes, amperometric or potentiometric scanning probe techniques, and flow cytometry have been developed for monitoring cell viability and proliferation on artificial surfaces, the effects of biomaterials on the proliferation and apoptotic processes of cells have not been studied in great detail, and the biomaterials with better biocompatibility for cytobiological study need to be further explored.

In this study, we report a new highly hydrophilic zwitterionic monolayer film (zwi-film) constructed by grafting \( N,N \)-dimethyl (\( \beta \)-hydroxyethylxoyethoxy) ammonium propanesulfonate (DHAPS) to hydroxy groups of glass (Scheme 1). In brief, the product DHAPS was first synthesized by the reaction of propane sulfone (PS) and 2-[2-(dimethylamino)ethoxyethyl] ethanol (DMAEE) and then added to the mixture of hexamethylene diisocyanate (HDI) and dibutylin dilurate (DBTDL), which were referred to as a coupling agent and a catalyst, respectively. Finally, this designed zwitterionic compound was introduced to glass with hydroxy groups to form the zwi-film. The zwi-film possessed excellent biocompatibility and good stability in solutions of high ionic strength, such as cellular media; thus, the adhered viable K562 cells could maintain their morphologic and physiological activities for 5 days.

**Experimental Procedures**

**Preparation of Zwitterionic Surfaces.** The synthesis of DHAPS was as described in ref 21. In brief, a propane sulfone (PS)/Acros solution was added dropwise to 2-[2-(dimethylamino)ethoxy) ethanol (DMAEE)/Aldrich] at 60 °C in 3 h and maintained for 24 h. The product of DHAPS was filtered and recrystallized with 2-propanol. Yield: 19.0 g (74.5%). Mp 107–109 °C.

While being stirred at 50 °C, DHAPS was added dropwise to the mixture of HDI and DBTDL solutions for 30–45 min. After the reaction was kept for 4 h at 50 °C, pretreated glass was immersed. This system was maintained under an nitrogen atmosphere at 50 °C for 4 h to form a zwitterionic surface on the glass.

**Characterizations of Zwitterionic Films.** 

**H NMR spectra** were obtained by using a Bruker AM 500 MHz spectrometer, and the chemical shifts were referenced in D2O with respect to external sodium 4,4-dimethyl-4-silapentane-1-sulfonate. Attenuated total reflection Fourier transform infrared spectra (ATR–FTIR) were recorded on a Nicolet 170 sx FTIR equipped with an omni sampler over 32 scans. X-ray photoelectron spectroscopy (XPS) analysis was carried out on an ESCALAB MK spectrometer (VG Scientific Co Ltd, UK) using MgKo (1253.6 eV) as the radiation source. Atomic force microscopy (AFM) experiments were performed on a SPA-300 HV atomic force microscope with a SPI 3800 controller (Seiko, Japan). Scanning electron microscopy (SEM) photos of adhesion cells were obtained with a Hitachi X-650 scanning electron microscope (Hitachi Ltd, Tokyo, Japan). The static water contact angle was measured at 25 °C by employing drops of deionized water. For blood platelet attachment, a zwi-film modified glass slide was contacted with fresh human PRP at 37 °C for 20 min and then rinsed with PBS and fixed with 2.5% glutaraldehyde solution for 30 min. After being dehydrated in a series of ethanol–water solutions, the obtained sample was examined by SEM.

**Cell Proliferation and Determination of Cell Apoptosis and Differentiation.** The 1.0 × 10⁵ cells/mL K562 cells were introduced onto the zwitterionic surface and cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Sigma), penicillin (100 µg/mL), and streptomycin (100 µg/mL) at 37 °C in a humidified atmosphere containing 5% CO₂. The cell number was determined using a Petroff-Hauser cell counter.

After the K562 cells were cultured on the zwi-film for 72 h, they were collected and evaluated by a Trypan blue and ANNEXIN V-FITC apoptosis detection kit I (BD Science). Briefly, 0.5 mL of 0.1% Trypan blue solution was added to cell suspensions for incubation for 10 min. The images were recorded on an
inverted phase contrast microscope. ANNEXIN V-FITC detection was performed after adding 10 μL of Annexin V-FITC and 10 μL of propidium iodide solutions to collected cell suspensions and incubating at 25 °C in the dark for 15 min. The obtained cells were characterized on a FACStar Plus flow cytometer (Becton Dickinson) within 1 h. When more than 40% of the apoptotic to total cells ratio was positive, the result was accepted as positive for apoptosis.

The differentiation of the K562 cells was assessed by the determination of specific cell surface antigen expressions. Briefly, the cells were incubated in a solution containing 10 μL of 50 μg/mL monoclonal antibodies of anti-CD13, anti-CD15, and anti-CD33 at 4 °C for 30 min and immediately analyzed on a flow cytometer after being washed twice with pH 7.4 PBS containing 10% FCS.

Electrochemical Measurements for Cell Proliferation. A glass carbon electrode was pretreated electrochemically to obtain surface containing abundant hydroxyl groups by applying a potential of +1.25 V in 0.1 M pH 5.0 PBS for 300 s and was scanned between +0.3 and +1.25 V then +0.3 and −1.3 V until a steady-state current–voltage curve was observed. The pretreated electrode was then immersed into a reaction solution to form the zwitterionic monolayer. The proliferation of cells was accomplished by dropping 5 μL of a 1.0 × 10^6 cells/mL suspension on the surface. The electrochemical signal was recorded on a CHI 730 electrochemical analyzer (CHI Co.) and a PGSTAT30/FRA2 system (Autolab, Netherlands), respectively, in 1.0 mL of pH 7.0 PBS containing 10 mM Fe(CN)₆³⁻/⁴⁻.

Results and Discussion

Characteristics of DHAPS Compound and the Zwitterionic Film. The synthesized zwitterionic molecule of DHAPS was characterized by ¹H NMR (Figure 1A). The ratio of the peak areas for a, (b + c + d), and e–g in the ¹H NMR spectrum was 1:4:3:1:1, which was exactly consistent with the ratio of protons in the DHAPS molecule. On the basis of the peak positions, the corresponding ascriptions were shown in Figure 1B. The zwi-film prepared on glass surface by a grafting reaction was confirmed by ATR–FTIR (Figure 2). The zwi-film showed the peaks ascribed to N–H stretching vibration at 3330 cm⁻¹, -CH₂- symmetric and asymmetric stretching at 2919 and 2853 cm⁻¹, a C=O stretching vibration of -NHCOO- and -COO- at 1620 and 1547 cm⁻¹, respectively, and SO₃⁻ absorption at 1038 cm⁻¹. When the K562 cells proliferated on the zwi-film (K562 cell/zwi-film), two absorption peaks occurred at 1644 and 1543 cm⁻¹ similar to the bands of proteins for amide I groups at 1610–1690 cm⁻¹ corresponding to the C=O stretching vibration of peptide linkages and amide II groups around 1500–1600 cm⁻¹ from a combination of N–H bending and C–N stretching, which verified the adhesion of K562 cells on the zwitterionic surface.

Figure 3 shows the corresponding C₁s, O₁s, N₁s, and S₂p core-level spectra of the zwitterionic surface. The C₁s XPS peak of the zwi-film consisted of three component peaks of hydrocarbons (–C–C–) at 284.8 eV, ammonium carbon (–C–N⁺(CH₃)₃⁻) at 286.3 eV, and urethane (–NH–COO–) and ester carbon (–COO–) at 288.1 eV. The N₁s chart showed two peaks for urethane nitrogen (–NH–COO–) at 401.1 eV and quaternary ammonium nitrogen (–N⁺(CH₃)₃⁻) at 402.7 eV. A little sulfurous content could be verified from the peak of SO₃⁻ at 168.1 eV (Figure 3A). The K562 cell/zwi-film showed a higher carbon content (Table 1) and wider O₁s peak than the zwi-film due to a more complex configuration of cells (Figure 3B). A new small peak observed at 133.1 eV was verified from the peak of -SO₃⁻.


assigned to P2p since lecithoid proteins are the main component of the cell membrane.

An AFM image of the pretreated glass displayed an even surface with a height less than 1 nm (Figure 4A), while the zwi-film showed a uniform distribution and a height of a short oligomer as expected for a densely packed monolayer (Figure 4B). The contact angle of the zwi-film was less than 5°, indicating a high hydrophilicity, which was in favor of promoting cell adhesion and growth.\(^\text{16,24}\) Furthermore, the zwi-film could be assessed as a bio-compatible material by comparing the adhesion of fresh human platelet rich plasma (PRP) on the zwi-film and bare glass. Obvious platelet activation occurred on the glass surface, as evidenced by pseudopodia extension, and a large number of adhered platelet aggregations was observed (Figure 4C). With the increasing platelet adhesion time, the number of adhered platelets and the degree of platelet distortion increased. Contrarily, no platelet attachment occurred on the zwi-film (Figure 4D), indicating that this film provided a good biocompatible interface for biomolecules. The excellent biocompatibility is due to the introduction of sulfobetaine in the zwi-film, which could substantially maintain the normal conformation of proteins of biomolecules.\(^\text{25}\) Thus, cell proliferation and cytobiological study could be achieved on this designed biocompatible interface.

Table 1. Surface Elemental Composition from XPS

<table>
<thead>
<tr>
<th>materials</th>
<th>atomic percentage (%)</th>
<th>atomic ratio</th>
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<tr>
<td></td>
<td>C_{1s}</td>
<td>O_{1s}</td>
</tr>
<tr>
<td>zwi-film</td>
<td>57.9</td>
<td>35.8</td>
</tr>
<tr>
<td>K562 cells on zwi-film</td>
<td>81.7</td>
<td>13.8</td>
</tr>
</tbody>
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Figure 4. AFM images of (A) bare glass slide and (B) zwi-film sputtered on the slide. SEM photos of (C) bare glass (D) and zwi-film incubated in human PRP for 300 min. Acceleration voltage: 10 kV.

Proliferation and Apoptosis of Cells on Zwitterionic Films. After incubation for 72 h, the adhered K562 cells on the zwi-film showed an obvious spreading appearance and good activity concluded from the morphology of distinguishable filopodia (Figure 5A), which is the evidence of cell adhesion to material surfaces and the indication of a good viability of cells.\(^\text{26}\) The cells proliferated on the zwi-film rapidly at the early stage of their growth in the culture, as shown in Figure 5B. The number of cells increased significantly, and the spreading of filopodia became more obvious. After the cells covered the whole surface over 120 h, longer incubation times made the cells become flat and then gradually shrink with a disappearance of filopodia, indicating that a majority of the K562 cells was already dead. Thus, the adhered K562 cells retained their normal cytomorphology and viability on the zwi-film for almost 5 days. The presence of the zwi-film did not have a deleterious effect on cell growth in normal conditions.

The pictures of viable (Trypan blue negative) and dead (Trypan blue positive) cells by Trypan blue staining were shown in Figure 6A. At an incubation time of 120 h, a few visual apoptotic cells were observed. The increase of incubation time led to a significant increase of apoptotic cells. The apoptosis rate of K562 cells during the proliferation process on the zwi-film was quantitatively assessed with Annexin V-FITC and PI staining. As shown in Figure 6B, the apoptotic curves during proliferation on the zwi-film were smooth and consistent with the results of the above study.


film indicated that more than 95% of K562 cells was viable within 72 h, approximately 8% of the cells showed early apoptosis at 96 h, and 72% of the cells was still viable at 120 h. Further incubation resulted in more cells changing from viability to early apoptosis and then to late apoptosis. After 168 h, only 15% of the cells was viable on the zwi-film (Figure 6C). The movement of cells through these three stages exhibited obvious proliferating and apoptotic processes.

Examination of Cell Differentiation on Zwitterionic Films. No significant differentiation of K562 cells adhered on the zwitterionic monolayer was detectable as revealed with surface marker expressions by means of a flow cytometric assay (Figure 7). The expression of CD13, CD15, and CD33 surface antigens on K562 cells was analyzed. The mean fluorescent intensities of various antigens on the K562 cells proliferated on the zwi-film were nearly the same as those in normal medium before adhesion and surface proliferation. Thus, this biomimetic interface not only promoted cell proliferation but also remained undifferentiated.

Electrochemical Monitoring of Cell Proliferation on Zwitterionic Film Modified Electrodes. Electrochemical methods were used for further confirming the change of support surface resulting from cell proliferation. Theoretically, the electron-transfer rate or electron-transfer resistance of an electroactive probe on the
When an electrode was modified with the zwi-film, the cyclic voltammograms of Fe(CN)_6^{3-}, serving as a probe, displayed a slight change. However, after the K562 cells were adhered on the zwitterionic surface and proliferated for 48 h, a decrease of ~50% in peak current and a drastic increase in peak separation were clearly observed. The further proliferation of the adhered cells resulted in a larger decrease in peak current and increase in peak separation (Figure 8A). These results implied the attachment and proliferation of the living cells on the zwi-film, which blocked the electron transfer due to the increase of the electrode interface resistance.

As seen from Figure 8B of the electrochemical impedance spectra (EIS), the electron-transfer resistances of Fe(CN)_6^{3-} at bare, zwi-film modified, and cell proliferated electrodes were in the range of 26–2808 Ω (Figure 8C). No obvious change of resistance was observed after the electrode was modified with the zwitterionic monolayer. The significant change occurred after the adhesion and further proliferation of the cells on the electrode surface. The cells adhered on the electrode introduced a barrier to electrochemical processes. With the increasing culture time after 120 h, a drastically increasing resistance was observed (Figure 8D). The change might be related to the apoptosis of cells, which were congregated on the electrode surface.

**Conclusion**

This work demonstrates a newly designed artificial interface, which possesses very high hydrophilicity, good stability, and excellent biocompatibility and promotes cell adhesion and proliferation without a deleterious effect. The K562 cells cultured on this zwi-film can retain their viability for 5 days. The current techniques for monitoring cell spreading and proliferation are applicable to the study of cell growth and biomaterial–cell interaction. The constructed material has potential applications in medicine, cytobiology, and the design of medical devices for interventional therapy.

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