ACS APPLIED MATERIALS & INTERFACES

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Introducing Mixed-Charge Copolymers As Wound Dressing Biomaterials

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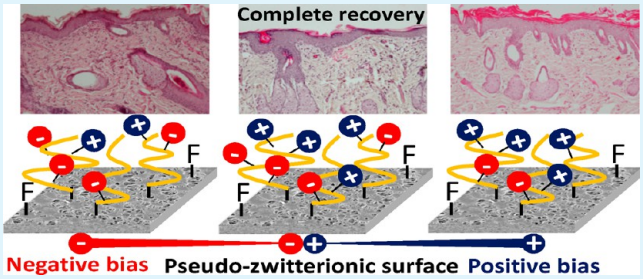
Supporting Information

ABSTRACT: Herein, a pseudozwitterionic structure bearing moieties with mixed positive and negative charges is introduced to develop a potential biomaterial for wound dressing applications. New mixed-charge matrices were prepared by copolymerization of the negatively charged 3-sulfopropyl methacrylate (SA) and positively charged [2-(methacryloyloxy)ethyl] trimethylammonium (TMA) onto expanded polytetrafluoroethylene (ePTFE) membranes. The charge balance was effectively regulated through the control of the initial SA/TMA ratio. The focus was then laid on the assessment of a variety of essential properties of efficient wound dressings including, hydration property, resistance to fibrinogen adsorption, hemocompatibility, as well as resistance to fibroblast attachment and bacteria colonization. It was found that the pseudozwitterionic membranes, compared to those with charge bias in the poly(SA-*co*-TMA) structure, exhibited the best combination of major properties. Therefore, they were further tested for wound healing. Histological examination of mouse wound treated with the pseudozwitterionic membranes exhibited complete re-epithelialization and total formation of new connective tissues after 14 days, even leading to faster healing than using commercial dressing. Results presented in this work suggest that the mixed-charge copolymers with a perfect balance of positive and negative moieties represent the newest generation of biomaterials for wound dressings.



KEYWORDS: *mixed-charge copolymer, ePTFE-g-poly(SA-co-TMA) membrane, charge bias, antibiofouling biomaterial, wound healing*

INTRODUCTION

The design of new wound dressings has caught the attention of many scientists since no current dressing presents the complete set of properties that a bandage should have to ensure a fast and efficient healing.[1−4] An ideal wound dressing should accelerate one or several stages of the healing process including the inflammatory phase, the migratory phase, the proliferative phase, and the remodeling phase. Even though the properties of ultimate wound dressings are well identified, major difficulties arise when it comes to combine them within the same material. For instance, to maintain a moist environment, a well-known strategy is to use hydrogels.[5−8] Nevertheless hydrogels do not permit exchanges of gases as efficient as in a dry porous film, due to lower diffusion coefficients in the gel matrix. But, if dry porous films are used, gas exchange will be promoted and the dressings will present good mechanical properties. But the environment above the wound will not be as ideal as that created by a gel because of the dryness. Furthermore, there might be interactions between the polymer network of the film and the wound, which is less likely to occur with a hydrophilic gel. Therefore, realizing optimal combination of the desired properties still remains challenging, and there is a need for the development of new materials that would fulfill all design requirements, despite extensive research.[1−9] Wwith the global aging of populations, bed sores and resultant chronic wounding of skin as well as the underlying tissues are more commonly seen, urging scientists to provide better solutions through the development of new biomaterials.

Over the past 10 years, the design of biomaterials enhancing hydrophilicity of hydrophobic polymeric surfaces in the field of membrane engineering or biomedical engineering has been the objective of lots of far-reaching studies.[10−14] Indeed, many

Received: April 19, 2014
Accepted: June 2, 2014
Published: June 2, 2014

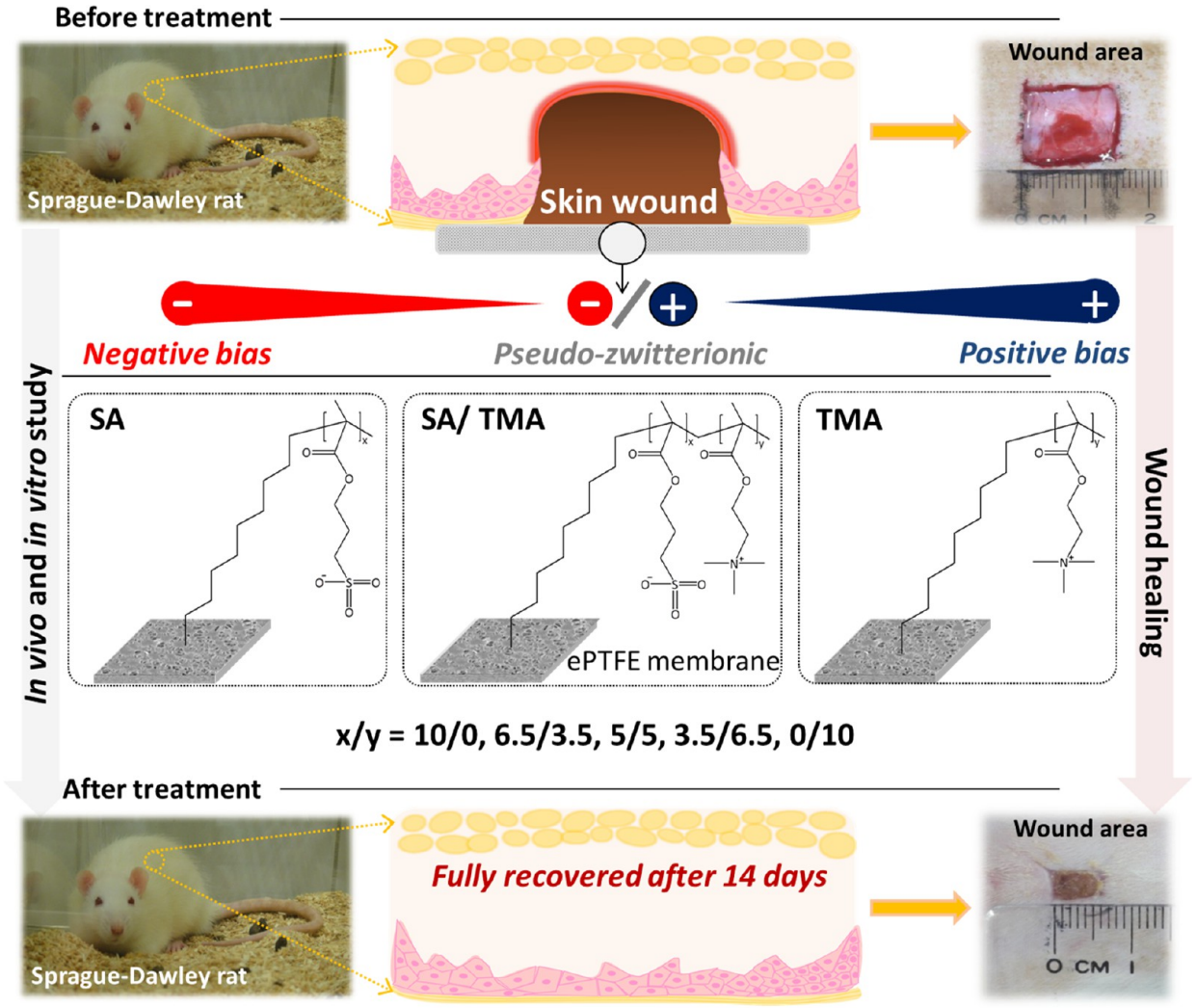


Figure 1. Schematic illustration of the use of SA and TMA monomers in controlled ratios for surface modification of ePTFE membranes, in order to yield ePTFE-*g*-pSA/pTMA intended to be used as wound-dressings.

polymers of interest are hydrophobic and are used because of their excellent bulk properties. However, their hydrophobic nature prevents wetting and promotes their interactions with biofoulants. In the case that we are interested in, the use of such polymers would irreversibly lead to the adsorption of blood cells, fibroblasts, proteins, and possibly bacteria, which must be avoided. New biomaterials able to significantly increase surface wettability of hydrophobic polymers therefore are needed for wound healing. Some of the best among these new materials are those with zwitterionic structures. The essential structures leading to their antifouling properties were identified in the early 2000s by Whitesides' group.[15−17] They are electrically neutral, possess polar functional groups, incorporate hydrogen bond accepting groups, and do not contain hydrogen bond donating groups. In addition, they are more stable than antifouling PEGylated materials, which ethylene glycol groups can be oxidized leading to a partial loss of their properties.[18,19]

In our group, we have recently reported the synthesis of zwitterionic copolymers for various applications including low-biofouling nanofiltration membranes[20] and antifouling matrices for blood filtration.[21] In each case, the use of zwitterionic copolymers led to significant improvement of the polymer matrix wettability. Also, the dry porous polymer membrane exhibited mechanical properties good enough for a potential use as a wound dressing, and could favor gas exchange. However, aside for the carboxybetaine-based materials, the partial loss of antiadhesive properties of zwitterionic materials has been reported.[22] This can be the case for instance when the surface has to be modified to add a supplementary conjugated bioactive function. It may be an issue in the design of wound dressing materials, which can be elaborated from several biomacromolecules fulfilling distinct functions. Therefore, efforts need to be oriented toward the generation of materials that could mimic zwitterionic polymers and ensure an even better stability of their antifouling properties when incorporated in complex molecular design. Pseudozwitterionic polymers made of mixed-charge brushes constitute this newest generation of antibiofouling materials.[22−24] They can be grown onto a surface with controlled net electric charge as well as a controlled bias (i.e., deviation from electroneutrality, either negative or positive), to lead to a matrix with improved wettability and antiadhesive properties, and could then be tested as materials for wound dressings. In addition, many cells in the bloodstream are electrically charged. It would then be of interest to investigate the role of the net electric charge of the surfaces on the resistance to the adhesion of human cells, as well as on their wound healing ability. This can be done by intentionally introducing a charge bias in the grafted surface

layer, through the control of the initial monomer ratio. We reported recently the use of mixed-charge copolymer brushes for the design of surfaces resisting nonspecific plasma protein fouling.[23] By changing the formulation and the process parameters, the charge bias was readily controlled and either negative, positive or pseudozwitterionic brushes were formed. Though extremely promising for the development of antibiofouling matrices and important for the understanding of interactions between surfaces and biomolecules/bio-organisms, this strategy has yet to be tested in the design of wound dressings and in the assessment of their essential properties, including their compatibility toward blood cells or fibroblasts.

In this study, inspired from the above knowledge in the design of antibiofouling materials and in the formation and characterization of copolymer brushes from mixtures of positive and negative monomers in one pot, we present our investigations on the critical role of the electric charge and charge bias on the properties of new wound dressings. As displayed in Figure 1, the paper investigates the novel usage of copolymers, made from a combination of the positively charged [2-(methacryloyloxy) ethyl] trimethylammonium chloride (TMA) and negatively charged 3-sulfopropyl methacrylate potassium salt (SA), for the modification of hydrophobic expanded polytetrafluoroethylene (ePTFE) matrix, with an intention of being used as a wound dressing.

EXPERIMENTAL SECTION

Materials. ePTFE microporous membranes (average pore size: 0.24 μm, thickness: 500 μm) were purchased from YMT Co., Taiwan. Poly(ethylene glycol) methacrylate (PEGMA) macromonomer with molecular weight of about 500 Da and average number of ethylene glycol units of about 10 was bought from Aldrich. 2-(Methacryloyloxy) ethyl]trimethylammonium chloride (TMA) and 3-sulfopropyl methacrylate potassium salt (SA) were purchased from Sigma Chemical Co. Isopropanol (IPA) was obtained from Aldrich and used as a solvent for the plasma-induced graft-polymerization. In each experiment, deionized water was obtained using a Millipore water purification system with a minimum resistivity of 18.0 MΩ.cm.

Surface Copolymerization. Plasma-induced surface copolymerization process was used to modify ePTFE membranes. The ePTFE microporous membrane was first treated by low pressure plasma with hydrogen flow (30 sccm). The input power was set to 100W and controlled by a 13.56 Hz RF generator (Cesar 136, Dressler). The post-treated ePTFE membrane with a surface area of 2.5 cm^2 was then incubated in a solution of IPA and DI water (8:2 v/v) containing 30 wt % of a mixture of TMA and SA monomers for 12 h. The SA/TMA molar ratio was 10/0, 6.5/3.5, 5/5, 3.5/6.5 or 0/10. For the preparation of the PEGylated membranes, the mixed IPA/water solvent contained 30 wt % of PEGMA macromonomer. Afterward, membranes were dried at ambient temperature (25 °C) for 24 h. Subsequently, the coated membranes were treated by low pressure plasma with argon flow rate of 30 sccm and input power of 150W. After plasma treatment, membranes were transferred into methanol and soaked for 60 min. Then, they were treated with DI water for 60 min in an ultrasonic device to strip off homopolymers and unreacted monomers. Residual solvent was finally removed by drying membranes in a vacuum oven under reduced pressure for 1 day and then in a freeze-dryer at −45 °C for 1 day. The final coating density was ∼2.0 mg/cm^2.

Surface Characterization. X-ray photoelectron spectroscopy (XPS) was used to characterize the chemical composition of the surface-modified ePTFE membranes, according to a protocol previously described.[21] The surface grafting yield was determined by the weight increase compared to the virgin ePTFE membrane, normalized to the membrane surface area. Before the measurements, membranes were dried in a vacuum oven at 50 °C for 3 days. Static water contact angles were measured with an angle-meter (Automatic Contact Angle Meter, Model CA-VP, Kyowa Interface Science Co., Ltd. Japan) at 25 °C. The DI water was dropped on each sample surface at 10 different sites and the average of the measured values taken as the membrane water contact angle. Hydration capacities (mg/cm^2) of materials were also determined in DI water and PBS. Dry weights of the 0.85 cm-diameter membranes were first measured using a 1×10^{-5} g precision balance (Mettler, Toledo Pac Rim AG, Taiwan Branch). Subsequently, membranes were immersed in DI water for 24 h. Surface water or PBS solution was then gently wiped out with tissue, and membranes were weighed. Hydration capacity was evaluated based on the weight increase per unit surface area of the membranes. For each membrane, the average of values from five independent measurements was taken as the final membrane hydration capacity.

Protein Adsorption On the Mixed-Charge ePTFE Membranes. The adsorption on the sample surfaces of fibrinogen from human plasma was evaluated using the enzyme-linked immunosorbent assay (ELISA), following a standard procedure that we detailed in several previous work.[14,21]

Bacterial Attachment Onto the Virgin and Grafted ePTFE Membranes. *Staphylococcus epidermidis* (SE) and *Escherichia coli* (EC) were used to investigate bacterial adhesion on membranes. SE and EC were first cultured, according to a procedure earlier reported.[14] Before incubation with bacterial solutions, membranes (0.4 cm^2) were placed in a 24-well plate and incubated with 75 wt % ethanol for 1 h at 25 °C, before being washed 3 times with phosphate buffered solution (PBS). Afterward, 1 mL of bacterial suspension was added to each well and incubated with the samples for 24 h at 37 °C. The bacterial solution was refreshed every 6 h. After the incubation, membranes were washed 3 times with PBS. Bacteria adhering to the sample surfaces were stained with 200 μL of Live/Dead BacLight for 10 min. After another triple washing with PBS, samples with stained bacteria were observed with a CCD camera mounted on Olympus BX51 with a 10× objective lens. Epifluorescent illumination was used for observation, through a blue fluorescence filter at an excitation range of 450−490 nm.

Thrombocyte Adhesion. Membranes (surface area: 0.4 cm^2) were placed in a 24-well tissue culture plate and equilibrated with 1 mL of PBS for 2 h at 25 °C. Platelet rich plasma (PRP), containing approximately 10^5 cells/mL, was prepared by centrifugation at 1200 rpm for 10 min of human blood. To test the adhesion of nonactivated platelets on the membranes, 200 μL of PRP were added on the surfaces in each well. For the adhesion of activated platelets, 200 μL of PRP recalcified by the addition of calcium (1 M $CaCl_2$, 5 μL) were used instead. In both cases, incubation at 37 °C lasted 2 h. Subsequently, membranes were rinsed twice with 1 mL of PBS and immersed for 48 h into 2.5% (v/v) glutaraldehyde in PBS at 4 °C, to fix the adhered platelets and adsorbed proteins. The membranes were then washed twice with PBS and gradient-dried with ethanol in 100% v/v PBS, 90% v/v PBS, 75% v/v PBS, 50% v/v PBS, 25% v/v PBS, 0% v/v PBS, and 0% v/v PBS for 20 min each time. Membranes were finally dried in air. The samples were sputter-coated with gold prior to the observation with a JEOL JSM-5410 scanning electron microscope (SEM) operating at 7 keV.

Erythrocyte Adhesion. Membranes (surface area: 0.4 cm^2) were placed in a 24-well tissue culture plate, and each well was equilibrated with 1 mL of PBS for 24 h at 37 °C. Red blood cell (RBC) concentrates were prepared by centrifugation of 250 mL of fresh blood (obtained from a healthy human volunteer) at 1200 rpm for 10 min. To test the erythrocyte adhesion, RBCs were placed on the surface of the membrane in each well and incubated for 120 min at 37 °C. Afterward, membranes were rinsed twice with 1 mL of PBS, and then immersed in 300 μL of 2.5% glutaraldehyde in PBS for 10 h at 4 °C to fix the adhered RBC. Samples were then rinsed five times with 1 mL of PBS. Morphology of the blood cells adhering on the substrates immersed in PBS was observed using a Nikon A1R laser scanning confocal microscope (LSCM, A1R, Nikon, Japan) at 200X on the same chip. Images were taken at λ_{ex} = 488 nm/λ_{em} = 520 nm and a *z*-step of 1 μm to depths of 30 μm from the optical horizontal sections of three different areas on each membrane.

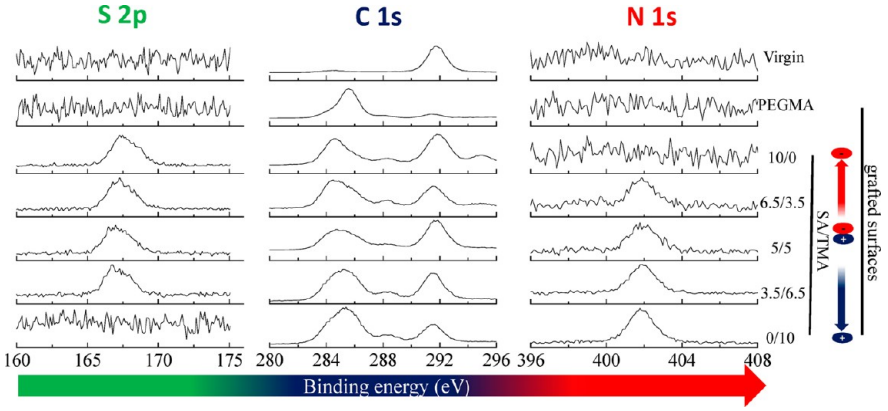


Figure 2. XPS analysis of virgin ePTFE and modified membranes.

Table 1. Assessment of Surface Charge of Mixed-Charge Membranes from XPS Analysis

	PTFE-*g*-10SA	PTFE-*g*-6.5SA/3.5TMA	PTFE-*g*-5SA/5TMA	PTFE-*g*-3.5SA/6.5TMA	PTFE-*g*-10TMA
electropositive moieties EP (%)	0	35.96 ± 0.22	50.48 ± 1.37	66.23 ± 0.54	100
electonegative moieties EN (%)	100	64.06 ± 0.24	46.19 ± 3.97	33.8 ± 0.49	0
charge bias: (EP-EN)/(EP+EN) x 100 (%)	−100	−28.09 ± 0.49	0.95 ± 2.76	32.42 ± 1.01	100

Leucocyte Adhesion. White blood cell (WBC) concentrate was prepared by centrifugation of blood at 500 rcf for 30 min. Then, WBC was placed on the surface of the membranes (0.4 cm^2) in each well of the tissue culture plate and incubated for 120 min at 37 °C. After that, membranes were rinsed twice with 1 mL of PBS, immersed into 2.5% glutaraldehyde in PBS for 10 h at 4 °C to fix the adhered WBC, and rinsed five times with 1 mL of PBS. Samples were observed by confocal microscope using the same instrument and settings as those used for the observation of erythrocytes.

Red Blood Cell Hemolysis. Cell membrane disruption of erythrocytes was estimated to further determine the hemocompatibility of the virgin and modified ePTFE membranes. The protocol used to isolate and purify red blood cells and to quantify the extent of hemolytic activity is the same as that earlier reported.[21]

Human Cell Attachment. Human HT-1080 fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM, Gaithersburg, MD), supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% nonessential amino acids, and 1% penicillin streptomycin at 37 °C. This medium was maintained on tissue culture polystyrene flasks in a humid atmosphere containing 5% CO_2. Before culturing the fibroblasts onto the membranes placed in a 24-well plate, samples were incubated at 25 °C with 75 wt % ethanol for 1 h and washed three times with PBS. Next, 1 mL of fibroblast suspension (10^4 cells/mL) was added to each well and incubated with the samples for 24 h at 37 °C in a similar atmosphere as that used to grow the cells. Finally, proliferation of cells was observed at 1, 2, and 3 days of incubation, with a Nikon TS100 phase contrast microscope equipped with a digital camera using a 10× objective lens.

Wound Healing Properties of Grafted Mixed-Charge ePTFE Membranes. Wound healing efficiency of pseudozwitterionic materials was evaluated using rats, obtained from BioLasco Taiwan Co., and weighing 400 ± 25 g. 0.15 mL tranquilizer (Rompun 2%, Bayer Healthcare A.G.) was first injected subcutaneously. Rats were then anesthetized with 0.2 mL of Zoletil 50 (Virbac Taiwan Co., Ltd.). Thereafter, an electrical razor was used to shave the surgical trauma area. Rats were then wounded with a surgical scalpel, over a surface of about 1.5 × 1.5 cm^2. The wound was then covered with either ePTFE-*g*-poly(TMA-*co*-SA) membrane or commercial dressing (3MTM TegadermTM hydrocolloid dressing, Minnesota Mining and Manufacturing Company) and materials were fixed to the wound with bandage. After 14 days, wound recovery was observed and histological analysis performed to compare the efficiency of the various dressings. In order to perform the histological analysis by microscopy (Nikon Eclipse 80i), the wound tissues were first immersed in formalin and then stained with hematoxylin-eosin (HE) reagents.

RESULTS AND DISCUSSION

Characterization of Grafted ePTFE Membranes. XPS analysis was performed to determine whether the surface modification of ePTFE membranes had been successfully performed and results are presented in Figure 2 and Table 1. The characteristic peaks of ePTFE,[25] and ePTFE-*g*-PEGMA[26] were assigned referring to the literature. The C 1s spectrum of the ePTFE surface revealed one major peak at 291.8 eV, attributed to the [F-C-F] species. As for ePTFE-*g*-PEGMA, the C 1s spectrum also revealed the presence of two peaks at 285.6 and 291.5 eV. The major peak (BE = 285.6 eV) was attributed to the [C-H] and [C-O] species brought by the PEGMA brushes, while the smaller peak was due to the [C-F] species. Compared to the virgin membrane, the intensity of the peak at 291.5 eV of ePTFE-*g*-PEGMA was lower. A possible explanation is that if density and cross-linking of the PEGylated layer are significant, the native ePTFE matrix may hardly be detected by XPS, which indicates a very high surface coverage of ePTFE membranes by PEG derivative and optimal plasma operating conditions.

The C 1s spectra of the mixed-charge membranes are also presented in Figure 2. Compared to the virgin ePTFE, a second major peak arose at 284.5, 285.4, 284.5, 284.8, and 285.2 eV, for ePTFE-*g*-10SA, ePTFE-*g*-10TMA, ePTFE-*g*-6.5SA/3.5TMA, ePTFE-*g*-5SA/5TMA and ePTFE-*g*-3.5SA/6.5TMA, respectively. It corresponded to the [C-O] species brought by polySA, polyTMA or poly(SA-*co*-TMA) . Note that a smaller peak, attributed to the [C-OO] species, was also found in the range of 288.2−288.4 eV in the spectra of ePTFE-*g*-10SA, ePTFE-*g*-10TMA and ePTFE-*g*-poly(SA-*co*-TMA) membranes. Its presence also further supported that an efficient surface grafting of ePTFE membranes with SA/TMA monomers had been achieved. Moreover, a N 1s peak appeared on the spectra of ePTFE-*g*-10TMA (BE = 401.8 eV) and ePTFE-*g*-poly(SA-*co*-TMA) (BE: 401.7−401.9 eV) membranes, attributed to the quaternary ammonium cations. As for the S 2p spectra, they revealed the occurrence of a peak at 167.4, 167.2, 166.8, and

dx.doi.org/10.1021/am502382n | ACS Appl. Mater. Interfaces 2014, 6, 9858−9870

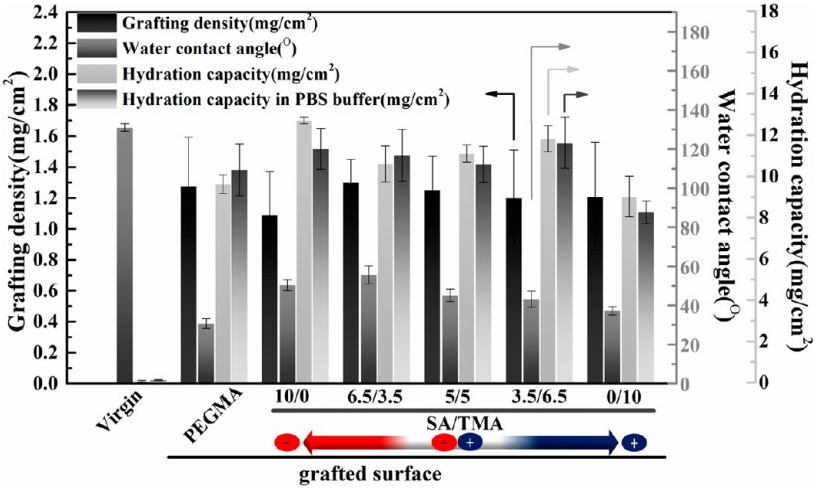


Figure 3. Hydration properties and grafting density of virgin ePTFE and grafted ePTFE membranes.

166.7 eV, corresponding to the sulfonate groups carried by ePTFE-*g*-10SA, ePTFE-*g*-6.5SA/3.5TMA, ePTFE-*g*-5SA/5TMA and ePTFE-*g*-3.5SA/6.5TMA membranes, respectively. Finally, XPS analysis allowed the determination of the charge bias of the copolymer brushes grafted onto the ePTFE membranes by plasma treatment (Table 1).

The surface treatment led to a grafting density of 0.9−1.3 mg/cm^2, depending on the sample (Figure 3), with the surface coverage optimized by adjusting the plasma treatment time. Such treatment led to major improvement of membranes hydrophilicity, as evidenced by water contact angle measurements as well as by hydration capacity tests (Figure 3). The water contact angle dropped from about 131° for the virgin ePTFE membrane to 45° for the pseudozwitterionic membranes. Hydration capacity, a measurement of water uptake by the whole matrix, increased from 0 to a value in the range of 10−12 mg/cm^2. In this respect, not only top surfaces of the modified membranes were made hydrophilic, but also water could be easily entrapped within the copolymer brushes, regardless of the overall net charge carried by the copolymers. Notice that hydration of negative species is a little higher than that of positively charged polymer surfaces. Indeed, according to the study of Yang et al. in which molecular dynamics simulations were used,[27] water molecules can approach closer to the anions than to the cations and allows for stronger electrostatic interaction between the anions and water.

To conclude on the characterization of the prepared membranes, it was shown that the electric charge carried by copolymer brushes grafted onto ePTFE surfaces could efficiently be controlled through the use of different monomer ratios in the initial polymerization solution. Surfaces with an overall positive, neutral, or negative charge, could be achieved. By comparing results reported in Table 1 with the initial SA/TMA monomer ratios used prior to surface polymerization, it was shown that the atmospheric plasma-induced polymerization is a reliable and controllable surface modification process. This, along with optimal surface grafting densities, resulted in surfaces with ideal surface and bulk hydrophilic properties. In general, water entrapment occurs in several aspects including trapping of water molecules in the bulk and binding of water molecules around the ionic heads carried by copolymer brushes at the top-surface. Surface modification not only improved water diffusion and entrapment along the copolymer brushes and in deeper layers of ePTFE membranes, but also allowed the generation of a hydrophilic top-surface favorable to the creation of a wet interface. In this work, the increase of hydrophilicity indicated that membranes were suitable to maintain a moist environment above a wound, a first major condition to favor healing.[1]

Resistance of Grafted ePTFE Membranes to Protein Adsorption and Bacterial Adhesion. To test the antibiofouling properties of engineered surfaces and the effect of net charge on biofouling at a nanoscale, adsorption test of a sticky protein, fibrinogen, was performed (Figure 4). Human

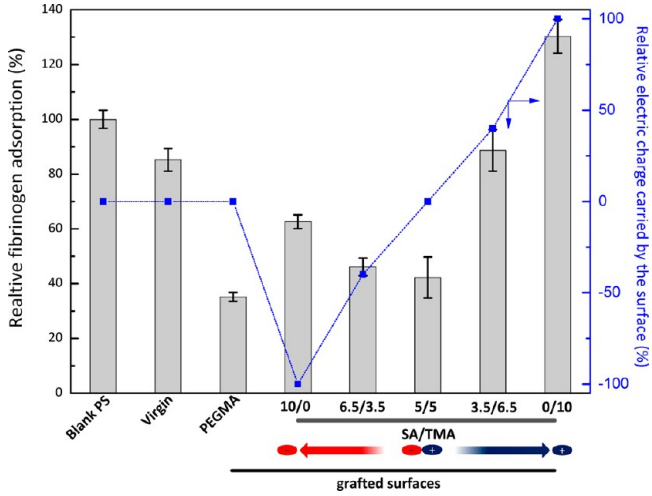


Figure 4. Evaluation of fibrinogen adsorption onto virgin ePTFE and grafted ePTFE membranes.

fibrinogen, a soluble plasma glycoprotein, is believed to easily interact with membrane materials. Owing to its large molecular weight, it possesses numerous hydrophobic groups that can readily interact with hydrophobic surfaces. Furthermore, it carries many local charges, implying that electrostatic interactions with charged surfaces can also occur. It was worth noting from Figure 4 that the trend for fibrinogen adsorption could be correlated with the charge carried by the copolymers (or SA/TMA ratio). It appeared that fibrinogen adsorption decreased up to 54% on negative surfaces, compared to the virgin ePTFE membranes while it was enhanced for surfaces with net positive charge. Minimal adsorption was

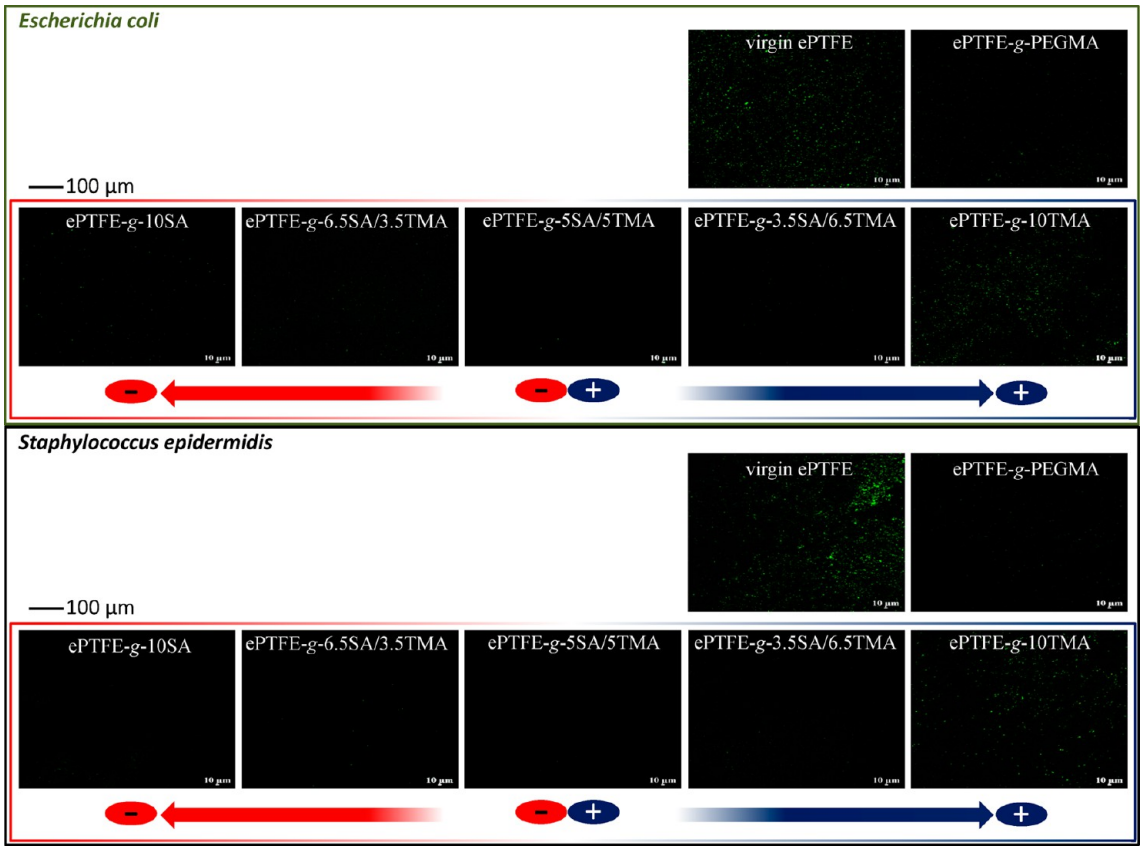


Figure 5. *Escherichia coli* and *Staphylococcus epidermidis* attachment onto virgin ePTFE and grafted ePTFE membranes. As only few green spots evidencing the presence of bacteria can be seen on the negatively charged and pseudozwitterionic membranes, one can further use the associated quantitative data, presented in Figure S1 in the Supporting Information, to classify the membranes with respect to their resistance to bacterial attachment..

found with the pseudozwitterionic membrane, with an adsorption of 49% of that for the virgin membrane. This surface had a N/S ratio of 1, and was assumed to carry no net electric charge, suggesting that low-bioadhesive surfaces could be obtained using neutral coatings formed from mixed-charge components.

Resisting fibrinogen adsorption is essential, as highlighted in literature and detailed later in this study, since fibrinogen is considered as a factor leading to blood clotting.[28] By controlling the N/S ratio to 1, and assuming that it is directly correlated to the net charge carried by mixed-charge brushes, we were able to control protein adsorption. This result also supported the earliest conclusion of Whiteside's group, regarding the importance of electroneutrality to provide a surface with antifouling properties.[15] However, copolymer brushes prepared in the present work were mixed-charge polymers, rather than zwitterionic. Somehow, it indicates that the distance between the electropositive site and the electronegative one is secondary in the design of low-biofouling materials, as long as the electric balance between positive and negative moieties is respected. Importantly, it was confirmed herein that a high hydrophilicity was clearly not enough to lead to an antibiofouling matrix. All surfaces prepared in this work had about the same hydrophilic properties (Figure 2). Yet, their tendency to adsorb fibrinogen differed totally. The charge carried by the hydrophilic moieties appeared to be more important than their ability to bind water, since both negatively charged and positively charged membranes (ePTFE-*g*-polySA and ePTFE-*g*-polyTMA) adsorbed fibrinogen. Adsorption was even more significant for the positively charged membranes. Different parts of fibrinogen molecule have different local charges, but the overall charge on fibrinogen (at pH 7.4) is negative, so the attractive electrostatic forces favored its adhesion onto the positively charged membranes.[29] Furthermore, adsorption onto the negatively charged surfaces was still possible, owing to the heterogeneous charge distribution along the fibrinogen backbone. Local sites bearing electropositive monomers could interact with fibrinogen, even though the protein was negatively charged. Minimum protein adsorption, an essential property of wound dressings, was therefore only possible for the pseudozwitterionic membranes.

Following protein adsorption tests, bacterial attachment onto virgin and surface-modified PTFE membranes was evaluated, using two model bacteria species commonly utilized in the studies of antibiofouling materials, *Escherichia coli* (EC) and *Staphylococcus epidermidis* (SE).[30,31] Typical fluorescence images are shown in Figure 5 while associated quantitative data are shown in Figure S1 (see the Supporting Information section). Similar trends were observed for both Gram-positive (SE) and Gram-negative bacteria (EC). In each case, the lowest adhesion was obtained for surfaces carrying no net charge; only a few spots were found on negatively charged surfaces, whereas positively charged surfaces were colonized by bacteria, with a maximal adhesion obtained for ePTFE-*g*-10TMA membranes.

Therefore, this work shows the importance of the surface electric charge on bacterial adhesion, with bacteria preferentially adhering onto the positively charged surfaces. One potential factor that can enhance bacterial adhesion and biofilm

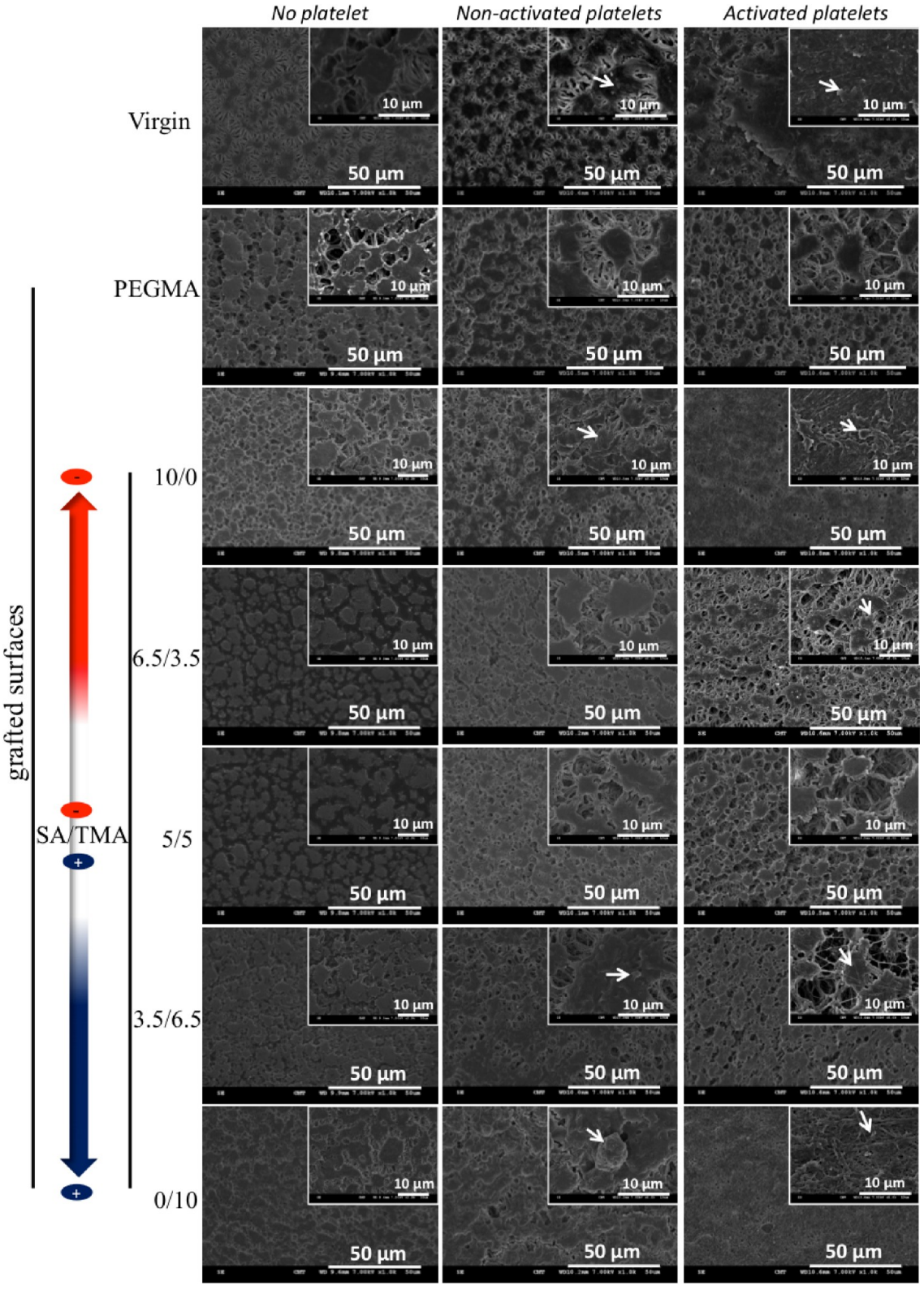


Figure 6. Evaluation of platelet adhesion onto virgin ePTFE and grafted ePTFE membranes. The white arrows point to the presence of platelets.

formation is the preadsorption of proteins, since they can lead to low-energy interactions with the bacterial cell wall. Herein, lowest bacterial attachment was found on those surfaces which also efficiently resisted protein adsorption. On the other hand, significant bacterial attachment was observed on positively charged surfaces, because electrostatic interactions could easily be established with negatively charged bacteria. Pseudozwitterionic membranes efficiently resisted colonization by model Gram-positive and Gram-negative bacteria, and therefore exhibited a second important property for wound dressings: the ability to resist micro-organism invasion, which is potentially responsible for wound infection.

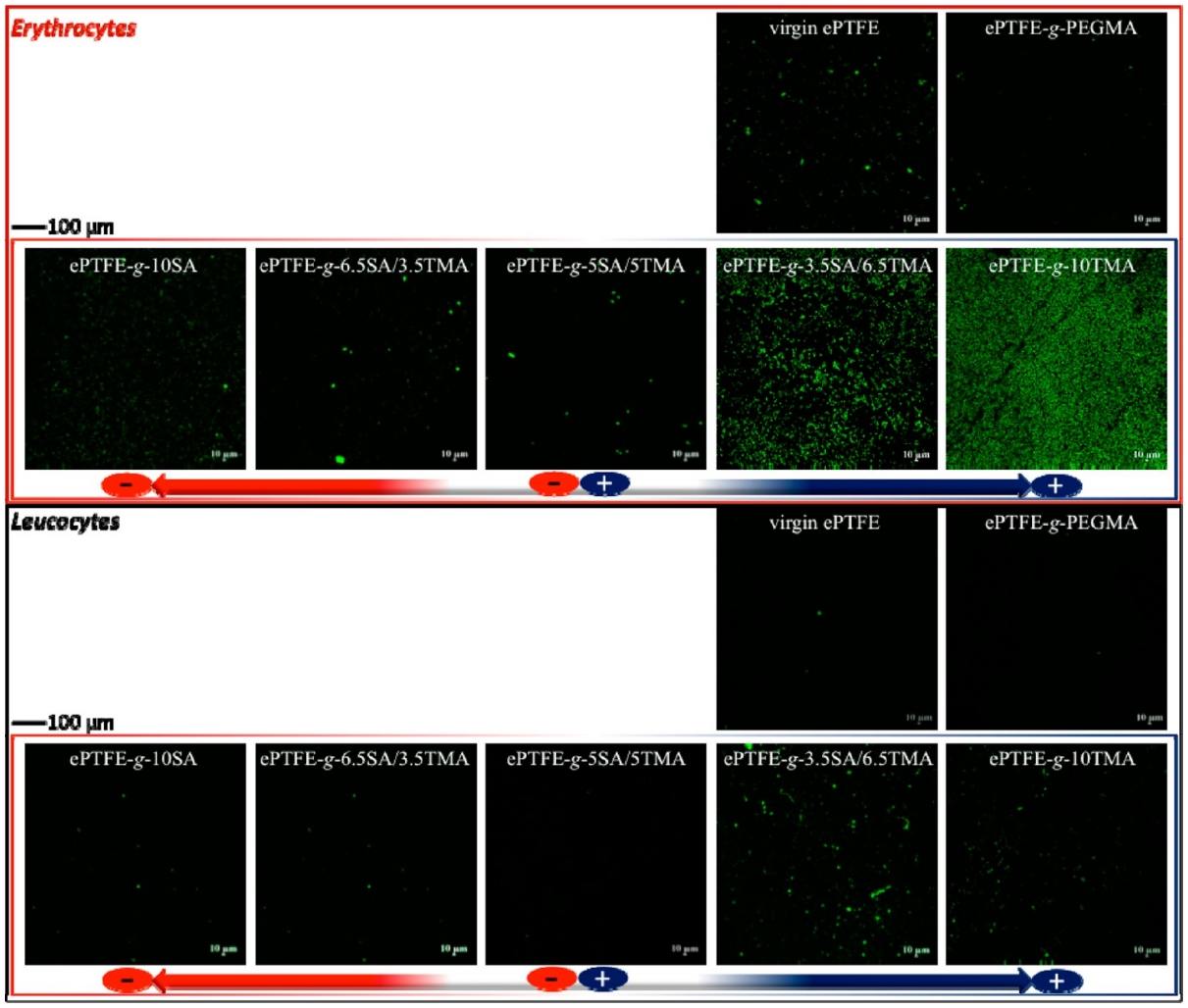


Figure 7. Erythrocyte and leucocyte adhesion onto virgin ePTFE and grafted ePTFE membranes.

Blood Compatibility of Grafted ePTFE Membranes. To test the hemocompatibility of membranes, an essential property of a wound dressing, the resistances to adhesion of thrombocytes, erythrocytes and leucocytes were evaluated. Ideally, membranes should not activate the blood defense systems, such as coagulation or fibrinolysis, and need to efficiently resist the adhesion and activation of blood cells. Furthermore, if the surface of the membrane surface interacts with any of the blood components, the wound surface will be at risk upon removal of the dressing.

SEM images of surfaces after incubation with blood platelets are presented in Figure 6. Notice that the background of images revealed a classic structure of polymer matrices prepared by stretching, with crystalline regions oriented parallel to the extrusion direction and a porous structure ideally causing physical entrapment of small cells such as platelets. It is also worth mentioning that the grafting of copolymers only led to a very small decreasing of the membrane porosity. Therefore, entrapment of platelets within the porous structure was still possible. Furthermore, platelets respond to minimal stimulation and are activated when they contact any thrombogenic surface such as an artificial surface.[32] It is generally accepted that rapidly adsorbed fibrinogen plays a critical role in platelet adhesion.[33,34] As fibrinogen was shown to easily adsorb onto ePTFE membranes (Figure 4), it was expected that fibrinogen in plasma would behave the same and eventually lead to adhesion of numerous platelets on the ePTFE surfaces. Moreover, nonactivated thrombocytes adhered significantly with the increasing electropositivity of the surfaces, whereas only slight adhesion was observed onto the negatively charged and pseudozwitterionic surfaces. As for the membranes incubated with the activated thrombocytes, it was found that adhesion of platelets occurred on all surfaces. However, aggregation, i.e., the formation of thrombosis with full-scale platelet adhesion and activation, only occurred for hydrophobic membrane (virgin ePTFE) or hydrophilic membranes with largest charge densities (ePTFE-*g*-10SA and ePTFE-*g*-10TMA).

In the vascular system, endothelial cells are responsible for the production, secretion, and binding of anticoagulants and procoagulants, which maintain blood fluidity and vascular integrity.[35] When blood is exposed to artificial surfaces, however, blood activation occurs. One important challenge in designing wound dressings is to avoid blood activation and blood cell adhesion on dressing surfaces, which may disrupt healing. In this study, aggregation of platelets was reduced for hydrophilic surfaces carrying no net charge (i.e., ePTFE grafted with PEGMA or 5SA/5TMA). When increasing the charge bias, regardless of the sign of the net charge, aggregation of platelets was favored. Platelets are negatively charged. So they can interact with positively charged surfaces via electrostatic

attraction. Furthermore, proteins in plasma could easily adhere onto both positively and negatively charged surfaces, as presented in previous section. These primary protein−surface interactions favored the adhesion of thrombocytes and explained the thrombosis on negatively charged surfaces (ePTFE-*g*-10SA) incubated with activated platelets, despite electrostatic repulsion between thrombocytes and surfaces. By designing membranes efficiently resisting the adsorption of plasma fibrinogen, platelets were less likely to interact with surfaces. Therefore, antithrombogenicity could only be improved using hydrophilic surfaces carrying no net charge.

The attachment of erythrocytes was subsequently tested and results are shown in Figure 7 and Figure S2 (Supporting Information section). Similar to platelets, attachment of erythrocytes depends on many factors such as the nature and extent of surface forces[36] or the topography of the surface.[37] Here, the topography of the different membranes was similar but electrostatic surface forces varied. A trend was identified: the adhesion of erythrocytes increased with the charge bias and in particular with the electropositivity of surfaces. Pseudozwitterionic membranes showed a similar level of adsorption as the virgin ePTFE and ePTFE-*g*-PEGMA membranes, which carry no net charge: only a few cells were observed on their surfaces.

Finally, leucocyte adsorption tests presented in Figure 7 and Figure S2 in the Supporting Information revealed that these cells barely interacted with surfaces carrying negative or zero net charge. On the other hand, an increase of surface electropositivity had an important impact on leucocytes adhesion. Overall, fewer leucocytes were spotted on the membranes surface compared to erythrocytes, but this was only due to the fact that the leucocyte concentration in blood is less than that of erythrocytes.

As a general observation, adhesion of erythrocytes and leucocytes was importantly enhanced onto electropositive ePTFE surfaces. However, by increasing the negative bias, erythrocytes could still importantly interact with membranes (ePTFE-*g*-6.5SA/3.5TMA; ePTFE-*g*-10SA). Negatively charged surfaces, as well as the pseudozwitterionic surface did not exhibit major leucocyte attachment. We first suspected that blood cells carried an overall negative charge, but the presence of numerous proteins in the concentrate prevented the determination of the electric charge carried by cell membranes through zeta potential measurements. It was reported earlier that the reduction of surface negative charge carried by erythrocytes led to an increase in their aggregation, due to the decreased electrostatic repulsion,[38] which would support our initial assumption regarding the overall negative charge of RBCs. Nonetheless, as with the platelets, surface electrostatic charge cannot explain alone the extent of erythrocytes adhesion. Otherwise, no cell would have been found onto ePTFE-*g*-6.5SA/3.5TMA and ePTFE-*g*-10SA membranes, negatively charged. Other secondary factors definitely play key roles. In particular, the ability of membranes to resist protein adsorption is critical as well, since red blood cell membranes contain dozens of proteins in their skeleton. Results of protein adsorption tests showed that negatively charged membranes were not as efficient as pseudozwitterionic membrane to resist protein adsorption, which supports results of red blood cells adhesion. As for the leucocytes, their greater adsorption onto positively charged surfaces was also mainly due to electrostatic interactions between the negatively charged cells and the positively charged polymer membranes. The electronegativity of the cells membrane was confirmed by a previous study in which polycations were used to attract leucocytes and favor their attachment on glass surfaces.[39]

To complete the hemocompatibility tests, a RBC hemolysis assay was performed and results are displayed in Figure 8. DI water and PBS solution were used as positive and negative controls, respectively. Hydrophobic polymers are capable of interacting with biological membranes, causing disruption. It was observed that the virgin ePTFE membrane exhibited ∼7% hemolytic activity. However, significant reduction of hemolytic activity was observed for grafted membranes, especially for ePTFE-*g*-5SA/5TMA membranes (only 2.5% hemolytic activity), further evidencing the improvement of blood compatibility using pseudozwitterionic coatings, with almost no measurable undesired reaction following the blood−membrane contact. A lack of hemocompatibility was again observed for positively charged surfaces, which had increased hemolytic activity.

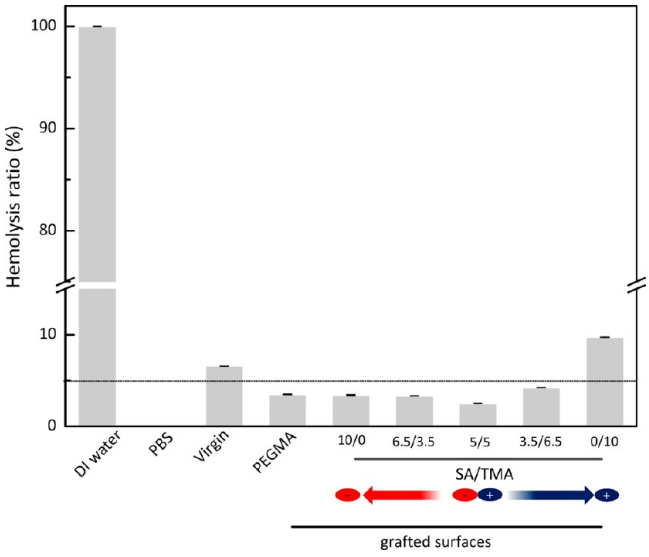


Figure 8. Hemolysis of RBC solution in the presence of virgin and modified ePTFE membranes at 37 °C. The dashed line at 5% corresponds to the threshold required by ISO 10993.

From all these considerations and results regarding the behavior of ePTFE membranes toward the adsorption of thrombocytes, erythrocytes, and leucocytes, hemocompatibility of membranes considering only the surface electrostatic charge can be summarized as follows. A negative bias tends to favor electrostatic repulsion of blood cells, with reduced hemolytic activity compared to the virgin membrane, but still permits platelet thrombosis and important erythrocytes adhesion owing to secondary factors mediating cells adhesion. A positively charged surface is clearly not hemocompatible, because the hemolytic activity is quite high and electrostatic attraction occurs with erythrocytes, leucocytes and thrombocytes. Most importantly, the pseudozwitterionic membrane leads to few interactions with all types of blood cells and lowest blood disruption from the hemolysis test, partly due to the absence of a surface charge (primary factor) but also to the resistance to protein adsorption (secondary factor) that positively influences cell attachment. In this respect, the most blood compatible membrane prepared in this work was the pseudozwitterionic ePTFE-*g*-5SA-5TMA membrane. The use of such pseudozwitterionic membrane can reduce the risk of blood disruption and

blood clotting, that is, improve the overall hemocompatibility, a third major property of ideal wound dressings.

Cell Adhesion Resistance of Grafted ePTFE Membranes. Another important property of a wound dressings is their ability to resist adhesion of human cells constituting epidermis. To promote the development of skin tissue, the adhesion of fibroblasts onto the wound dressing, which would otherwise delay healing and potentially cause supplementary trauma, must be minimized and if possible, annihilated. From previous studies, surfaces efficiently resisting adhesion of proteins and bacteria should also demonstrate cell resistance,[40,41] which was verified by our dedicated tests. Human fibroblasts (HS-1080) were cultured on membranes at 37 °C, and surfaces observed at 1, 2, and 3 days. The microscope images and corresponding quantitative data are shown in Figure 9 and Figure S3 (see the Supporting Information), respectively.

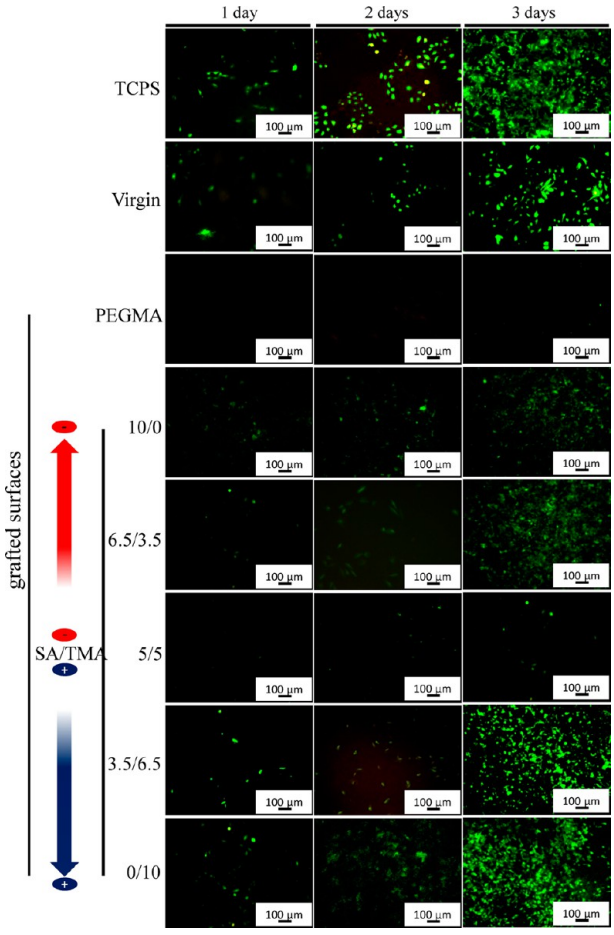


Figure 9. Fluorescence microscopic images of HT-1080 cell attachment onto different surfaces. Observations were made from 1 to 3 days after incubation of the samples with human fibroblasts. Cell culture was performed at an initial concentration of 1×10^4 cells/mL.

For the bare TCPS plate, fibroblasts readily adhered and spread over the surface into a confluent layer. Similarly, cells easily attached to the surface of the virgin ePTFE membrane, which provided a hydrophobic and porous environment for cell-membrane interactions. After 3 days, TCPS and ePTFE surfaces were almost entirely covered with fibroblasts. Similarly, the grafted ePTFE surfaces carrying electric charges offered suitable environment for cell growth. It is interesting noting that increasing the net surface charge led to strong adhesion of fibroblasts onto the grafted membranes with a maximum obtained on ePTFE-*g*-10TMA. On the contrary, cells barely adhered to surfaces of the pseudozwitterionic membranes, even after three-day incubation.

Results of fibroblast adhesion clearly exhibited a relationship between the electric charge carried by the surfaces and the ability of fibroblasts to grow, develop and spread on the polymer membranes. For the negative surfaces (ePTFE-*g*-10SA), cell adhesion was on the same order of magnitude as that for the virgin ePTFE membrane. By increasing the electropositivity, cells adhesion increased significantly, except for the pseudozwitterionic membranes, which displayed no cell adhesion. Therefore, a surface carrying net electric charge favored cell adhesion. Human fibroblasts, which extracellular matrix is rich of glucosaminoglycans, are negatively charged and consequently, easily adhere onto electropositive surfaces.[42] However, electric attraction is once again not the only factor responsible for fibroblast adhesion. Otherwise, negatively charged surfaces would not permit cell adhesion. Surface energy is another critical parameter that can affect cell adhesion.[43] Surface energy arises from a combination of several factors including surface chemical composition, net charge, and topography. By grafting negatively charged copolymer, surface energy has evolved in such a manner that an increase in cell adhesion occurred. Furthermore, once adhering onto a surface, cells secrete extracellular matrix proteins. These proteins, if possessing net electric charge, can easily adhere to the surface of the grafted ePTFE membranes, regardless of being positively or negatively charged as demonstrated earlier in this work. The adsorbed proteins can then interact with cell membranes via hydrophobic interactions, thus favoring the adhesion of fibroblasts. A clear correlation can be drawn between the ability of membranes to resist protein adsorption and cell attachment, i.e., if a surface is devoid of any adhesive proteins, cell−membrane interactions cannot occur. Notice that the proteins can come from cell culture medium (bovine serum) or from cell secretion. Therefore, it is essential to design a surface resisting protein adsorption to inhibit cell adhesion. In summary, pseudozwitterionic membranes led to minimal cell adhesion because (i) they carried no net electric charge, (ii) they resisted adsorption of proteins contained in the medium, (iii) they presented a minimal surface energy, and (iv) extracellular matrix proteins were almost not secreted onto these surfaces. Such improved resistance to cell adhesion is another property of efficient wound dressings. No supplementary trauma would be caused by a potential change of dressing, because cells of the new epithelium do not adhere to ePTFE-*g*-5SA-5TMA.

Wound Healing Efficiency of Grafted ePTFE Membranes. The main purpose of designing pseudozwitterionic membranes was to evaluate if they can efficiently promote the skin tissue reconstruction. The tests were carried out on SD rats, and wound healing efficiency of the mixed-charge membranes, after 7- and 14-day contact with the wound, was compared to that of a commercial dressing made of hydrocolloid gel. Notice that no negative control (without dressing) could be used, since uncovering the wound led to the death of the rat. Results of histological analysis are depicted in Figure 10, whereas Figure S4 (see the Supporting Information) presents the evolution of surface area along healing. Several histological indicators can be used to evaluate the efficiency of membranes, including the granulation level, the presence of fibroblasts, the collagen production, the presence and number

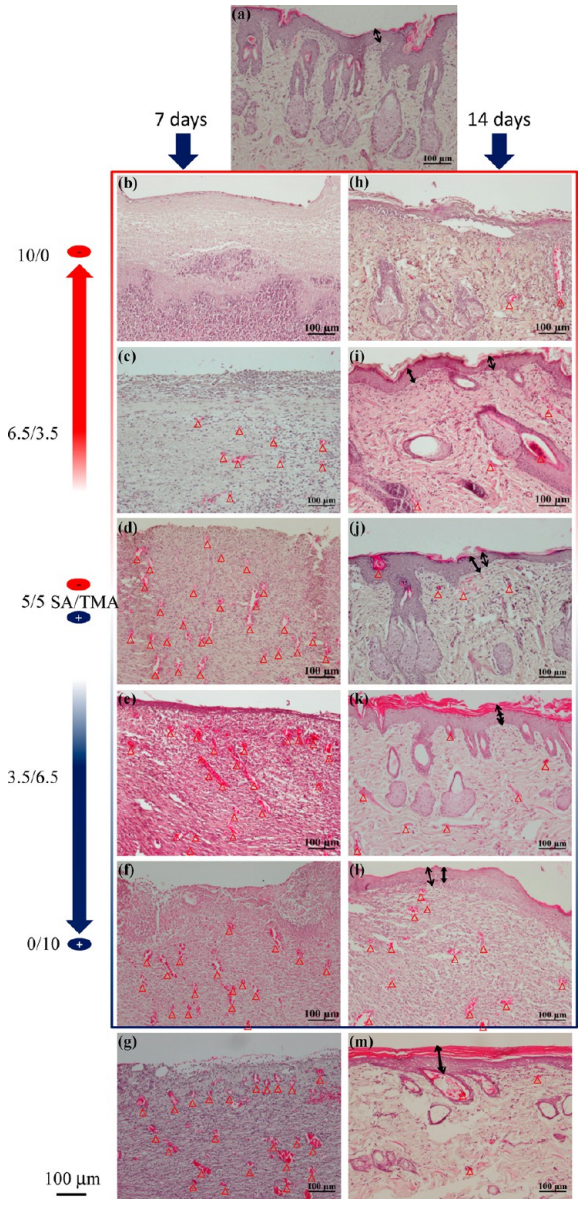


Figure 10. Evaluation of wound healing efficiency from in vivo tests after 7 and 14 days. (a) Normal skin tissue; wound covered with (b, h) ePTFE-*g*-10SA membrane, (c, i) ePTFE-*g*-6.5SA/3.5TMA membrane, (d, j) ePTFE-*g*-5SA/5TMA membrane, (e, k) with ePTFE-*g*-3.5SA/6.5TMA membrane, (f, l) ePTFE-*g*-10TMA membrane, (g, m) commercial product (3 M Tegaderm hydrocolloid dressing. All images were taken at a magnification of X 100. The black arrows indicate the granulation layer. The red triangles point to blood vessels and vascularization zones.

of blood vessels as well as extracellular proteins, the number and proliferation of derma papillae or the hair follicle development.[44−47]

Form Figure 10, wounds covered with pseudozwitterionic or positively charged membranes presented significant vascularization after 7 days. On the other hand, wounds covered with negatively charged membranes were still in the early stage of healing (inflammatory phase). Negative bias seemed to delay the wound recovery, compared to other dressings. After 14 days of contact with ePTFE-*g*-5SA/5TMA, thickness of the granulation layer was similar to that of the unwounded skin, indicating optimal healing. The granulation layer was neither thinner nor thicker, suggesting that the healing was almost complete with no visible differences between new and old tissues (thus no scar). This is confirmed by Figure S4 in the Supporting Information, which highlights the minimum surface area of the wound covered with the pseudozwitterionic membrane, even lower than that covered with the commercial dressing. Positively and negatively charged membranes exhibited either incomplete formation of the granulation layer or presence of numerous blood capillaries, indicating that the healing process was still ongoing. In particular, important vascularization was observed for wounds treated with positively charged membranes. Compared to wounds treated with positively or negatively charged membranes, fewer blood vessels were observed in the deeper layers of epidermis and dermis of the wound covered with the pseudozwitterionic membrane. Indeed, the reconstruction of epithelium and dermis had already occurred in the earlier stages of healing therefore significant vascularization of the wound was no longer necessary. Also, dermis of the wound covered with PTFE-*g*-5SA/5TMA was filled with fibroblasts and collagen, which play a pivotal role in maintaining skin structure. On the other hand, wounds covered with other dressings did not exhibit as many fibroblasts, therefore the wounds were still in the migratory/proliferative phase of healing.

Comparing normal skin tissue to wounded tissues covered with the commercial hydrocolloid and mixed-charge membranes, it could be concluded that pseudozwitterionic matrix led to the best wound recovery. Also, the wound covered with ePTFE-*g*-6.5SA/3.5TMA exhibited a recovery close to that treated with the commercial product, but the pseudozwitterionic membrane with no charge bias showed more densely packed collagen fibers and more fiber accumulation in the extracellular matrix. In contrast, loosely packed collagen (white regions) was observed when using ePTFE-*g*-6.5SA/3.5TMA.

Properties necessary to the use of new matrices in wound-healing applications were exhibited by the pseudozwitterionic membranes in previous sections. As shown by the histological data, pseudozwitterionic membranes increased the rate of re-epithelialization by providing an adequate environment in which epithelial, endothelial and fibroblast cells could survive and migrate. The surface carrying no net electric charge stimulated healing faster than the positively or negatively charged surfaces, suggesting the importance of electrical neutrality on healing. By minimizing the attachment of any cells using pseudozwitterionic wound dressing, skin re-epithelialization was favored since the bandage did not interact with cells (instead, it only provided the environmental conditions favorable to healing). In this respect, new connective tissue was formed within 14 days, evidence of a complete healing. One may have also noticed from Figure 10 that a slight positive charge bias (ePTFE-*g*-3.5SA/6.5TMA) led to an apparent fair re-epithelialization of the skin. Although skin recovery was definitely not as good as that treated with the pseudozwitterionic membrane, positive brushes can be of interest if serious bleeding occurs after injury because they can enhance blood clotting. In such case, one could first use ePTFE-*g*-3.5SA/6.5TMA to stop hemorrhage and then ePTFE-*g*-5SA/5TMA to promote healing. The use of ePTFE-*g*-10TMA, however, is not recommended, because of its high extent of cell disruption. Moreover, the in vivo on laboratory rats also highlighted a significant effect of charge bias on healing time-scale. Healing process was almost complete for wound recovered with pseudozwitterionic membranes after 14 days,

whereas ePTFE-*g*-10SA and ePTFE-*g*-10TMA clearly demonstrated a poor tissue reconstruction. The use of a single monomer, rather than a mixture of oppositely charged monomers in the polymerization solution, led to an incomplete healing. Wounds covered with ePTFE-*g*-10SA or ePTFE-*g*-10TMA membranes were still in the proliferative phase after 14 days, which implied that the underlying molecular and cellular activities such as formation of granulation layer, fibrogenesis, vascularization of new tissues, wound contraction, and re-epithelialization were still ongoing. Fibroblast development is essential to the remodeling of the skin, through the synthesis of extracellular matrix such as collagen. From cell-adhesion results, it was shown that fibroblasts could easily interact with surfaces presenting net charge which is not favorable to an efficient healing, because fibroblasts should grow and develop in the skin tissue without interacting with the bandage. Additionally, the hemolytic activity has to be minimal since erythrocytes play a fundamental role in tissue development through delivering oxygen, therefore ePTFE-*g*-10TMA membranes, which led to poor tissue reconstruction, could not be used as wound dressings.

CONCLUSION

Inspired from the design of zwitterionic membranes, pseudozwitterionic brushes with mixed charges were grown onto the virgin ePTFE membranes, by atmospheric plasma induced polymerization. We aimed at investigating the role of the membrane surface charge on essential wound dressing properties for wound healing. It was proven that the combination of optimal properties was only obtained with pseudozwitterionic membranes, formed by balancing positively and negatively charged monomers in the polymerization solution. They allowed the establishment of a highly hydrophilic interface, resisted fibrinogen adsorption and cell adhesion (including erythrocytes, leucocytes, thrombocytes, and fibroblasts), and resisted the attachment of *Escherichia coli* and *Staphylococcus epidermidis*. In vivo tests suggested that pseudozwitterionic surfaces promoted fast wound healing. In addition, negative and positive surfaces are not recommended for wound healing. Nonetheless, a positive bias can importantly enhance blood clotting, which can be of major interest when serious bleeding occurs. Pseudozwitterionic brushes arising from plasma polymerization, an easily controlled and regulated process, constitute a new and efficient strategy to the preparation of wound dressings for a complete and fast skin re-epithelialization of a wound after injury.

ASSOCIATED CONTENT

Supporting Information

Quantitative analysis of bacterial attachment, blood cell adhesion and human cell adhesion, wound area analysis. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Author Contributions

This manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors express their sincere gratitude to the Center-of-Excellence (COE) Program on Membrane Technology from the Ministry of Education (MOE), R.O.C., the Outstanding Professor Research Program in the Chung Yuan Christian University, Taiwan (CYCU-00RD-RA002-11757), and the National Science Council (NSC97-2622-E-033-003-CC3, NSC98-2622-E-033-017-CC3, and NSC100-2622-E-033-004-CC3) for their financial support. We acknowledge the International High Cited Research Group (IHCRG # 14-104), Deanship of Scientific Research, College of Science Research Center, King Saud University, Kingdom of Saudi Arabia. Y.C. and A.H. thank King Saud University, Riyadh, Kingdom of Saudi Arabia, for the Visiting Professorship.

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