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Surface Zwitterionization of Expanded Poly(tetrafluoroethylene) Membranes via Atmospheric Plasma-Induced Polymerization for Enhanced Skin Wound Healing

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ABSTRACT: Development of bioinert membranes to prevent blood clotting, tissue adhesion, and bacterial attachment is important for the wound healing process. In this work, two wound-contacting membranes of expanded poly(tetrafluoroethylene) (ePTFE) grafted with zwitterionic poly(sulfobetaine methacrylate) (PSBMA) and hydrophilic poly(ethylene glycol) methacrylate (PEGMA) via atmospheric plasma-induced surface copolymerization were studied. The surface grafting chemical structure, hydrophilicity, and hydration capability of the membranes were determined to illustrate the correlations between bioadhesive properties and wound recovery of PEGylated and zwitterionic ePTFE membranes. Bioadhesive properties of the membranes were evaluated by the plasma protein adsorption, platelet activation, blood cell hemolysis, tissue cell adhesion, and bacterial attachment. It was found that the zwitterionic PSBMA-grafted ePTFE membrane presented high hydration capability and exhibited the best nonbioadhesive character in contact with protein solution, human blood, tissue cells, and bacterial medium. This work shows that zwitterionic membrane dressing provides a moist environment, essential for "deep" skin wound healing observed from the animal rat model in vivo and permits a complete recovery after 14 days, with histology of repaired skin similar to that of normal skin tissue. This work suggests that the bioinert nature of grafted PSBMA polymers obtained by controlling grafting structures gives them great potential in the molecular design of antibioadhesive membranes for use in skin tissue regeneration.

KEYWORDS: *zwitterionic, bioinert, atmospheric plasma, poly(tetrafluoroethylene), sulfobetaine, wound healing*

INTRODUCTION

Skin wound healing is an intricate process in which the skin tissue repairs itself after injury. The classic model of wound healing is divided into four sequential phases, including hemostasis, inflammatory, proliferative, and remodeling. Once the tissue is wounded, bleeding occurs as well as the generation of exudates so that it is necessary to cover the wounded area with a suitable dressing, to improve the skin-tissue reconstruction and reduce scab formation. Healing can be favored by using various approaches. Recently, Murphy and Evans presented the latest advances in this field, from the use of silver, to treatments involving hyperbaric oxygen.[1] Moreover, Gauglitz and Jeschke reviewed the works performed on the use of cell-based treatments for cutaneous wound healing.[2] Advanced dressings in particular concern many studies since dressings should possess numerous properties to favor the wound healing process.[3−8] Importantly, they should promote the removal of excess exudates and toxic components, maintain high humidity at the wound/dressing interface, permit gaseous exchanges, provide thermal insulation, allow its removal without trauma at the dressing change, help the body achieve an ideal moist and warm state, and protect the wound from potential secondary infection as well as from possible mechanical stress. In sum, wound healing materials must protect the wound to favor quick tissue regeneration by providing an ideal environment. In some cases, dressings can even act as a bioactive material intended not only to protect the wound but also to accelerate the healing process.[9,10] However, although numerous efforts have been done by researchers worldwide, it remains challenging to prepare the perfect dressing, owing to the numerous required properties it should possess.

A set of biocompatible polymers are available to prepare wound dressings. Collagen is a major component of the extracellular and is therefore a good material for wound dressing applications.[11,12] It can serve as a natural substrate for

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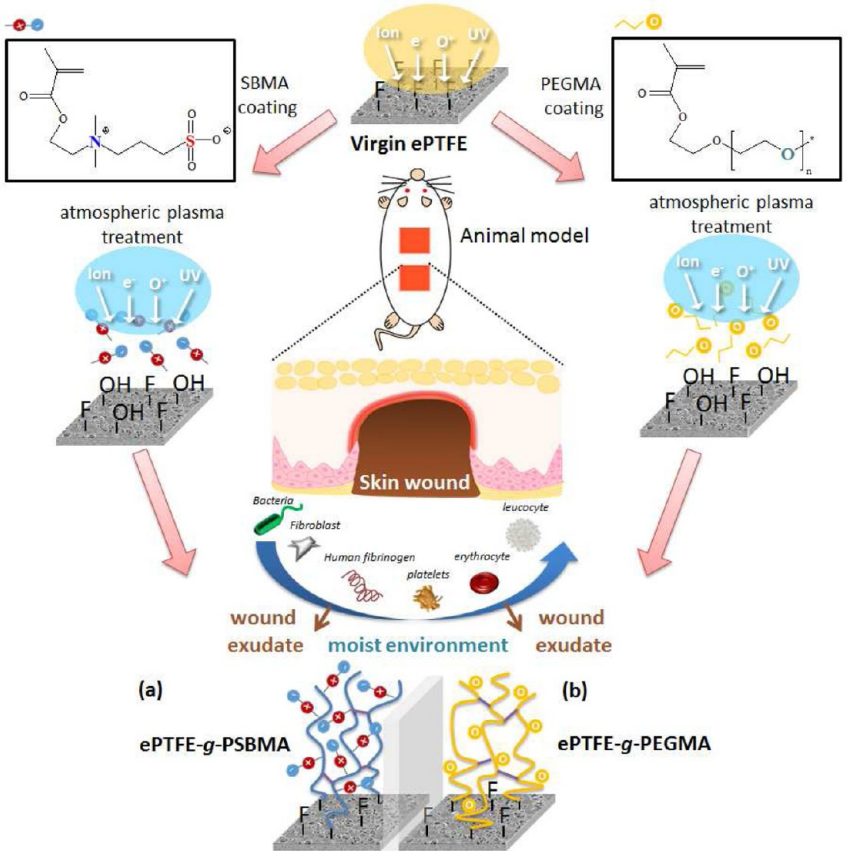

Figure 1. Schematic illustration of the preparation process of the (a) zwitterionic ePTFE-*g*-PSBMA and (b) ePTFE-*g*-PEGMA membranes via atmospheric plasma-induced surface copolymerization.

cell attachment, proliferation, and differentiation. Alginates have also been known for a long time as materials for wound dressings and still concern many investigations.[9,13,14] These are biocompatible polymers that can, for instance, form a gel on absorption of wound exudate. Knuill et al. reminded that this absorption process promotes the removal of fiber entrapment in the wound, which is a major cause of patient trauma/discomfort during dressing removal.[13] Gels prevent the surface of the wound from drying out by maintaining a moist environment and therefore promote wound repair as demonstrated in the early 60s by Winter.[15] Chitosan is a natural polymer that is biocompatible, bioactive, and bioresorbable and is also a potential candidate for wound dressings.[16−18] Among the nonexhaustive list of polymer materials that can be used as wound dressings, supplementary important examples concern aromatic diisocyanate, historically employed where degradation was not desirable, styrene-based rubbers physically cross-linked serving as barrier scaffolds,[10] poly(glycerol sebacate-co-acrylate) (PGSA)-based tissue adhesive used as hemostatic patches,[19] or poly(methacrylates) and polyvinylpyrrolidone synthetic polymers, used because of their swellable nature so that they can entrap water upon contact with suppurating wounds.[20]

The inhibition of bacterial attachment is one of the important characteristics for wound dressings since microorganisms can be responsible for wound infection. Several strategies are available to prepare a matrix resisting attachment of bacteria. These methods also lead to the preparation of matrices efficiently resisting the adhesion of blood cells and fibroblasts, important properties of efficient dressings. The preparation of absorbent dressings such as hydrogels is a way to go. On the other hand, dry polymer films usually provide very good mechanical properties, so that it can be interesting to functionalize these films, often made of hydrophobic polymer materials. This results in a hydrophilic surface applied as a dressing onto the wound and which is not likely to be colonized by bacteria. To achieve it, there are several possible approaches, concerning the choice of the process as well as that of materials. Starting with the materials and regardless of the intended application, two kinds of polymer additives are popular to provide a hydrophobic matrix with a hydrophilic interface: the PEGylated systems and the zwitterionic systems.[21−28] The first ones are built around ethylene glycols units, easily binding water via the establishment of hydrogen bonding. The zwitterionic systems possess the characteristic of antifouling materials identified by Whitesides and co-workers: they possess hydrophilic groups, are hydrogen-bond acceptors rather than donors, and present no net charge.[29,30]

Concerning the processes enabling the increased hydrophilicity of a polymer matrix, either the polymer matrix is modified after its preparation, which makes it a two-step process usually achieved by coating, surface chemical reaction, or grafting, or the hydrophobic polymer material is blended with an appropriate additive in a common solvent, and a process applied to the blend leading to the final modified-polymer matrix.[31−33] For preliminary studies, one may consider that two-step processes are more convenient. Indeed, the original structure is well controlled and defined in the first stage, and there is no action of additive on polymer matrix formation. On the other hand, when polymer and additive are

blended, it is sometimes hard to anticipate whether the matrix will be more porous or denser, than without additive, so that it is not possible to only focus on hydrophilic and low-fouling properties. Especially, in the field of wound dressings, porosity control is important, to favor drainage of exudates and gas exchanges with the external environment, so that it is definitely more convenient to begin with a given matrix having a well-controlled porosity and then modify it to improve its hydrophilicity. Among the surface modification processes, plasma treatment is a recognized technique permitting us to efficiently modify a membrane's surface and control its surface coverage.[34−36]

The preparation in controlled conditions of a suitable dressing for wound healing, evidencing the retainment of a highly moist environment and combining resistance to bacteria colonization, cell adhesion, and blood compatibility, to favor a quick tissue recovery, remains a great challenge. This is the scope of this paper, which aims at presenting the preparation by atmospheric plasma treatment of zwitterionic expanded polytetrafluoroethylene (ePTFE) membranes for wound dressing applications. The zwitterionic skeleton is based on repeat units of sulfobetaine methacrylate (SBMA), yielding to ePTFE-*g*-PSBMA films. The focus will be put on the important hydrophilic properties of such matrices and on their resistance to cell attachment including blood cells and human cells, essential for biocompatibility of the dressing material, as well as on their ability to resist bacteria adhesion, an important feature to prevent potential infection by microorganisms. Wound healing property will be assessed by application of membranes onto mice wounds, and healing efficiency will be compared to that of the virgin ePTFE membrane, gauze, PEGylated ePTFE film, and commercial hydrocolloid dressing.

EXPERIMENTAL SECTION

Materials. ePTFE microporous membranes (average pore size, 0.24 μm; thickness, 500 μm; diameter, 120 mm) were obtained from YMT Co., Taiwan. The [2-(methacryloyloxy)ethyl] dimethyl(3-sulfopropyl)-ammonium hydroxide (sulfobetaine methacrylate, SBMA) macromonomer was purchased from Monomer-Polymer & Dajac Laboratories Inc. The poly(ethylene glycol) methacrylate (PEGMA) macromonomer was bought from Aldrich. Its molecular weight was about 500 Da and its average number of ethylene glycol units was about 10. Isopropanol (IPA) was obtained from Aldrich and used as a solvent for the plasma-induced graft polymerization. Fibrinogen was purchased from Sigma Chemical Co. Human blood and plasma solution were both obtained from Taiwan Blood Services Foundation. Phosphate buffer saline (PBS) was purchased from Sigma-Aldrich. Deionized water used in experiments was purified using a Millipore water purification system with a minimum resistivity of 18.0 MΩ m.

Surface Copolymerization. The plasma-induced surface copolymerization of zwitterionic PSBMA under the control of low-pressure plasma is presented in Figure 1. The virgin ePTFE microporous membrane was first treated by low-pressure plasma with a hydrogen flow rate of 30 sccm and input power of 100 W 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 incubated in IPA/DI water (8/2, v/v) mixed solution containing 30 wt % SBMA monomer. Afterward, the monomer-coated membrane was dried at 25 °C for 24 h, and the coating amount of SBMA monomer onto ePTFE membrane was about 2.0 mg/cm^2. Subsequently, the membrane was treated by atmospheric plasma with an argon flow rate of 30 sccm and input power of 150 W controlled by a 13.56 Hz RF generator. Afterward, the modified ePTFE membrane was immersed in pure methanol and then extracted with deionized water and methanol for 60 min using an ultrasonic device to strip off PSBMA, as well as unreacted monomers. The residual solvent was removed in a vacuum oven under reduced pressure for 1 day and the membrane dried in a freeze-dryer at −45 °C for 24 h. As for the ePTFE-*g*-PEGMA membrane, a similar pretreatment procedure was followed. The virgin ePTFE membrane was incubated in an IPA/DI water mix solution containing 30 wt % of PEGMA monomer. The coating amount was also about 2.0 mg/cm^2. Subsequent plasma treatment, extraction, and drying procedures were the same as those used for the preparation of the zwitterionic membrane.

Surface Characterization. In this work, we used surface characterization methods that were detailed in our previous studies.[37−39] As testing conditions were similar to those used in previously published works, one should refer to these works to obtain further details. The chemical composition of surface-modified ePTFE membranes with a grafted copolymer layer was characterized by X-ray photoelectron spectroscopy (XPS) using a PHI Quantera SXM/Auger spectrometer with a monochromated Al KR X-ray source (1486.6 eV photons). The surface grafting yield of PSBMA and PEGMA on the ePTFE membrane was determined by the extent of weight increase per unit surface area, compared to the virgin ePTFE membrane. Membranes were dried for 3 days and at 50 °C in a vacuum oven before the measurements. Static water contact angles were determined at 25 °C using an angle meter (model CA-VP, Kyowa Interface Science Co., Ltd. Japan). The average of 10 measured values was taken as the final water contact angle of the membrane. Hydration capacities (mg/cm^2) were determined by weighing 0.85 cm diameter dried membranes using a 10^{-5} g precision balance (Mettler, Toledo Pac Rim AG, Taiwan Branch). Then, membranes were immersed in deionized water for 24 h. Afterward, surface water was wiped out, and wet weights were recorded. Hydration capacity was evaluated based on the unit surface area of membranes. The average of five independent measurements was taken as the final value for membrane hydration capacity.

Protein Adsorption on the Zwitterionic and PEGylated ePTFE Membranes. Fibrinogen adsorption of human plasma solution on the sample surfaces was evaluated using the enzyme-linked immunosorbent assay (ELISA) according to the standard protocol previously described.[37−39]

Bacterial Attachment on the Zwitterionic and PEGylated ePTFE Membranes. *Staphylococcus epidermidis* (SE) and *Escherichia coli* (EC) were used to investigate bacterial adhesion behavior on the surface of grafted ePTFE membranes. Both species were cultured at 37 °C in a medium containing 3.0 mg/mL of beef extract and 5.0 mg/mL of peptone. Cultures were shaken at 100 rpm until the stationary phase was reached (SE concentration, 10^9 cells/mL obtained after 15 h; EC concentration, 10^6 cells/mL obtained after 12 h). Membranes (0.4 cm^2) were placed in a 24-well plate. Then, they were immersed in 75 wt % ethanol for 1 h at ambient temperature before being washed 3 times using PBS. An amount of 1 mL of bacterial solution was added to each well. Incubation of samples in bacteria suspension lasted 24 h, and temperature was controlled to 37 °C. Bacterial media were changed every 6 h. After 24 h incubation with bacterial solution, membranes were washed with PBS 3 times at 37 °C. A volume of 200 μL of Live/Dead BacLight was used to stain bacteria adhering to the sample surfaces. Incubation with staining agent lasted 10 min. Samples were then washed three times with PBS and observed with a CCD camera mounted on Olympus BX51 with a 10× objective lens. Epifluorescent illumination was used, through a blue excitation fluorescence filter at the excitation range 450−490 nm.

Blood Platelet Activation and Blood Cell Adhesion on the Zwitterionic and PEGylated ePTFE Membranes. ePTFE membranes of 0.4 cm^2 surface area were equilibrated at 25 °C in a 24-well tissue culture plate with 1000 μL of phosphate-buffered solution (PBS) for 2 h. Platelet-rich plasma (PRP) containing about 1×10^5 cells/mL was prepared by centrifugation of blood at 1200 rpm and for 10 min. In addition, 200 μL of PRP, first recalcified by the addition of calcium (1 M $CaCl_2$, 5 μL), was placed on ePTFE surfaces to test the adhesion of activated platelets. Incubation lasted 120 min and was performed at 37 °C. Samples were then rinsed twice with 1000 μL of PBS and then immersed into 2.5% glutaraldehyde of PBS for 48 h at 4

°C to fix the adhered platelets and adsorbed proteins. Subsequently, membranes were rinsed twice with 1000 μL of PBS, before being gradient dried with ethanol in 0% v/v PBS, 10% v/v PBS, 25% v/v PBS, 50% v/v PBS, 75% v/v PBS, 90% v/v PBS, and 100% v/v PBS. Each step lasted 20 min, and membranes were finally dried in air. Samples were sputter-coated with gold prior to SEM observation (JEOL JSM-5410 SEM).

Red blood cell concentrates (RBCs) were prepared by centrifugation of 250 mL of fresh blood at 1200 rpm for 10 min. To test red blood cell adhesion, membranes were incubated with RBC at 37 °C and for 120 min. 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 RBCs. Then, samples were rinsed five times with 1000 μL of PBS. The morphology of blood cells adhering on the surfaces immersed in PBS was observed by confocal microscopy (LSCM, A1R, Nikon, Japan). Images were taken at λ_{ex} = 488 nm/λ_{em} = 520 nm, at five different places on the same chip and at a 200× magnification.

To test white blood cell (WBC) adhesion, a WBC, prepared by centrifugation of whole blood, was placed on the membranes' surface (0.4 cm^2) and incubated for 120 min at 37 °C. Then, substrates were rinsed twice with 1000 μL of PBS, immersed into 2.5% glutaraldehyde of PBS for 10 h at 4 °C, and rinsed five times with 1000 μL of PBS. Samples were observed by confocal microscopy using the same instrument and settings as that used for RBC adhesion assay.

Red Blood Cell Hemolysis of the Zwitterionic and PEGylated ePTFE Membranes. The membrane disruption of RBC was also determined, and the detailed procedure was reported elsewhere.[38] Therefore, one may refer to a previous study to obtain details on this specific procedure.

Human Skin Cell Adhesion on the Zwitterionic and PEGylated ePTFE Membranes. Human skin cell adhesion tests were performed as follows. First, Fibroblasts HT-1080 were grown in Dulbecco's modified Eagle medium (DMEM, Gaithersburg, MD). Afterward, cells were supplemented with a solution containing 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% nonessential amino acids, and 1% penicillin streptomycin solution. The temperature of the cell culture was maintained at 37 °C. Before carrying out the cell adhesion test, membrane disks were immersed in 75 wt % ethanol for 1 h at 25 °C and then washed three times by PBS in a 24-well plate. Subsequently, 1 mL of cell suspension (cell concentration: 104 cells/mL) was added to each well. Incubation of cell suspension with the samples was performed at 37 °C in a humidified atmosphere of 5% CO_2 and for 24 h. Cells' morphology and proliferation were then observed using a phase contrast microscope (Nikon TS100) using a 10× objective lens, at 1, 2, and 3 days of incubation.

Wound Healing Properties of the Zwitterionic and PEGylated ePTFE Membranes in Vivo. The wound healing property of membranes was evaluated using an animal (rat) model. Adult Sprague–Dawley (SD) rats weighing approximately 400 g ± 25 g were obtained from BioLasco Taiwan Co., Ltd. First, subcutaneous injection of 0.15 mL of tranquilizer (Rompun 2%, Bayer Healthcare A.G.) was performed. Then rats were anesthetized with 0.2 mL of Zoletil 50 (Virbac Taiwan Co., Ltd.). Afterward, the surgical trauma area was shaved with an electric razor, and rats were wounded using a surgical scalpel. The wound area of approximately 2 × 2 cm^2 was covered with either gauze, virgin ePTFE membrane, ePTFE-*g*-PEGMA membrane, ePTFE-*g*-PSBMA membrane, or commercial dressing (3M Tegaderm hydrocolloid dressing, Minnesota Mining and Manufacturing Company). The dressing was then fixed with a bandage. After 7 and 14 days, histological observations were performed to observe the wound recovery and compare the efficiency of the various dressings. To do so, wound tissues were first immersed in formalin and stained with hematoxylin-eosin (HE) reagent, before being observed using a microscope (Nikon Eclipse 80i).

RESULTS AND DISCUSSION

Surface Copolymerization and Characterization. The efficiency of surface grafting of a copolymer onto a polymer matrix can be assessed using several various physicochemical methods. In this work, because the prepared membranes were intended to be used in wound dressing applications requiring a very hydrophilic external surface at the membrane–tissue contacting side, water contact angle and hydration capacity measurements were assessed. In parallel, chemical analysis of the surface was performed via XPS characterization. Coverage of the grafted layer on the ePTFE membrane can be first controlled during the coating step of virgin membrane by SBMA monomer and then by regulating the plasma treatment time. As for the PEGMA-grafted layer on the ePTFE (ePTFE-*g*-PEGMA) membrane, a similar treatment procedure was followed. The ePTFE membrane coated with SBMA and PEGMA monomer of ∼2.0 mg/cm^2 was directly treated by atmospheric plasma. Figure 2 presents the surface grafting yield

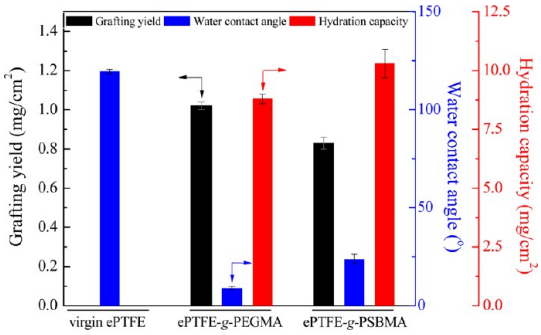

Figure 2. Surface grafting yield, water contact angle, and hydration capacity of virgin ePTFE membrane, ePTFE-*g*-PEGMA membrane, and ePTFE-*g*-PSBMA membrane.

and water contact angle of the virgin ePTFE membrane and grafted membranes. It was found that the atmospheric plasma-induced grafting process was efficient since the water contact angle dropped from about 120° for the ePTFE membrane to 10° for the ePTFE-*g*-PEGMA membrane and to 22° for the zwitterionic ePTFE-*g*-PSBMA membrane. The water contact angle was associated to a similar grafting density of about 1.02 and 0.85 mg/cm^2 for the ePTFE-*g*-PEGMA and ePTFE-*g*-PSBMA membranes. While the water contact angle measures the surface hydrophilicity, the hydration capacity gives information about the whole top-layer thickness wettability. The latter was assessed after immersing the membrane samples into water for 24 h. It was verified that water penetration within the matrix as well as its binding by either the ethylene glycol groups of PEGMA or the zwitterionic structures in SBMA head groups was promoted, and efficient. Indeed, the hydration capacity was increased from 0 to 8.8 and 10.3 mg/cm^2 for the ePTFE-*g*-PEGMA and ePTFE-*g*-PSBMA membrane. It appeared that the zwitterionic membrane was slightly more effective to entrap water, highlighting that grafting of zwitterionic PSBMA layers is at least as efficient as, or even more efficient than, grafting of the PEGMA copolymer, to increase the hydrophilicity of a surface and the amount of water finally trapped within the polymer matrix. These results also evidenced the ability of grafted membranes to retain water and therefore provide a moist environment essential for an effective healing.

To go further in the surface modification analysis, XPS characterization was performed, and results are shown by Figure 3. The C 1s, N 1s, and S 2p core level spectra are provided, and identification of species was performed according

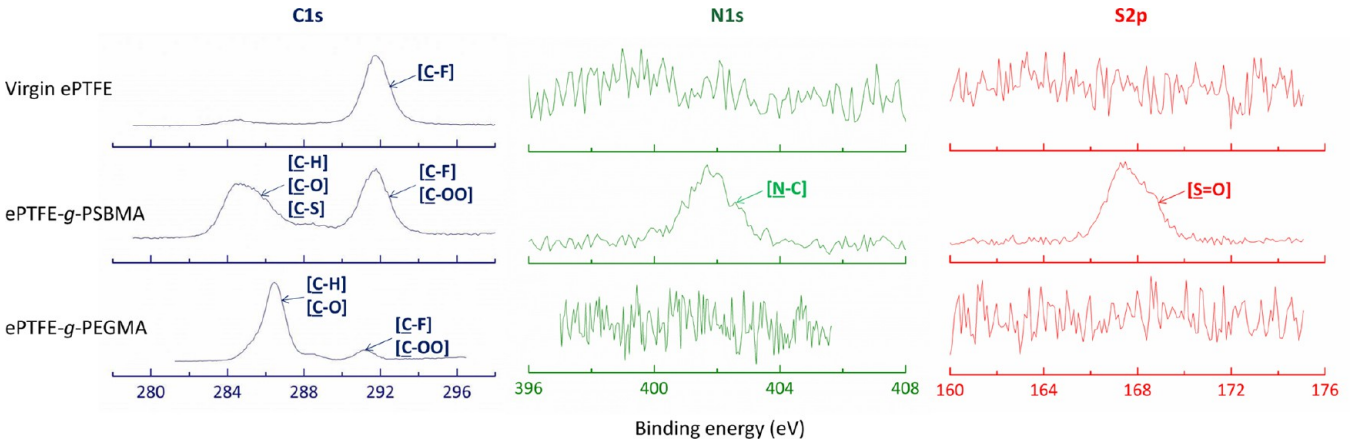

Figure 3. X-ray photoelectron spectroscopy analysis of virgin ePTFE membrane, ePTFE-*g*-PEGMA membrane, and ePTFE-*g*-PSBMA membrane.

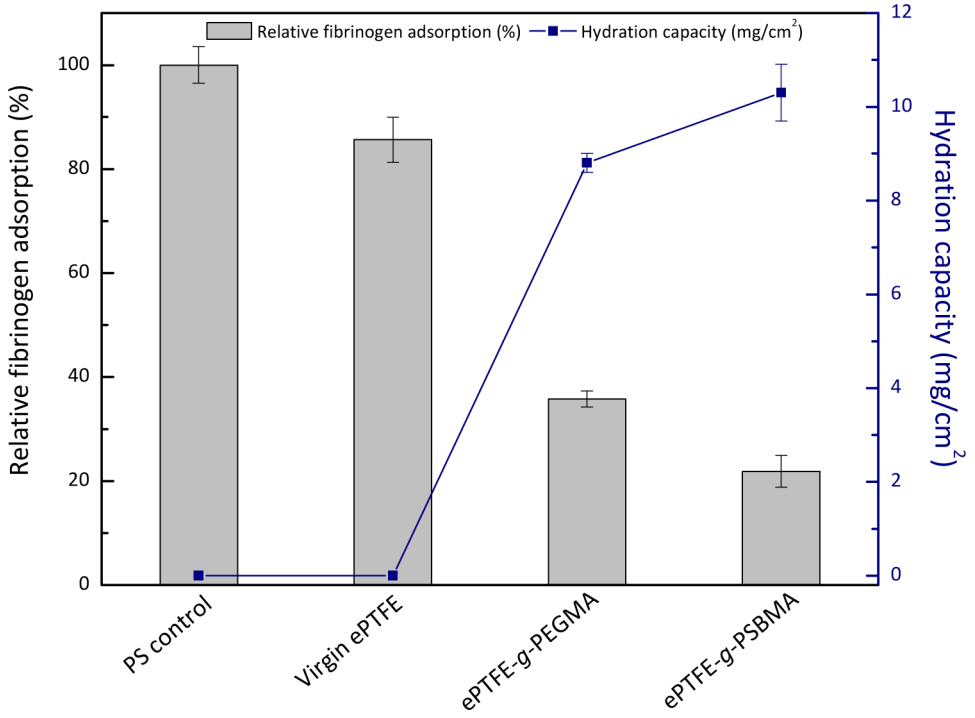

Figure 4. Relative human fibrinogen adsorption from 100% platelet-poor plasma solution on the virgin ePTFE membrane, ePTFE-*g*-PEGMA membrane, and ePTFE-*g*-PSBMA membrane. All data were determined from ELISA with each virgin sample as a reference, where the amount of fibrinogen adsorbed on the virgin ePTFE sample was normalized to unity. Data are expressed as the means ± SD of three independent measurements.

to previous studies.[40−43] The virgin ePTFE membrane presented one single peak at binding energy (BE) of 291.7 eV on the C 1s spectrum, attributed to C−F species, and of course, no peak was found on the N 1s and S 2p core level spectra. Concerning the ePTFE-*g*-PSBMA membranes, the C 1s spectrum evidenced two main peaks at 291.7 and 284.7 eV. The first one was due to [C−F] and [C−OO] species, while the second peak arose from the contribution of [C−H], [C−O], and [C−S] species, respectively. The presence of SBMA groups was further assessed through the analysis of N 1s and S 2p spectra, each one showing the presence of one single peak at a BE of 401.7 and 167.4 eV, respectively, due to quaternary ammonium cations and sulfonates in the SBMA backbone. Concerning the PEGylated membrane, the C 1s spectrum also provided useful information to identify successful grafting. Indeed, it displayed the presence of one main peak at a BE of 285.6 eV, attributed to the contribution of [C−H] and [C−O] species brought by the PEGMA segments. Moreover, the peak related to [C−F] species could still be found at 291.5 eV. However, this peak was somewhat very small in terms of intensity and area. In comparison, it was worth noticing that the intensity of the same peak was still very high for the ePTFE-*g*-PSBMA membrane. The conformation of the PEGylated layer as well as the important cross-linking between the copolymer chains may explain this result. If both density and cross-linking are important, the native ePTFE matrix may hardly be detected by XPS, which also further supports a very efficient surface coverage of the ePTFE membrane by a PEG derivative, and so optimal plasma operating conditions.

Resistance of Grafted Membranes to Protein Adsorption and Bacterial Attachment. It is generally acknowledged that an increase of hydrophilic properties of a matrix is a first

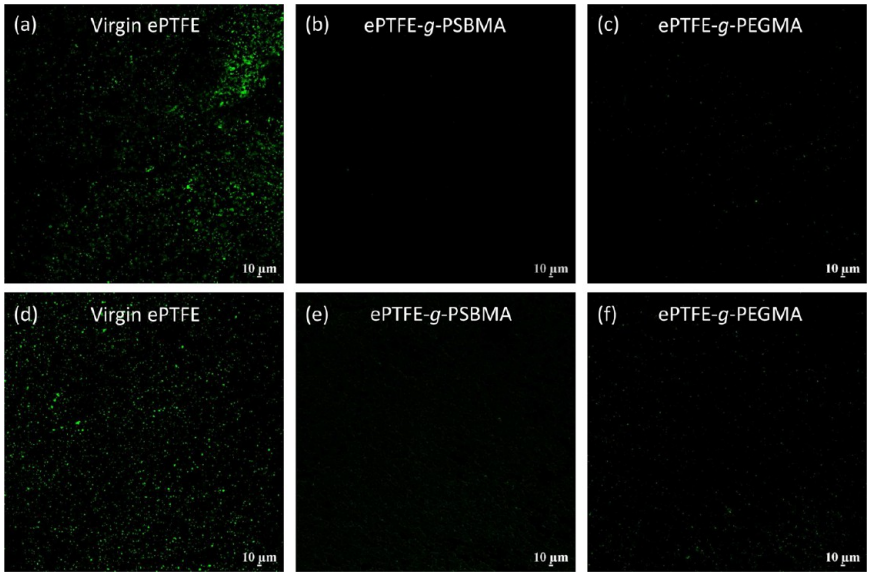

Figure 5. Confocal images of bacteria adhering onto the surface of the virgin and modified ePTFE membranes. Membranes were contacted with *Staphylococcus epidermidis* (a) to (c) and *Escherichia coli* (d) to (f). All images were taken at a magnification of 200×.

indication for a decrease of biofouling.[44,45] Biofouling is often due to favorable interactions between hydrophobic matrix interfaces and hydrophobic segments of proteins or to interactions with the outer cell walls of bacteria made of peptidoglycan. Actually, the adsorption of any substance different from the feed solvent permits to thermodynamically stabilize the interface and explains for instance the occurrence of nonspecific adsorption of proteins. By making the interface hydrophilic, protein/membrane or bacteria/membrane interactions are no longer thermodynamically stable, so that hydrophilic membranes are less likely to be fouled by bioorganisms. In our case related to wound dressing application, it was important to obtain low-biofouling properties, since the prepared dressings must efficiently resist bacteria attachment and biofilm formation, blood cell attachment, and human cell adhesion. Bacteria may be responsible for potential infection, while any attachment of blood cells or skin cells may lead to tissue damage upon removal of the dressing. A surface-resisting protein adsorption does not necessarily resist bacterial attachment[46] but a surface on which nonspecific adsorption of protein generally induces bacterial attachment. In addition, because plasma protein such as fibrinogen can be responsible for sets of reactions leading to plasma clotting, therefore evidencing a lack of blood compatibility, the efficiency of grafted membranes to resist fibrinogen, a sticky protein, was first assessed through ELISA tests.

Figure 4 presents the fibrinogen adsorption onto grafted membranes from 100% PPP solutions. In this study, the positive control was the polystyrene surface constituting the well plate. The virgin ePTFE membrane, owing to their hydrophobic nature, adsorbed about 90% of the limitation of polystyrene. A strong correlation existing between hydration capacity and protein adsorption could be seen from the results related to grafted membranes. Hydrophilic ePTFE-*g*-PEGMA and ePTFE-*g*-PSBMA membranes efficiently resisted fibrinogen adsorption. Especially, the best result was obtained with the zwitterionic membrane, the most hydrophilic one, with an adsorption reduced to 20% the limitation of the positive control (or 23% the limitation of the virgin ePTFE matrix). This result confirmed that hydrophilicity is a prerequisite for membranes to resist protein adsorption and shows that zwitterionic structures are somewhat more efficient than PEGylated systems. This first test therefore evidenced that nonspecific adsorption of fibrinogen plasma protein was drastically reduced, allowing us to further investigate the low-biofouling properties and biocompatibility of ePTFE membranes.

An important required property of wound dressings is the inhibition of wound infections due to bacterial colonization. Therefore, the surface directly in contact with the wound should ideally resist the attachment of bacteria. In this study, two bacteria were tested, *Staphylococcus epidermidis* (SE) and *Escherichia coli* (EC), the first one being gram-positive and the latter one gram-negative. Results related to SE are shown in Figure 5a to 5c, while those concerning EC are presented in Figure 5d to 5f. If ePTFE membranes presented a favorable surface to bacteria attachment—that is, both hydrophobic and rough, favoring low-energy chemical interactions and physical interactions with the infectious agent—almost no bacteria could be found on the zwitterionic ePTFE-*g*-PSBMA membrane whether incubated with SE or EC, while only a few stains remained on PEGylated ePTFE-*g*-PEGMA membranes. Even though the matrices were still porous and, therefore, more suitable to bacteria attachment via physical interactions than smoother surfaces, almost no microorganism could be found on the modified membranes. It highlighted the successful chemical role of the zwitterionic layers on membrane low-biofouling properties. Furthermore, zwitterionic membranes were again more efficient than the PEGylated ones, owing to a greater capacity to entrap water. One should note that Susanto and Ulbricht investigated the photografting of thin polymer hydrogel layers on polyethersulfone ultrafiltration membranes. They used both PEGMA monomers and zwitterionic monomers, as grafted materials, and compared performances of membranes with respect to fouling.[47] Interestingly, PEGylated membranes performed better than zwitterionic ones, which the authors explained by a better degree of swelling reached of membranes grafted with PEGMA monomers. Even though comparisons with the present study are difficult owing to the different matrix polymer and zwitterionic monomer, the hydration capacity of the ePTFE-

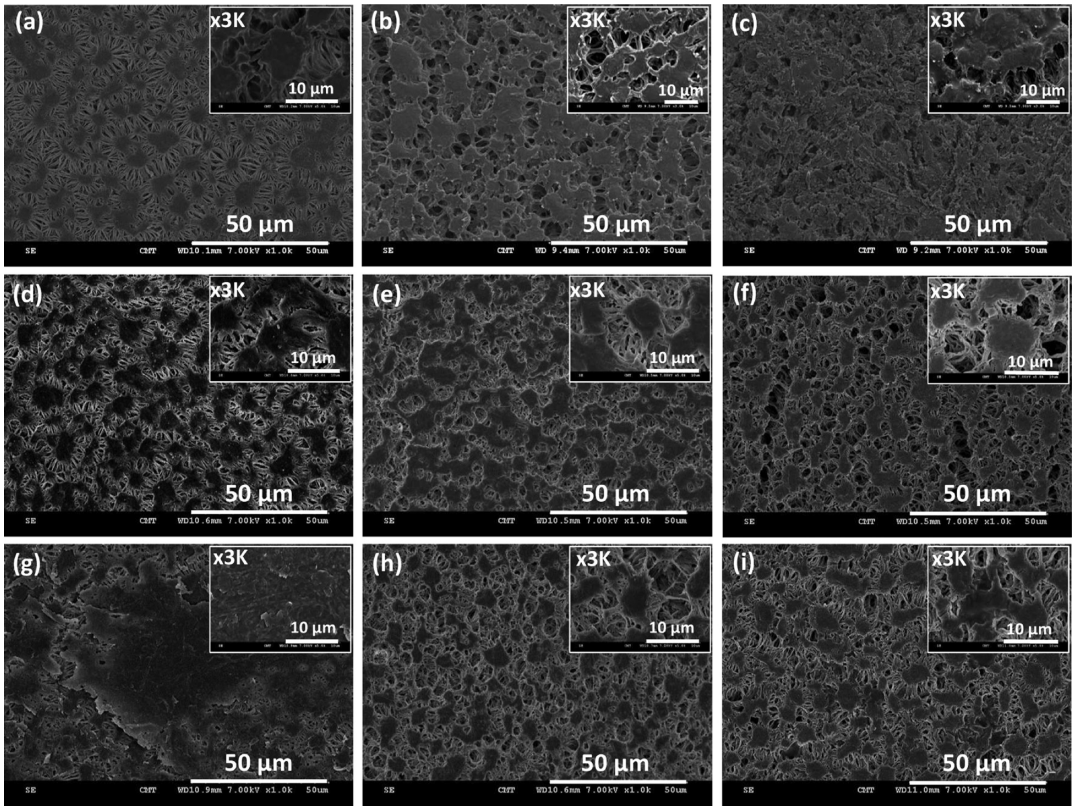

Figure 6. SEM images of platelets adhering onto the surface of the virgin and modified ePTFE membranes. (a), (d) and (g) Virgin ePTFE membrane; (b), (e), and (h) ePTFE-*g*-PSBMA membrane; (c), (f), and (i) ePTFE-*g*-PEGMA membrane. (a) to (c): Membranes before incubation with platelets; (d) to (f) membranes were contacted with nonactive platelet PRP solution; (g) to (i) membranes were contacted with recalcified PRP solution. All images were taken at a magnification of 1000× and 5000×.

g-PSBMA membrane was herein found to be higher than that of ePTFE-*g*-PEGMA (Figure 4), which surely was the reason why zwitterionic membranes performed somewhat better than PEGylated ones: hydration of a surface is a prerequisite to provide the material with antibiofouling properties.[48]

Blood Compatibility of Grafted Membranes. Blood compatibility is an important characteristic that a wound dressing must possess. Ideally, a dressing should not activate the blood defense systems, such as coagulation or fibrinolysis, and has to resist efficiently the adhesion and activation of blood cells including platelets, red blood cells (erythrocytes), and white blood cells (leucocytes). Blood platelet adhesion results in the formation of thrombosis and embolism at the blood contact side of the membrane, which must be avoided. In this study, the adhesion of platelets, an important technique to estimate the hemocompatibility of a surface, was evaluated. It was achieved using both nonactive platelets and recalcified platelets with calcium ions, and results are presented in Figure 6. First, it was important noting that SEM images of membranes revealed a classic structure of polymer matrices prepared by stretching, with crystalline regions oriented parallel to the extrusion direction, and giving rise to a porous structure suitable for the entrapment of small cells via physical interactions. It was also worth noting that grafting of PSBMA (Figure 6b) and PEGMA (Figure 6c) only led to a very small decrease of surface porosity, owing to a partial blockage of surface pores by the hydrophilic moieties. So, entrapment of platelets within the porous structure was believed to still be possible. Figure 6d evidenced the presence of numerous platelets (white spots of about 3 μm in diameter) onto the surface of the virgin ePTFE membrane, highlighting its obvious lack of blood compatibility. On the other hand, neither the zwitterionic membrane (Figure 6e) nor the PEGylated surface (Figure 6f) could reveal the presence of thrombocytes. Therefore, there was no interaction established between the platelet and the modified surface material established, which is one characteristic of blood compatible membranes. The improved antithrombogenicity could be attributed to the hydrophilic moieties, either zwitterionic structure or PEGylated chains, that is, to a very efficient chemical modification, since the physical effect (porous structure) was not favorable at all as aforementioned in previous section. This result had to be correlated to fibrinogen adsorption results. In general, fibrinogen is considered as a major protein conveying the surface chemical feature to platelets in material−platelet interactions.[49−51] By reducing its adsorption, platelets were less likely to interact with ePTFE membranes. Surely, there still was some fibrinogen that could interact with the surfaces (Figure 4). Also, Liu and co-workers reminded that the conformation of fibrinogen in media, rather than the amount, was responsible for platelet adhesion and activation.[51] Therefore, it was quite reasonable that despite fibrinogen could still be adsorbed no platelet was found on the modified ePTFE membranes. Tests were also carried out using recalcified platelets with calcium ions (Figure 6g to 6i). The SEM results showed the formation of thrombosis on the virgin ePTFE membranes with full-scale platelet adhesion and activation at the blood contact site. On the other hand, even though calcium ions were used to promote clotting and therefore attachment of platelets onto the membranes, activated platelets did not appear

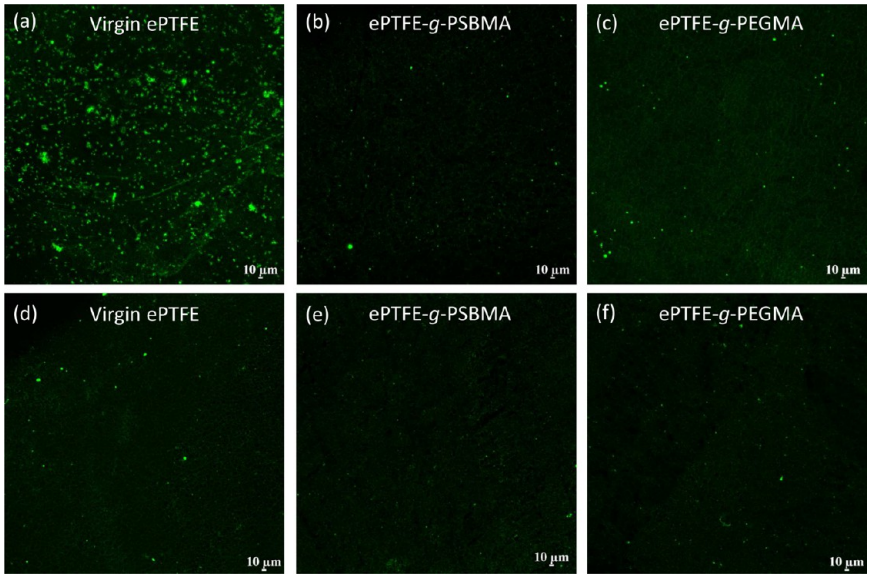

Figure 7. Confocal images of blood cell adhering onto the surface of virgin and modified ePTFE membranes. Membranes were contacted with red blood cell concentrates (a) to (c) and white blood cell concentrates (d) to (f). All images were taken at a magnification of 200×.

on the ePTFE-*g*-PEGMA nor on the ePTFE-*g*-PSBMA membrane surfaces. Indeed, it was shown in a previous work that no activated platelets could adhere to the PVDF-*g*-PSBMA membrane surface with overall electric neutrality.[38] Herein, the ePTFE-*g*-PSBMA membrane was designed to ensure electric neutrality on the surface. In other words, the N to S ratio was controlled, from the knowledge and experience of our previous studies dealing with the design of similar zwitterionic surfaces.[38,39] The plasma treatment operating parameters were ideal to ensure neutrality. As for the PEGylated membrane, their good performance can also be explained by their ability to catch and bind water as well as by their electric neutrality inherent in their nature.

Following the tests carried out with platelets, attachment of red blood cells and white blood cells was investigated. As pointed out earlier, dressings must be blood compatible and not interact with any type of blood cell. Figure 7 presents the confocal images obtained after contacting the virgin and modified membranes with erythrocytes on one hand (Figure 7a to 7c) and leucocytes on the other hand (Figure 7d to 7e). Virgin ePTFE membranes constituted an ideal surface for blood cell adhesion, for reasons similar to those explaining bacterial attachment (hydrophobic and porous surface), but it was worth noting that if red blood cell were easily attached, only fewer white blood cells could be observed on the surface of the ePTFE membrane, probably because of their lower amount in human blood and despite that concentrates were used. Grafted membranes resisted efficiently the adhesion of blood cells, whether erythrocytes or leucocytes. Especially, ePTFE-*g*-PSBMA performed better, once again showing the excellent blood-inert nature of the zwitterionic sulfobetaine structure. This result further supported our previous study in which polyprolyene was used as the matrix substrate and demonstrated that an excellent blood-inert membrane surface could be achieved by atmospheric plasma-induced surface zwitterionization.[38]

To complete the blood compatibility tests of zwitterionic ePTFE membranes, a RBC hemolysis assay was performed. The observed hemolysis of RBCs in DI water and PBS solutions at 37 °C was used as positive and negative controls, respectively. The observed hemolytic activity of ePTFE-*g*-PSBMA and ePTFE-*g*-PEGMA membranes at 37 °C was normalized to that of the positive control DI water. Figure 8

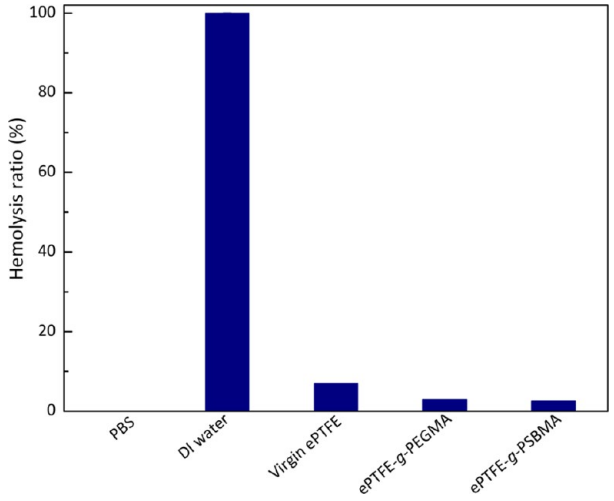

Figure 8. Hemolysis of RBC solution in the presence of virgin and modified ePTFE membranes at 37 °C. *The error limits on data do not appear because they are lower than 0.06%.*

displays the results. In general, hydrophobic polymers are capable of interacting with biological membranes, causing disruption. Thus, it was observed that the virgin ePTFE membrane exhibited ∼7% hemolytic activity. On the other hand, an important reduction of hemolytic activity was observed for both grafted membranes (2.5% for ePTFE-g-PSBMA and 3% for ePTFE-*g*-PEGMA), further evidencing an improvement of blood compatibility using modified membranes. Almost no unwanted reaction to blood/membrane contact could then be measured, and the experimental error associated to this measurement was each time lower than 0.06%, showing the reliability and accuracy of the test. Therefore, it could be concluded with certainty that modified membranes, and especially the zwitterionic membrane,

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exhibited a good nonfouling nature with antihemolytic activity to resist the disruption of blood cell membranes.

In addition to this blood compatibility study, the ability of membranes to resist the attachment of human skin cells, fibroblasts, was evaluated. Human fibroblasts (HT-1080) were cultured on the membranes in 24-well tissue culture polystyrene (TCPS) plates at 37 °C for 1 to 3 days, and the samples were then observed under a microscope equipped with a digital camera using a 10× objective lens. The images are shown in Figure 9 at a magnification of 100×. HT-1080 cell

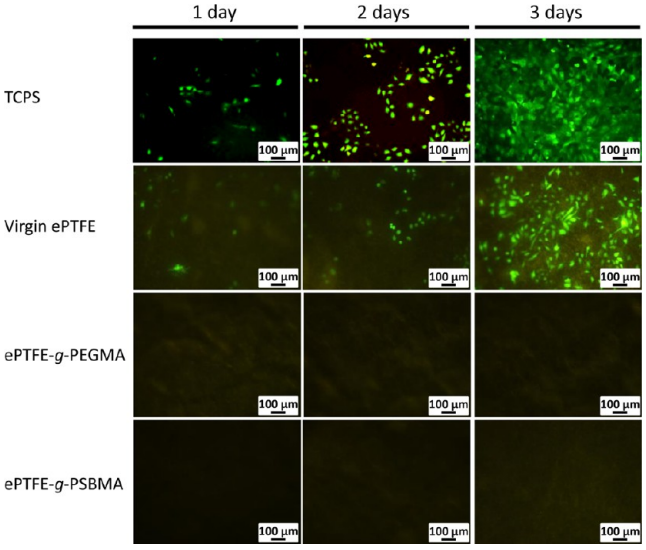

Figure 9. Fluorescence microscopic images of HT-1080 cell attachment on the TCPS surface, virgin ePTFE membrane, ePTFE-*g*-PEGMA membrane, and ePTFE-*g*-PSBMA membrane. Observations were made from 1 to 3 days after incubation of the samples with the human fibroblasts. Cell culture was performed at an initial concentration of 2×10^4 cells/mL.

adhesion and growth on the membranes was compared with cell adhesion and growth on a flat TCPS surface. For the bare TCPS plate, fibroblasts adhered and spread over the TCPS surface into a confluent-like layer, and they also easily attached to the surface of virgin ePTFE membranes, providing an adequate hydrophobic and porous environment for cell–membrane interactions. After 3 days, TCPS and ePTFE surfaces were almost entirely recovered with fibroblasts. On the contrary, cells did not adhere to the surface of both zwitterionic and PEGylated ePTFE membranes, even after a 3-day incubation time. A possible explanation for this antiadhesive behavior is that surfaces of modified membranes did not permit the adsorption of proteins: indeed, if devoid of any adhesive proteins, there cannot be cell–membrane interactions.[52] Especially, this excellent resistance to cell attachment for surfaces modified with zwitterionic polymers has been previously reported[53−56] and is inherent to the zwitterionic structure. It was important to note that this cell antiadhesive behavior remained for at least 3 days (the maximum duration tested in the present work). This behavior is important regarding the wound dressing application. Membranes must favor wound repair, without adhering to the newly formed tissue, to avoid potential trauma during replacement of the dressing. Also, because dressings may not be changed daily, they must resist efficiently the adhesion of cells for several days, a behavior exhibited by the grafted ePTFE membranes prepared herein.

Wound Healing Efficiency of Zwitterionic ePTFE Membranes from in Vivo Tests. The main objective of this work was to demonstrate that the ePTFE-*g*-PSBMA membrane could be potentially employed as wound dressing materials. The goal was to reach a fast and complete recovery of the skin issue. We therefore aimed at designing wound dressings that would help at improving the overall wound healing process by accelerating the different phases during healing including the inflammatory phase, the migratory phase during which the epithelial cell invaded the wound area, the proliferative phase, and the remodeling phase. We showed recently that zwitterionic grafted membranes exhibited antifouling character for plasma-protein and blood-platelet resistance,[38] which was also demonstrated in the present study using ePTFE as a substrate. Optimized hydration of the PSBMA-grafted layer led to excellent blood-inert property and anticoagulant activity in human blood, attributed to the formation of a strong interfacial hydration layer due to the binding of water molecules around electrically neutral PSBMA chains. This, along with the excellent bacterial and cell resistance, formed some of the important properties required for the design of advanced wound dressings. First results were encouraging enough to carry out in vivo studies on laboratory rats to evaluate the wound healing efficiency of membranes. After being anesthetized, rats were wounded using a surgical blade, and wounds were covered with gauze, membrane, or commercial hydrocolloid dressing. To assess the efficiency of ePTFE-*g*-PSBMA membranes, wound recovery was analyzed through histomorphology data 7 and 14 days after injury. Several histological indicators can be used to judge the effectiveness of the healing process.[6,7,17,20,57−61] Some important indicators are the granulation level, the presence of fibroblasts, the collagen production, the presence and number of blood vessels, as well as extracellular proteins, the number and proliferation of derma papillae, or the hair follicle development.

In Figure 10, the histology of wounds showed that the epithelization of incisions, that is, the recovery process, increased in the order ePTFE < gauze < ePTFE-*g*-PEGMA < commercial product < ePTFE-*g*-PSBMA, indicating that materials covered with a grafted zwitteironic copolymer could provide a moist environment as good as or even better than that offered by commercial dressing, to accelerate healing of wounds. It was interesting to note that after 7 days the zwitterionic membrane led to a better reconstruction of the wound than the commercial hydrocolloid (Figure 10d vs Figure 10f) as shown by the extent of the nascent granulation layer and the regeneration and development of hair follicles in the wound. Indeed, the wound covered with zwitterionic membrane had evolved into the proliferative phase, while that covered with commercial dressing was still in the late stage of the inflammatory phase. After 7 days, the skin covered with the zwitterionic membrane presented numerous blood vessels that will further permit the reconstruction of the epithelium and dermis, including derma papillae and hair follicles, already visible at the early stage of healing and completely reformed after 14 days of healing. Therefore, Figure 10 demonstrated that the zwitterionic grafted membranes could stimulate the wound healing process, compared to other dressings, and prevented the loss of evaporative water and overall wound dehydration. In addition, from Figure 10a and 10i, it was

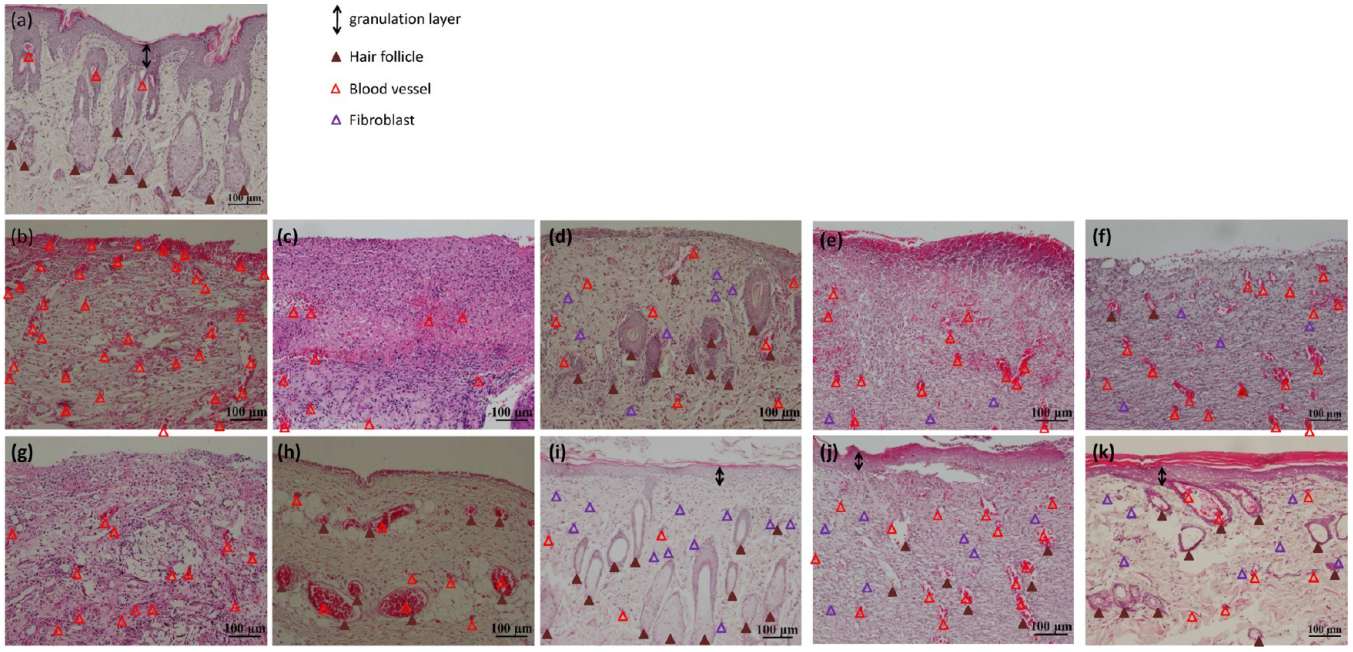

Figure 10. Evaluation of wound healing efficiency from in vivo tests. (a) Normal skin tissue; (b) and (g) wound covered with gauze; (c) and (h) wound covered with virgin ePTFE membrane; (d) and (i) wound covered with the ePTFE-*g*-PSBMA membrane; (e) and (j) wound covered with the ePTFE-*g*-PEGMA membrane; (f) and (k) wound covered with commercial product (3MTM TegadermTM hydrocolloid dressing). (b) to (f) Wound sections at 7 days and (g) to (k) wound sections at 14 days. All images were taken at a magnification of 100×. *Only regions where apparent fibroblast density was relatively high are pinpointed.*.

observed that the surface of the ePTFE-*g*-PSBMA-covered wound showed complete formation of new orderly arranged epithelium after 14 days, with a granulation layer thickness comparable to that of uninjured skin. Furthermore, histomorphology indicated densely packed keratinocytes in the epidermis and numerous hair follicles in the wounds treated with ePTFE-*g*-PSBMA, compared to that on the wounds treated with other dressings. Also, there were much less blood vessels, and the dermis was filled with fibroblasts and collagen fiber. The great formation of blood vessels in the early stage of healing, the development of hair follicles at 14 days, the well-proliferated fibroblast, and apparent comparable production of collagen to that of wound before injury indicated that the ePTFE-*g*-PSBMA membrane contributed to significantly enhance granulation tissue formation and therefore could be used as a novel type of wound dressings allowing a fast and complete wound recovery.

CONCLUSION

In this work, zwitterionic ePTFE-*g*-PSBMA membranes designed for wound dressing applications were prepared by atmospheric plasma treatment. The membrane exhibited numerous characteristics essential for wound dressings. Indeed, they showed superior water absorption, therefore favoring a moist environment suitable for fast healing. Their low protein, low adhesive property was also highlighted, and the membrane could resist bacterial adhesion, essential to preventing wound potential infection by micro-organisms. Membranes presented excellent blood compatibility and resisted cell attachment, which is important to avoid supplementary trauma when changing the dressing. As for their wound healing property, it was shown that the ePTFE-*g*-PSBMA membrane led to a faster wound healing than ePTFE-*g*-PEGMA or commercial dressings, with a complete re-epithelization of the wound after 14 days. As the new membranes presented in this work promote healing, and do not adhere to the skin wound, they are suggested to be potential outstanding dressings aiming at enhancing wound healing.

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Notes

The authors declare no competing financial interest.

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