Preparation of Polymeric Films of PVDMA–PEI Functionalized with Fatty Acids for Studying the Adherence and Proliferation of Langerhans $\beta$-Cells

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ABSTRACT: This study reports the synthesis of thin polymeric films by the layer-by-layer deposition and covalent cross-linking of polyvinyl dimethylazlactone and polyethylene imine, which were functionalized with lauric (12-C), myristic (14-C), and palmitic (16-C) saturated fatty acids, whose high levels in the bloodstream are correlated with insulin resistance and the potential development of type 2 diabetes mellitus. Aiming to assess the effect of the fatty acids on the adhesion and proliferation of Langerhans $\beta$-cells, all prepared films (35 and 35.5 bilayers with and without functionalization with the fatty acids) were characterized in terms of their physical, chemical, and biological properties by a battery of experimental techniques including $^1$H and $^1$C NMR, mass spectrometry, attenuated total reflectance–Fourier transform infrared spectroscopy, field emission scanning electron microscopy, atomic force microscopy, cell staining, and confocal laser scanning microscopy among others. In general, the developed films were found to be nanometric, transparent, resistant against manipulation, chemically reactive, and highly cytocompatible. On the other hand, in what the effect of the fatty acids is concerned, palmitic acid was found to impair the proliferation of the cultured $\beta$-cells, contrary to its homologues which did not alter this biological process. In our opinion, the multidisciplinary study presented here might be of interest for the research community working on the development of cytocompatible 2D model substrates for the safe and reproducible characterization of cell responses.

INTRODUCTION

The incidence of type 2 diabetes mellitus (T2DM) has increased globally because of the sedentary life style and obesity of the population; therefore, prevention based on the modification of the diet as well as other healthcare habits is recommended.1 T2DM is a metabolic disease that is produced by high blood glucose levels related to a low insulin production of the body, a resistance to its action, an increase in the production of glucose, or a combination of them. The increase in glucose production constitutes a state prior to the development of T2DM called prediabetes. At this stage, the $\beta$-cells are able to adapt to this hostile environment by increasing their insulin production, but only for a limited time. Gradually, the function of insulin deteriorates up until it is depleted, so that blood glucose levels begin to increase. As the $\beta$-cells defect progresses, blood glucose levels remain steadily high, and this increased glucose acts as a toxic agent to the pancreas, consuming the insulin the $\beta$-cells have stored inside them, and thus aggravating it. Other factors such as the increase in plasmatic fatty acids and chronic inflammation associated with obesity also act against the pancreas, being toxic for the $\beta$-cells. In different models, high levels of free fatty acids have been proposed like a determinant factor of apoptosis, which, connected to the degree of saturation, could also induce an inhibition of insulin biosynthesis and secretion. In particular, saturated fatty acids such as palmitic and stearic acids have shown to reduce the cell viability and activate the unfolded protein response pathway, which is important to maintain cellular homeostasis.2,3 Therefore, methods that allow us to deepen the grasp on the damage phenomena in $\beta$-cells through the implementation of novel strategies/techniques are critical for the understanding of the physiology of $\beta$-cells and, as a consequence, for the potential development of therapeutic protocols. One such method is the preparation of 2D platforms with particular architecture and/or functionality capable of interacting with and ruling the response of $\beta$-cells. An elegant example of this approach is the design of thin polymeric films with on-demand surface...
functionalization using the layer-by-layer (LBL) technique. The LBL methods allow the alternate deposition of polymer layers, resulting in being useful for the coating of different substrates such as glass, silicon, gold, and other materials. The main focus is to control with precision the molecular architecture of the deposited layers, thus controlling as well the physical, chemical and mechanical properties of the prepared films. In the present work, 2D polymeric films with a series of terminal fatty acids (lauric, myristic, and palmitic acid) were synthesized using the LBL technique upon the deposition and covalent cross-linking of alternate layers of polyvinyl dimethylazlactone (PVDMA) and polyethylene imine (PEI), leaving PEI at the uppermost layer which was functionalized with the selected fatty acids. The purpose of the study was to assess the effect of the fatty acids on the surface adhesion and proliferation of Langerhans β-cells. The developed systems and polymer precursors were characterized using a battery of experimental techniques including 1H and 13C NMR, mass spectrometry, attenuated total reflectance–Fourier transform infrared spectroscopy (ATR–FTIR), field emission scanning electron microscopy (FESEM), atomic force microscopy (AFM), cell staining, and confocal laser scanning microscopy (CLSM) among others.

### RESULTS AND DISCUSSION

**Overview of the Synthesis Process.** The LBL method allows the generation of multilayer films wherein different groups can be incorporated within each formed bilayer, which can also be surface-functionalized. Moreover, it allows the assembly of alternating layers of polymers on different substrates, providing additional stability and the possibility to control the film thickness at the micro and nanometric scales. As such, the LBL assembly constitutes a convenient coating strategy to modify the surface properties of materials for a plethora of applications, such as the deposition of paints against corrosion or graffiti, the development of semi-permeable membranes, and the fabrication of antifouling, superhydrophobic, and slippery liquid-infused porous surfaces. Of interest to this study, the use of surfaces prepared by LBL for seeding and growth of model cells has been implemented for different biomedical purposes, such as in biomaterial implants, for diagnosis, cell culture, and assessing of unwanted adsorption of proteins and bacteria on implantable devices, among others. Figure 1 schematizes the synthesis process of our films, by which 35 and

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**Figure 1.** (A) Schematic drawing of the prepared LBL assembly, (B) LBL film with PVDMA at the uppermost layer (35 bilayers) and (C) LBL film with PEI at the uppermost layer (35.5 bilayers).

**Figure 2.** ATR–FTIR spectra of the substrate films: (A) 35 bilayers and (B) 35.5 bilayers.
35.5 bilayer films were developed, as recommended in previous publications.37,38,41

**Validation of Film Reactivity and Covalent Cross-linking.** Surface reactivity is an important feature of materials toward their chemical functionalization with bioactive molecules (fatty acids in our case) and subsequent application as substrates for cell seeding.41 The surface reactivity of the developed films was characterized by ATR−FTIR and CLSM at the PVDMA and PEI layers (upon formation of 35 and 35.5 bilayers, respectively); the obtained results are shown in Figures 2 and 3. Figure 2 shows representative ATR−FTIR spectra of the films at both layers of interest. The 35 bilayer spectrum (panel A) shows distinctive bands of PVDMA, PEI, and the PVDMA−PEI cross-linking, specifically the amine (at ca. 3275 cm⁻¹, from PEI), azlactone (at ca. 1822 cm⁻¹, from PVDMA), and amide groups (at ca. 1647 cm⁻¹, from the PVDMA−PEI system). Meanwhile, the 35.5 bilayer spectrum (panel B) shows the distinctive bands of PEI (3275 cm⁻¹) and PVDMA−PEI crosslinking (1647 cm⁻¹) merely. Interestingly, the absence of azlactone bands from PVDMA in the latter spectrum (35.5 bilayers) shows that most of these groups (not to say all) reacted with the amine groups of PEI during the covalent cross-linking; on the other hand, the presence of residual amine groups from PEI reveals the films as reactive for the desired immobilization of the fatty acids to be carried out in a following step (see below).

Figure 3 shows representative CLSM micrographs of a 35 bilayer film functionalized with BODIPY-NCH₂CH₂NH₂, a fluorescent dye that emits in the 430−480 nm region (blue). The portrayed images show a highly ordered multilayered pattern resulting from the covalent cross-linking between PVDMA and PEI, which was colored in blue because of the emission of the dye upon reaction with residual azlactone groups of PVDMA onto the substrate surface. This even ordering reveals a highly controlled and uniform reaction process. In general, strong and uniform

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**Figure 3.** Representative CLSM images of a 35 bilayer film functionalized with the BODIPY-NCH₂CH₂NH₂: (A) LBL noncoated glass interface region. (B) LBL region (C) zoom in of the LBL region. The scale bars stand for 50 μm (A,B) and 200 μm (C).

**Figure 4.** Representative images of the film and glass slide substrate at different length scales: (A) Photograph of a glass slide (control), (B) photograph of the coated glass slide (film), (C) FESEM image of a 35 bilayer thin film, and (D) AFM image of a 35 bilayer thin film.

**Figure 5.** AFM images: (A) reactive thin film and (B) thin film functionalized with palmitic acid, (C,D) mean topography profiles of reactive and functionalized films.
interactions between the reacting species give rise to ordered and flat LBL coatings. By contrast, when weak interactions take place, the coating process results in the formation of thick layers wherein polymeric chains are not tightly bound one to each other at the reacting interface, adopting in consequence a loose and globular surface conformation. Interestingly, whenever nonregular structures are formed, they become exaggerated upon further LBL cycles. As compared to previous publications,40 the wavy structures typically described for weakly-interacting LBL systems were not detected all along our experimental process (see FESEM and AFM images shown above). Accordingly, these images provide evidence of both the proper cross-linking between PVDMA and PEI and the reactivity of the azlactones groups of PVDMA at the uppermost surface of the 35 bilayer film.

**Appearance, Thickness, and Topography of the Films.** Thin and transparent films with smooth and homogeneous surface features were obtained, as demonstrated by optical photography, FESEM, and AFM (see Figure 4). The thickness of the film was determined by FESEM by measuring in at least in five different points, giving an average value of 524 nm (Figure 4C, black label). With respect to the surface features, an even topography was observed by FESEM and also confirmed by AFM (Figure 4C,D), revealing an average roughness ($S_a$) of 1.613 $\pm$ 0.008 nm, with a mean value ($S_m$) of $-18.63$ fm, and a peak-valley height ($S_y$) of 15.11 nm. It is worth mentioning that the surfaces were also validated as highly resistant against the routine manipulation by hands and with forceps all along and after the synthesis process.

**Changes in Topography and Film Thickness upon Functionalization with Fatty Acids.** PVDMA–PEI films (35.5 bilayers) were functionalized with palmitic, lauric, and stearic acids in order to assess their effect on the adhesion and proliferation of Langerhans $\beta$-cells. These fatty acids are believed to be involved in metabolic pathways of Langerhans $\beta$-cells related to insulin resistance and the potential development of T2DM. The obtained functionalized films were characterized in terms of their topography, roughness, and water contact angle (WCA), properties that have been proved to regulate the early protein adsorption onto materials and their following affinity to cells.42 Figure 5 shows representative 2D AFM images and topography profiles of nonfunctionalized (panels A,C) and palmitic acid-functionalized films (B,D). The direct comparison of these panels demonstrate that the topography of the film remains smooth and homogeneous after functionalization, yielding a roughness increase of ca. 0.33 nm, a rather small noticeable change. Moreover, as derived from the topography profile (Figure 4D), it was found that an increment of $S_a$ is close to 21%, the $S_m$ and $S_y$ values being calculated as $-18.23$ fm, and 30.35 nm, respectively. The increase in peak-valley height value is due to the projections observed in the topography image, which can be assumed as zones of accumulation of palmitic acid. With respect to the thickness, the functionalization of the films led to an increment of about 50% from ca. 500 to 750 nm. This increase in thickness is ascribed to the 16-C chain of palmitic acid, and this was evaluated with FESEM analysis (see the Supporting Information).

**WCA Measurement.** Protein adsorption and subsequent cell adhesion have been proved to be dependent on the surface wettability of solid substrates.43 When an exogenous material comes into contact with a biological system, integrins spontaneously adsorb onto the surface. With increased integrin recruitment, the early cell–protein contact leads to the formation of anchoring complexes at the lamellipodium leading edge that are reinforced intracellularly to form larger focal adhesion plaques upon increased intra- and extracellular tension. In general, cells effectively adhere to substrates
presenting moderate wettability with WCA values in the range of 40°–90°.42,43 Taking into account this information, we proceeded to characterize the WCA of the functionalized surfaces. Table 1 shows the results obtained from the measurement of the WCA on the different surfaces: glass, reactive film (35.5 bilayers), and films functionalized with the fatty acids. The obtained results reflect good surface lipophilicity of the functionalized films, all of which exhibiting WCA values higher than those of glass. Interestingly, the surface functionalization with palmitic acid led to the highest surface lipophilicity among the tested fatty acids (the highest WCA value), which can fairly be attributed to the longer chain length of this acid relative to its homologues.

Evaluation of the Effect of Films on β-Cell Proliferation. Functionalized PVDMA–PEI films have been used in other reports to improve the cell function and regulate the cell proliferation, proving to be robust and stable in physiological environments.44–46 However, to the best of our knowledge, the effects of lipid structures attached to these films have not yet been evaluated. Previous results by our group on the culture of pancreatic β-cells in the presence of saturated and unsaturated fatty acids in solution demonstrated that saturated fatty acids, including palmitic and stearic acid, induced lipoapoptosis, whereas unsaturated fatty acids showed the opposite effect, not interfering with β-cell proliferation.47–49 As a step forward in our research, in the present work, we evaluated the adhesion and proliferation of β-cells onto PVDMA–PEI films functionalized with lauric, myristic, and palmitic saturated fatty acids, employing nonfunctionalized surfaces as a control. As revealed by optical microscopy and CLSM (Figure 6), the developed PVDM–PEI films with PEI at the uppermost layer (employed as a control in these experiments, 35.5 bilayers) proved to be cytocompatible and optimal for cell culture in as much as they adhered and spread onto the bi films, all of which exhibiting WCA values in the range 40°–90°.

Figure 6. Schematic representation of the produced films with PEI at the uppermost layer (35.5 bilayers) before (A) and after functionalization with lauric acid (E), myristic acid (I), and palmitic acid (M). Optical (B,F,J,N) and CLSM images (C,D,G,H,K,L,O,P) of β-cells cultured onto the corresponding surfaces. Coomassie was employed for cell staining of cells for optical microscopy characterization. Aza-BODIPY-(OH)2 and Hoescht were employed for the staining of the cytoplasm and cell nuclei for CLSM characterization, respectively. Scale bars in panels (C,G,K,O) and (D,H,L,P) correspond to 100 and 20 μm, respectively.

CONCLUSIONS
Thin polymeric films were prepared by the LBL deposition and covalent cross-linking of PVDM A and PEI first and functionalized with lauric, myristic, and palmitic saturated fatty acids next. The physical, chemical, and biological properties of all prepared films were evaluated in order to assess the effect of fatty acids on the adhesion and proliferation of Langerhans β-cells. In general, all films proved to be transparent and resistant against manipulation by hand and with forceps, showing thickness and topography profiles in the nanometric scale. On the other hand, the surface reactivity of the PVDM A and PEI layers, together with the proper cross-linking between them, was validated by the presence of characteristic ATR–FTIR bands of the azlactone (from PVDM A), amine (from PEI), and amide groups (from the PVDM A–PEI crosslinking), as also confirmed using the CLSM images upon postproduction treatment with BODIPY-NCH2CH2NH2 (staining in blue the VDPMA layers). Finally, the biological characterization by optical microscopy and CLSM evidenced an impairing effect of palmitic acid on the cell proliferation, opposite to lauric and myristic acids, which did not alter this biological process. Taken together, the multidisciplinary approach here reported reveals the potential of PVDM A–PEI films functionalized with saturated fatty acids as cytocompatible 2D model substrates to assess the effect of the acids on the adhesion and proliferation of β-cells. Moreover, the validation of the impairing effect of palmitic acid on the cell proliferation, elsewhere ascribed to be lipotoxicity, might be of interest for the development of clinical protocols for the treatment of T2DM. The last but not the least, given the proven reactivity of both PVDM A and PEI layers (holding active azlactone and amine groups, respectively), the developed films might also be functionalized with a broad range of biomolecules to study their effect on additional cell lines. Accordingly, these findings pave the way for future studies wherein versatile 2D and even 3D substrates might be considered for deepening the surface effects of functionalized biomaterials for cell culture.

EXPERIMENTAL SECTION

General Materials and Methods. Acryloyl chloride (97% containing 400 ppm phenothiazine stabilizer), 2-methylalanine (98%), ethyl chloroformate (97%), triethylamine (99%), NaOH (reagent grade), HCl (reagent grade, 37%), 1, 4-
dioxane (99.8%), 2,2′-azobis (2-methylpropionitrile) (98%), branched poly(ethylene imine) (PEI, \(M_w = 25,000\)) reagent grade, dimethyl sulfoxide (DMSO), hexane, acetone, glass microscope slides, lauric, myristic, and palmitic acids were purchased from the commercial source (Aldrich) and used without further purification. Anhydrous solvents were obtained using standard methods. BODIPY was prepared in our laboratory. Glass (76 × 25 mm) substrates were cleaned with acetone, ethanol, methanol, and deionized water and dried under a stream of compressed air prior to the manufacture of multilayered films. The thicknesses of the films (deposited on glass substrates) were determined by FESEM upon their measurement at five different standardized locations. CLSM images were acquired with a Leica TCS SP8 confocal microscope (Mannheim, Germany). Thin-layer chromatography was performed on silica gel \(F_{254}\) plates (Merck). All compounds were detected using UV light. Melting points were obtained in an Electrothermal 88629 (Merck). All compounds were detected using UV light. Melting points were obtained in an Electrothermal 88629 (Merck). All compounds were detected using UV light.

**Synthesis of Poly(2-vinyl-4,4′-dimethylazlactone) (PVMDA).** The initiator, 2,2′-azobisobutyronitrile (AIBN) (3.0 mg, 0.02 mmol, 0.01 equiv) was added into a 10 mL Schlenk flask equipped with a stir bar, then anhydrous 1,4-dioxane (1.5 mL) was added to the flask, and the mixture was stirred until AIBN was dissolved completely. After that, VDMA (0.8 g, 5.55 mmol, 1 equiv) was added to the flask, and the flask was capped with a septum and purged with argon for 5 min. The reaction solution was stirred at 70 °C for 16 h. The viscous reaction mixture was cooled in an ice bath to stop polymerization, acetonitrile (~1 mL) was added to the flask, the flask was stirred, and then, hexane was added to precipitate the product. The polymer was precipitated three times into hexanes to yield a white solid (0.7 g, 90% yield). GPC: \(M_n = 67,300\) g/mol; PDl = 1.03. FT-IR (ATR, neat): 2981, 2933 (C-H), 1818 (C=O), 1666 (C=N) cm\(^{-1}\). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 8.35 (s, 1H, NH), 6.26 (dd, Htrans, \(J = 17.0\) Hz, Htrans−Hcis = 10.0 Hz, H1, Vin), 6.02 (dd, Htrans, \(J = 17.2\) Hz, Htrans−Hgem = 2.4 Hz, 1H, vinyl), 5.55 (dd, Hcis, \(J = 10.0\) Hz, Hcis−Hgem = 2.4 Hz, 1H, vinyl), 1.34 (s, 6H, C\((\text{CH}_3)_2\)) \(^13\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 176.0, 132.0, 126.0, 125.9, 55.7, 25.6. EIMS (m/z): 157.2 (4%), 112.2 (100%), 58.2 (100%).

### 4,4-Dimethyl-2-vinylxazol-5(4H)-one

N-Acryloyl-2-methylalanine (6.0 g, 0.0382 mol), triethylamine (8.0 mL, 0.0573 mol), and acetone (130 mL) were combined in a two-neck flask round-bottomed flask. The reaction mixture was purged with argon while cooling in an ice bath for 10 min. Ethyl chloroformate (5.5 mL, 0.0573 mol) was added dropwise over 5 min using a pressure equalizing addition funnel. Once the addition was complete, the reaction was stirred on ice under an inert atmosphere for 3 h. The solution was then filtered using a Buchner funnel, and the precipitate was washed with cold acetone. The filtrate was concentrated via rotary evaporation and purified by vacuum distillation (bp ~ 70 °C, 60 mbar) to give a clear liquid. The purified monomer was stored at 4 °C until further use. (1.8 g, 0.013 mol, 34% yield). \(R_t = 0.83\) ethyl acetate 100%. FT-IR (ATR, neat): 2984 (C= H), 2938 (C-H, CH\(_2\)), 2868 (C-H, CH\(_3\)), 1818 (C=O, ester), 1666 (C=N imine), 1596 (C=C cm\(^{-1}\)). \(^1\)H NMR (DMSO-\(d_6\)): \(\delta\) 6.30 (dd, Htrans, \(J = 17.6\) Hz, Htrans−Hcis = 9.6 Hz, 1H, Vin), 6.23 (dd, Htrans, \(J = 17.6\) Hz, Htrans−Hgem = 2.0 Hz, 1H, vinyl), 5.92 (dd, Hcis, \(J = 9.6\) Hz, Hcis−Hgem = 2.0 Hz, 1H, vinyl), 1.47 (s, CH\(_3\)), 6H). \(^13\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 180.6, 159.1, 129.0, 124.1, 65.5, 24.4. EIMS (m/z): 139.2 (5%), 111.2 (45%), 95.2 (43%), 55.2 (100%), 42.2 (22%). Spectral data were identical to those reported previously.

**2-Acrylamido-2-methylpropanoic Acid.** 2-Methylalanine (10.0 g, 0.0970 mol) in NaOH (8.9 g, 223 mmol) and 2,3-dihydroxybenzyl-4-methoxphenol (2.0 mg, 0.009 mmol) were weighed in a 250 mL round-bottomed flask equipped with a magnetic stir bar and dissolved in ultrapure water (25.0 mL). The solution was stirred in an ice bath until the internal temperature reached ~4 °C. Acryloyl chloride (10.9 g, 123 mmol) was added dropwise using an addition funnel over approximately 15 min. The reaction was stirred for 3 h in an ice bath. Concentrated HCl (~6.5 mL) was added slowly to the reaction solution until the solution reached pH 2, resulting in the formation of a white precipitate. The solution was stirred for additional 30 min on ice. The white precipitate was filtered in a Buchner funnel and rinsed with 125 mL of cold water. The white solid was dried under ambient temperature. (9.2 g, 0.0587 mol, 60.5% yield), mp 187−189 °C. FT-IR (ATR, neat): 3340 (NH), 3073 (C−H), 2991 (C−H, CH\(_2\)), 1705 (C=O), 1649 (C=O), 1599 (C=C cm\(^{-1}\)). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 8.35 (s, 1H, NH), 6.26 (dd, Htrans, \(J = 17.0\) Hz, Htrans−Hcis = 10.0 Hz, H1, Vin), 6.02 (dd, Htrans, \(J = 17.2\) Hz, Htrans−Hgem = 2.4 Hz, 1H, vinyl), 5.55 (dd, Hcis, \(J = 10.0\) Hz, Hcis−Hgem = 2.4 Hz, 1H, vinyl), 1.34 (s, 6H, C\((\text{CH}_3)_2\)) \(^13\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 176.0, 132.0, 126.0, 125.9, 55.7, 25.6. EIMS (m/z): 157.2 (4%), 112.2 (100%), 58.2 (100%).
stored in a vacuum desiccator until use. All films were manufactured at ambient room temperature.

Characterization of Thin Film Reactivity. To evaluate the reactivity of the films by CLSM, a bilayer film was immersed in a solution of 10 mg of BODIPY-NHC\textsubscript{3}CH\textsubscript{2}NH\textsubscript{2} (see the Supporting Information) in 50 mL of DMSO at room temperature for ∼16 h. The film was washed for 1 h in DMSO, washed with ETOH, and dried with compressed air before being analyzed by CLSM.

Postfabrication Functionalization of Thin Films. PEI–PVDMA films with 35.5 bilayers were functionalized postfabrication by immersing film-coated substrates in solutions of either stearic, myristic, or palmitic acid 20 mM in (DMSO, EDC, and HOBT) at room temperature for ∼24 h. Films were soaked in DMSO for ∼1 h after functionalization, and DMSO was changed at least once during soaking, washed with ETOH, and dried with compressed air before being analyzed or used to grow beta cells.

WCA Estimations. The WCA estimations were carried out at room temperature employing a 12 MP, f/1.8, 28 mm (wide), PDAF, OIS 12 MP, f/2.8, 57 mm (telephoto) camera, working with 2× optical zoom. Static WCA was measured with the Image J software upon deposition of 5 μL of droplets of deionized water onto the selected films (2.5 × 2.5 cm\textsuperscript{2}) at three different locations. Data are reported as the average.

Cell Culture onto PEI–PVDMA Films. β-Cells of the islet of Langerhans were seeded onto reactive and fatty acid-functionalized films (35.5 bilayers). Briefly, the films were washed with 70% ETOH, placed in Petri dishes, and put under ultraviolet light for 30 min prior to cell seeding. Subsequently, 5 mL of phosphate-buffered saline (PBS) was added to each film and removed under vacuum (two times) to eliminate the excess of ETOH. Then, 8 mL of RPMI-1640 medium supplemented with 10% FBS, 10 U/mL penicillin, 10 μg/mL streptomycin, and 25 μg/mL amphotericin B were placed in the Petri dish. Subsequently, the cells with a density of 163,000 cells/mL were seeded onto the films and incubated for 96 h to allow their adhesion and proliferation. The cell culture was carried at standard culture conditions in all cases (37 °C, 5% CO\textsubscript{2} in a humidified atmosphere).

Optical Microscopy. Right after the cell incubation time onto the films was reached (96 h), a batch of the samples were stained with the Coomassie dye and observed under a photo zoom inverted microscope (Cambridge Instruments) employing a 10× magnification lens. Briefly, 5 mL of methanol at −70 °C was added and under gentle shaking for 10 min. Then, 5 mL of Coomassie (0.03 g/200 mL) was added to each film and incubated for 10 min at 4 °C under gentle orbital shaking. Finally, the cells were observed after rinsing twice with cold PBS (1×) and addition of 5 mL of PBS (1×) inside a Petri dish. Langerhans β-cells were chosen as a model for the characterization under adherent conditions. This cell line is of human physiopathological relevance associated with metabolic overload.\textsuperscript{31} The cells were bought from the American Type Culture Collection (ATCC).

Confocal Laser Scanning Microscopy. CLSM experiments were carried out to characterize both the LBL produced films and the adhered cells. A LEICA TCS-SP8 confocal microscope (LEICA Microsystems Heidelberg GmbH) was used. Regarding the surfaces, they were characterized by operating the microscope in the fluorescence and bright field modes. The excitation/emission wavelengths for the fluorescence mode were 405/430–480 nm (for detection of BODIPY-NHC\textsubscript{3}CH\textsubscript{2}NH\textsubscript{2}, blue channel). Meanwhile, the excitation wavelength for the bright field mode was 488 nm. On the other hand, concerning the cells, they were characterized by operating the microscope in the fluorescence mode exclusively. The experiments were carried out to characterize the cellular distribution of Aza-BODIPY-\textsubscript{(OH)}\textsubscript{2}. For these experiments, a second bath of the cell-seeded films was washed with PBS (1×), incubated with Aza-BODIPY-\textsubscript{(OH)}\textsubscript{2} (4 μL/mL in DMSO) for 30 min, and washed again twice with PBS (1×). Then, the films were added with Hoechst dye (0.2 μL/mL in PBS), incubated for 30 min, and washed again twice with PBS 1X. Finally, the films were washed four times with cold PBS (1X), the cells were fixed with paraformaldehyde (4% in PBS), and then subjected to observation. Macroscopically different zones were recorded, preferentially at the center of the specimens, in order to depict representative images. The images were recorded at the excitation/emission wavelengths of 405/430–550 (for detection of Hoechst, blue channel) and 638/650–750 (for detection of Aza-BODIPY-\textsubscript{(OH)}\textsubscript{2}, red channel).

■ ASSOCIATED CONTENT

† Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b04313.

○ H NMR spectra, FT-IR spectra, 13C NMR spectrum, 1H NMR spectra, FT-IR images, synthesis methodology of BODIPY-NHC\textsubscript{3}CH\textsubscript{2}NH\textsubscript{2} and Aza-BODIPY-\textsubscript{(OH)}\textsubscript{2}, AFM images, and evaluation of zones of reactive films with and without functionalization (35.5 bilayer) (PDF)

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M.E.A.-C., I.A.R., V.G.-G., M.A.-M., E.R.-V., J.C.C.-Y., K.A.E., and A.P.-C. performed the experiments, I.A.R. supervised the work, M.E.A.-C., I.A.R., V.G.-G., M.A.-M., and E.R.-V. wrote the manuscript. All authors reviewed the manuscript and made intellectual contributions.

Notes
The authors declare no competing financial interest.

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