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Original Article

The role of surface energy of technical polymers in serum protein adsorption and MG-63 cells adhesion

Jordi Comelles, MSc[a,*], Maruxa Estévez, MSc[a,b], Elena Martínez, PhD[a,c], Josep Samitier, PhD[a,b,c]

[a]*Nanobioengineering Group, Institute for Bioengineering of Catalonia (IBEC), Barcelona, Spain*
[b]*Department of Electronics, University of Barcelona, Barcelona, Spain*
[c]*Centro de Investigación Biomédica en Red en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Spain*

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Abstract

Polymeric materials are widely used as supports for cell culturing in medical implants and as scaffolds for tissue regeneration. However, novel applications in the biosensor field require materials to be compatible with cell growth and at the same time be suitable for technological processing. Technological polymers are key materials in the fabrication of disposable parts and other sensing elements. As such, it is essential to characterize the surface properties of technological polymers, especially after processing and sterilization. It is also important to understand how technological polymers affect cell behavior when in contact with polymer materials. Therefore, the aim of this research was to study how surface energy and surface roughness affect the biocompatibility of three polymeric materials widely used in research and industry: poly (methyl methacrylate), polystyrene, and poly(dimethylsiloxane). Glass was used as the control material.

***From the Clinical Editor*:** Polymeric materials are widely used as supports for cell culturing in medical implants and as scaffolds for tissue regeneration. The aim of this research is to study how surface energy and surface roughness affect the biocompatibility of three polymeric materials widely used in research and industry: poly(methylmethacrylate) (PMMA), polystyrene (PS), and poly(dimethylsiloxane) (PDMS).

Key words: Surface energy; Polymer; Osteoblast; Wettability; Protein adsorption

Polymeric materials are widely used in orthopedic surgery, biosensors, and tissue engineering. This is because they are compatible with simple manufacturing techniques and have a wide range of physical properties that make them desirable materials for biological applications.[1] Moreover, during the past decade new fabrication technologies have been successfully applied to polymeric materials, which has led to new developments such as lab-on-a-chip,[2] micro-and nanostructures for cell culturing,[3-6] and scaffolds for tissue engineering.[5]

Since 1960, when Craig et al studied the wetting properties of poly(methyl methacrylate) (PMMA) and polystyrene (PS) as a model for denture adhesion problems,[7] the interaction between polymeric materials and biological agents has been a key topic in biomaterials science. Consequently, different methods for improving polymer biocompatibility have been proposed. For instance, it has been shown that oxygen-plasma treatment on poly(dimethylsiloxane) (PDMS) modulates protein adsorption and improves cell adhesion[8,9]; Welle and Gottwald applied ultraviolet (UV) radiation to PMMA substrates to modulate cell response[10]; Patel et al functionalized PMMA with peptide sequences,[11] and finally, UV-ozone treatment has been used on PS substrates to control protein adsorption.[12]

Williams first defined biocompatibility as the ability of a biomaterial to perform with an appropriate host response in a specific application.[13] However, the use of biomaterials in new strategies like tissue engineering, drug delivery, and biotechnology applications has recently been revealed as a key factor in understanding the mechanisms and consequences that drive the interactions between biomaterials and biological entities.[14] In vivo, cells interact with their extracellular matrix via biochemical and biophysical signals. This matrix, which mainly comprises proteins and polysaccharides, influences cell behavior and therefore determines cell adhesion, spreading, migration, and function.[15] Thus, to understand cell behavior on biomaterials it is necessary to research the interaction between the cells and the physical and chemical stimuli present in their surroundings. Several authors have addressed this problem[16-18] and propose that cell adhesion to synthetic surfaces often implies the binding of cellular receptors to proteins adsorbed on the material. Protein

*Corresponding author:
E-mail address: jcomelles@ibec.pcb.ub.es (J. Comelles).

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adsorption is an energy-driven process that involves the properties of both the substrate surface and the proteins themselves. This interaction determines the amount of proteins adsorbed on the material surface and their conformational structure, which leads to an altered or unaltered biological activity.[16] Therefore, finding correlations between the material surface properties and the response of the biological model systems could help us to understand and predict the degree of material biocompatibility.[19]

There have been various suggestions regarding the way in which surface properties influence material biocompatibility. One of the most relevant parameters is surface energy. Surface energy drives surface wettability and is a key factor in determining adsorption kinetics and the amount of proteins adsorbed on the surface.[20,21] Despite the importance of surface energy in determining material biocompatibility, there are only a few reports in the literature that address surface energy in PMMA, PS, and PDMS in the context of biomedical applications.[8,10,22] These reports illustrate that substrates with higher values of surface energy promote cell adhesion. With the exception of one study by Toworfe et al, the reports do not consider the role of the protein adsorbed layer in mediating the interaction between substrates and cells. Toworfe et al focused on the adsorption of fibronectin on PDMS substrates and found that fibronectin adsorption is higher for hydrophobic surfaces.[8]

The present study is focused on the performance of different polymeric substrates in cell culture applications. Surface energy and surface roughness of PMMA, PS, PDMS, and glass were characterized using contact-angle and atomic force microscopy (AFM) measurements before and after sterilization. The adsorption of serum proteins on these polymeric substrates and glass was quantified. Protein distribution and conformation on the different surfaces was then evaluated using AFM images. All experiments on cell adhesion and proliferation were carried out using a human osteosarcoma cell line as a cell model. This approach was used to relate substrate surface energy to protein adsorption and its consequences on cell adhesion and proliferation.

Methods

Substrate preparation

Sheets of PMMA and PS (Goodfellow Ltd., Huntingdon, United Kingdom) of 125-μm thickness were cut into 75 × 25 mm pieces and used as supplied. Glass slides (26 × 76 mm) were used as supplied (Deltalab, Rubi, Spain). PDMS (Sylgard 184 Silicon Elastomer; Dow Corning, Wiesbaden, Germany) prepolymer was prepared by mixing the base polymer with the curing agent in a 10:1 ratio and degassing under vacuum conditions for 1 hour. The prepolymer was then cast into a Petri dish and cured for 1 hour in an oven at 80°C. Finally, the obtained polymer was cut into pieces of microscope slide size. Polymeric substrates were ultrasonically cleaned for 10 minutes in ultrapure water (Milli-Q; MilliPore, Billerica, Massachusetts) and dried using a stream of nitrogen to remove any dust particles. Glass slides were ultrasonically cleaned by consecutive immersions in acetone, 2-propanol, and 70% ethanol (Panreac, Castellar del Vallés, Spain) (5 minutes each). They were then dried under a stream of nitrogen. All the substrates were sterilized by immersion in 70% ethanol and UV radiation for 15 minutes immediately before cell culture.

Measuring surface roughness

AFM images of the substrate surfaces were used to evaluate polymer roughness. Topography images (1 μm^2) were taken in tapping mode in air using a silicon tip (175 MHz, 5 N/m) with a Molecular Force Probe 3D (Asylum Research, Santa Barbara, California) apparatus. The root mean square (R_{RMS}) of the different surfaces was calculated using WSxM 4.0 software (Nanotec, Tres Cantos, Spain).[23] At least three images of random areas of each substrate were collected.

Measuring contact angle

Contact angles (CAs) were measured by the sessile-drop method with an OCA contact angle system (Dataphysics, Filderstadt, Germany). Substrate contact angles were monitored on dried substrates before and after sterilization. Three different liquids were used to carry out the measurements: Milli-Q ultrapure water (MilliPore), formamide (Panreac), and diiodomethane (Sigma-Aldrich, Munich, Germany). A 1-μL liquid droplet was carefully deposited on the sample surface, which was then placed into a hermetic chamber with a saturated atmosphere of the corresponding liquid. Images of the liquid droplets in contact with the surfaces were captured immediately after droplet stabilization. The droplet profile was automatically fitted with SCA20 software (Dataphysics) using a circular fitting method. At least six contact-angle measurements were collected from three different samples for each combination of substrate and liquid.

Determining surface energy

The method proposed by Owens et al[24] (Eq 1) was used to evaluate the dispersive and polar components of the surface energy values of the substrates.

$$(1+\cos\theta)\gamma_L = 2\left(\left(\gamma_S^D\gamma_L^D\right)^{1/2} + \left(\gamma_S^P\gamma_L^P\right)^{1/2}\right) \quad (1)$$

This approach considers that the dispersive component γ^D of the surface energy takes long-range interactions such as van der Waals forces into account. On the other hand, the polar component γ^P refers to hydrogen bonding, dipole-dipole, and other short-range interactions. The contact-angle measurements of the three liquids tested together with their corresponding surface tension values, which are reported in Table 1, were used to determine the surface energy of the studied substrates.

Measuring protein adsorption

Sterilized substrates were incubated in Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL Life Technologies, Paisley, UK) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Gibco, Carlsbad, California), 1% penicillin/streptomycin (Invitrogen, Carlsbad, California), 1% L-glutamine (Invitrogen), and 1% MEM sodium pyruvate (Invitrogen) at 37°C in 5% CO_2 atmosphere. After 30 minutes the medium was removed and the substrates were rinsed three times with phosphate-buffered saline solution (PBS) for 30 seconds. To desorb proteins from the sample surfaces the substrates were subsequently incubated in 10%

Table 1
Surface tension values of the liquids used in this study

	γ (mN/m)	γ^D (mN/m)	γ^P (mN/m)
Water	72.8	21.8	51.0
Diiodomethane	50.8	49.5	1.3
Formamide	58.2	39.5	18.7

γ, total surface tension; γ^D, dispersive component; γ^P, polar component. Data obtained from SCA20 software.

sodium dodecyl sulfate solution (Bio-Rad, Hercules, California) in PBS for 3 hours on a Unimax 1010 shaker (1 cycle/sec) (Heidolph Instruments, Kelheim, Germany) at 37°C. The protein–sodium dodecyl sulfate solution was stored at −18°C for further analysis. The protein concentration was measured using Bio-Rad's *DC* Protein Assay. Absorbance measurements were carried out at 650 nm using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad). A standard calibration curve was constructed with bovine serum albumin (BSA).

In a parallel experiment, substrate topography was evaluated by AFM measurements before and after protein adsorption. Topography images (30 × 30 μm, 5 × 5 μm, and 1 × 1 μm) of the substrates under liquid conditions (incubation with DMEM and DMEM containing 10% vol/vol FBS) were taken in tapping mode with a silicon nitride tip (8.6 kHz, 0.08 N/m).

Cell culture experiments

Osteoblast-like MG-63 cells (American Type Culture Collection, Manassas, Virginia) were expanded to grow to 90% confluence in a 75-cm^2 flask in culture medium. The cells were maintained at 37°C and 5% CO_2. When cells reached the desired confluence they were detached with trypsin (Invitrogen) and seeded on the sterilized substrates (10,000 cells, 185 cells/mm^2 cell surface density) to test their adhesion and proliferation. The FlexiPERM (Greiner Bio-One GmbH, Frickenhausen, Germany) system was used to create multiple growth chambers and perform independent cell culture studies. MG-63 cells were in culture for a period of 7 days.

Evaluation of cell adhesion

Cell attachment to the substrate surfaces was evaluated at 1 and 3 hours after seeding by means of immunostaining and cell nuclei counting. MG-63 cells were fixed in a solution containing 3% paraformaldehyde in 0.1 M phosphate buffer and 60 mM sucrose for a period of 20 minutes at room temperature (21-23°C). Cells were washed twice in PBS-glycine (Gly) (dilution buffer) for 5 minutes. The cell membrane was then permeabilized with 0.1% Triton-X100 (Sigma-Aldrich) for 10 minutes at room temperature and washed in PBS-Gly (2 × 5 minutes). Afterward, samples were blocked with 1% (vol/vol) BSA in PBS-Gly, and the antibody for nucleus staining was added (Hoechst; Invitrogen). The samples were then diluted to 1:1000 in 1% (vol/vol) BSA in PBS-Gly and incubated at 37°C for 45 minutes. Samples were then mounted with mowiol before fluorescence imaging. Nuclei imaging was performed with a Nikon E1000 microscope (Nikon, Tokyo, Japan), and evaluated using *Image J* software.

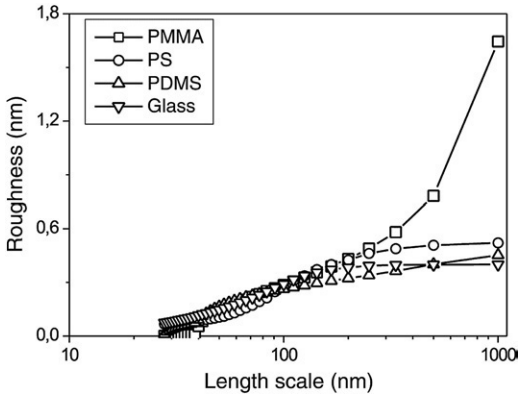

Figure 1. Surface roughness as a function of length scale, constructed from the power spectral density of 1 μm × 1 μm AFM images. All plots show the same roughness values for length scales ranging from 30 to 200 nm (corresponding to most protein sizes).

Measuring cell proliferation

Cell proliferation was evaluated using the WST test (Cell Proliferation Reagent WST-1; Roche Applied Science, Basel, Switzerland) at days 1, 3, and 7. Before the measurements the cell culture medium was removed and the WST reagent in a ratio 1:10 was added to complete culture medium, which had been prepared with DMEM without phenol red (Gibco/BRL Life Technologies). Cells were incubated for 1 hour at 37°C in a 5% CO_2 atmosphere. One hundred microliters of the dyed medium were placed into a microplate (tissue culture grade, 96 wells; NUNC, Roskilde, Denmark), and the absorbance was read at 450 nm in a spectrophotometer (ELX800 universal microplate reader; Bio-Tek Instruments, Winooski, Vermont).

Statistical analysis

All the results of this study presented here are the mean values and standard deviations of at least three independent experiments. Analysis of variance tests ($P < .05$) were performed using Microcal Origin 6.0 software, Northampton, Massachussetts.

Results

Surface roughness

The analysis of the AFM images showed that all the substrates had roughness values below 4 nm ($R_{RMS} < 4$ nm). PMMA and PS R_{RMS} values were 3.82 ± 1.33 and 1.21 ± 0.12 nm, whereas R_{RMS} values for glass and PDMS were lower (0.62 ± 0.38 and 0.58 ± 0.07 nm, respectively). The differences for surface roughness values between the different substrates were significant ($P < .05$) in all cases, except when comparing glass and PDMS. Because measurements for surface roughness depend on the length scale of observation, surface roughness was assessed by spectral analysis. Figure 1 plots surface roughness values as a function of an arbitrary length scale parameter with values from 10 to 1000 nm (also called spatial frequencies) constructed from the two-dimensional power spectral density of the topography images.[25] This spectral analysis illustrates the

Table 2
Mean contact angles (±SD) obtained on the pristine and sterilized (UV) substrates studied*

	PMMA		PS		PDMS		Glass	
	Pristine	UV	Pristine	UV	Pristine	UV	Pristine	UV
Water	83 ± 2	94 ± 1	91 ± 1	89 ± 1	107 ± 5	107 ± 1	35 ± 6	45 ± 4
Diiodomethane	41 ± 2	38 ± 1	27 ± 1	34 ± 2	67 ± 2	70 ± 1	52 ± 1	46 ± 2
Formamide	74 ± 3	80 ± 2	80 ± 3	73 ± 6	97 ± 2	103 ± 2	36 ± 5	30 ± 2

* Values in degrees.

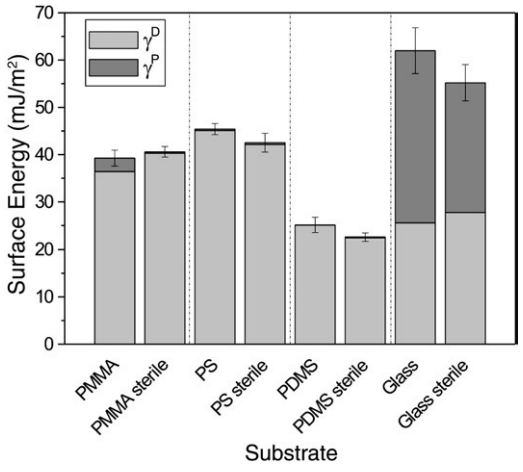

Figure 2. Total surface energy (total bar area), polar component (dark gray), and dispersive component (light gray) of the pristine and sterilized substrates.

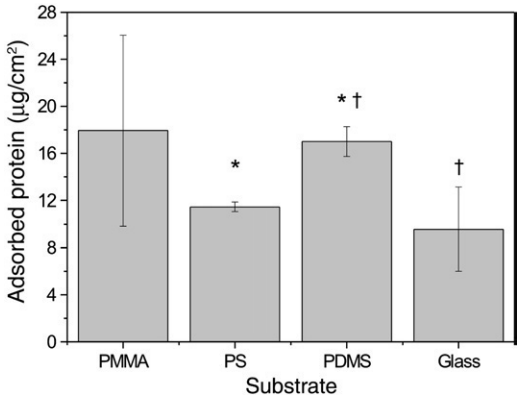

Figure 3. Amount of protein adsorbed (per unit area) for the studied substrates. Symbols ∗ and † indicate statistically significant differences ($P<.05$) between the signaled substrates.

increase in surface roughness as the observation scale increases. Roughness values below 0.6 nm were observed on all the substrates at length scale ranging from 10 nm to 200 nm, which includes most protein sizes. This analysis reveals that the substrates appear very similar for all protein sizes, and therefore as a parameter, surface roughness is not relevant to our protein adsorption measurements.

Table 3
Roughness root mean square (R_{RMS}) of the studied substrates in liquid DMEM before (DMEM) and after serum proteins were added (DMEM+10% FBS)

	PMMA	PS	PDMS	Glass
DMEM	2.60 ± 0.87	1.36 ± 0.14	0.91 ± 0.11	1.36 ± 0.04
DMEM+10% FBS	3.19 ± 0.53	1.56 ± 0.13	1.49 ± 0.85	1.02 ± 0.20

Contact angle

Results obtained from contact-angle measurements for the three tested liquids on the sterilized and nonsterilized substrates are shown in Table 2. For pristine nonsterilized samples the tested polymeric substrates (PMMA, PS, and PDMS) showed hydrophobic behavior (CA > 65 degrees),[26] whereas glass slides were hydrophilic. The same trend was observed after sterilization, although measurements showed that sterilization induced changes in surface wettability, which increases the hydrophobicity of PMMA and glass ($P<.05$). For PDMS and PS, changes were not significant. The results obtained with diiodomethane and formamide for the different substrates were used to calculate surface energy values (Table 2).

Surface energy

The Owens equation was used to calculate surface energy of the studied substrates. The values are shown in Figure 2 (values split into two components). The total surface energy of the polymeric substrates was lower than that obtained for glass slides (glass > PS > PMMA > PDMS). Based on the contact-angle measurements it could be expected that unsterilized polymeric substrates would show the main contribution to total surface energy due to the dispersive component ($\gamma^D \gg \gamma^P$), which was indeed the case. Actually, the dispersive component contributed approximately 90% of the total surface energy of PMMA and approximately 99% of that of PS and PDMS. In contrast, both dispersive and polar components ($\gamma^D \approx \gamma^P$) significantly contributed to total glass surface energy. After sterilization (Figure 2) the total surface energy values maintained the same trend as before (glass > PS > PMMA > PDMS). However, the polar component of the glass substrates decreased, which correlates with the increase in hydrophobicity observed in the contact-angle measurements. With regard to the polymeric substrates, PDMS and PS, there was an increase in the polar component of the surface energy but a decrease in total surface tension. In contrast, the total surface tension in PMMA increased, whereas the polar component decreased.

Protein adsorption

Figure 3 shows the amount of serum proteins adsorbed on the different substrates when incubated in cell culture media for 30

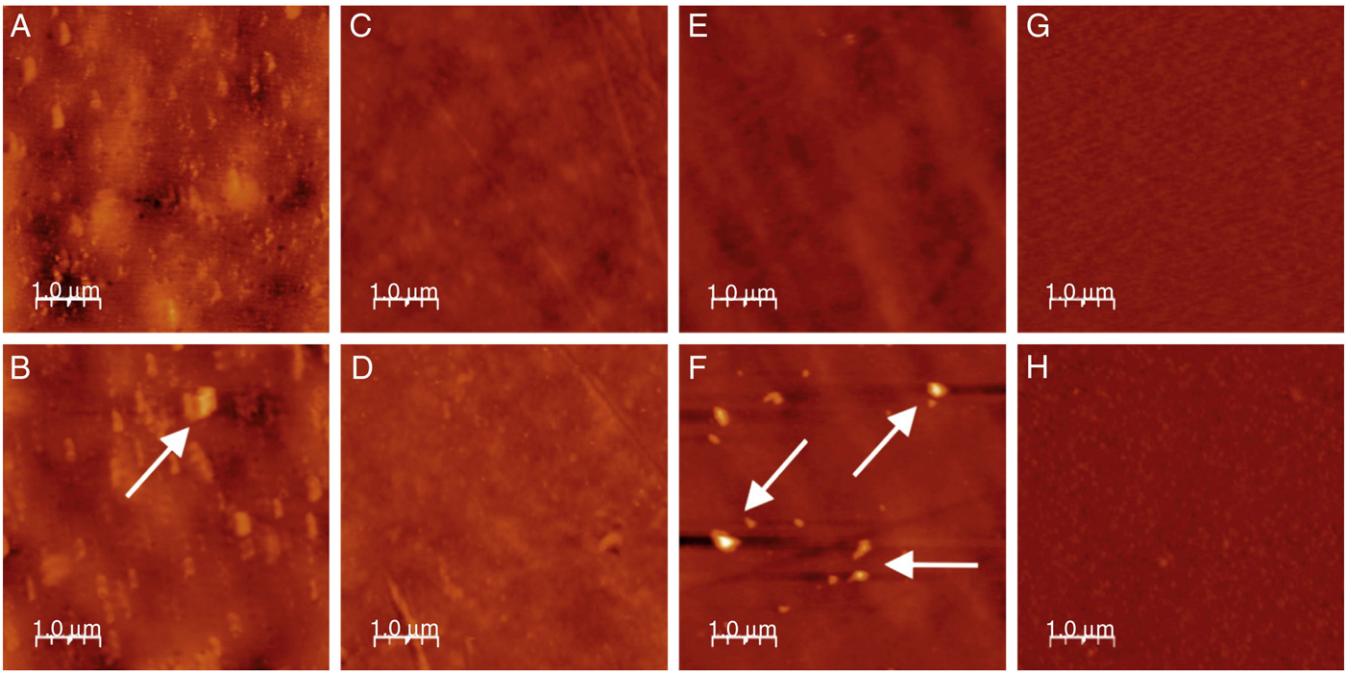

Figure 4. AFM topographic images of PMMA in DMEM before **(A)** and after **(B)** serum proteins were added; PS in DMEM medium before **(C)** and after **(D)** serum proteins were added, PDMS in DMEM medium before **(E)** and after **(F)** serum proteins were added, and glass in DMEM medium before **(C)** and after **(D)** serum proteins were added. *Arrows* in **B** and **F** indicate the presence of protein globular structures on the PMMA and PDMS surfaces after the addition of serum.

minutes. Protein adsorption values were higher for polymeric substrates than for glass (PMMA ≈ PDMS > PS > glass), although there were no significant differences between PS and glass. The highest mean values were obtained for PMMA (17.95 ± 8.11 μg/cm^2) and PDMS (17.00 ± 1.26 μg/cm^2), although the standard deviation in PMMA measurements was much higher. Mean values for protein adsorption on PS (11.47 ± 0.39 μg/cm^2) and glass (9.57 ± 3.57 μg/cm^2) surfaces were lower than with PDMS ($P < .05$).

There were no significant differences between the roughness values of the polymeric substrates before and after protein adsorption for large surface areas (>400 μm^2). However, AFM images for a smaller field of scanning (1 μm^2) showed that protein adsorption induced an increase in the local roughness values of polymers. In contrast, the surface roughness of glass decreased in the presence of serum (Table 3). AFM images (Figure 4) revealed large globular structures on PMMA and PDMS surfaces when serum was added. This was not true for glass (Figure 4, *G* and *H*) or PS (Figure 4, *C* and *D*). Therefore, these qualitative observations are consistent with the results obtained with the Bio-Rad *DC* Protein Assay.

Cell adhesion and proliferation

Figure 5, *A* shows the results of cell adhesion experiments performed on the studied substrates. After 3 hours of culture, the number of MG-63 cells attached to polymers was consistently lower than for glass ($P < .05$). Moreover, no significant differences were found between the values of adhered cells for PMMA, PS, and PDMS at 1 and 3 hours after seeding (at 1 hour, values obtained were 6.60% ± 2.60%, 8.15% ± 2.60%, and 10.49% ± 1.24% for PMMA, PS, and PDMS, respectively. After 3 hours results were 16.59% ± 1.38%, 16.41% ± 8.78%, and 25.65% ± 9.79% for PMMA, PS, and PDMS, respectively).

Measurements for MG-63 cell proliferation performed at days 1, 3, and 7 are shown in Figure 5, *B*. On the control surfaces (glass), cells proliferated with exponential growth until day 7. However, this was not the case for polymeric substrates. At day 1, no statistically significant differences were found for PMMA, PS, or PDMS, whereas at day 3 cells on PMMA started to proliferate linearly until day 7. The number of cells on PS remained constant from day 1 to 7. Finally, on PDMS, cell proliferation was minimal until day 7. A comparison of the results obtained from days 1 to 7 reveals that there were no statistically significant differences between the substrates at day 1. At day 3 cell proliferation on glass was higher than that observed on PDMS and PS ($P < .05$), whereas cell proliferation on PMMA was similar to that of the control.

Discussion

There were no statistically significant differences in surface energy before and after the sterilization process, which indicates that the sterilization process followed in this study does not induce any important changes in surface properties. However, although some changes were observed in contact-angle measurements (Table 2) as described above, the total surface energy of sterilized and of pristine substrates was very similar (Figure 2).

It is widely accepted that surface energy values of substrates have an important role in protein adsorption, cell adhesion, and

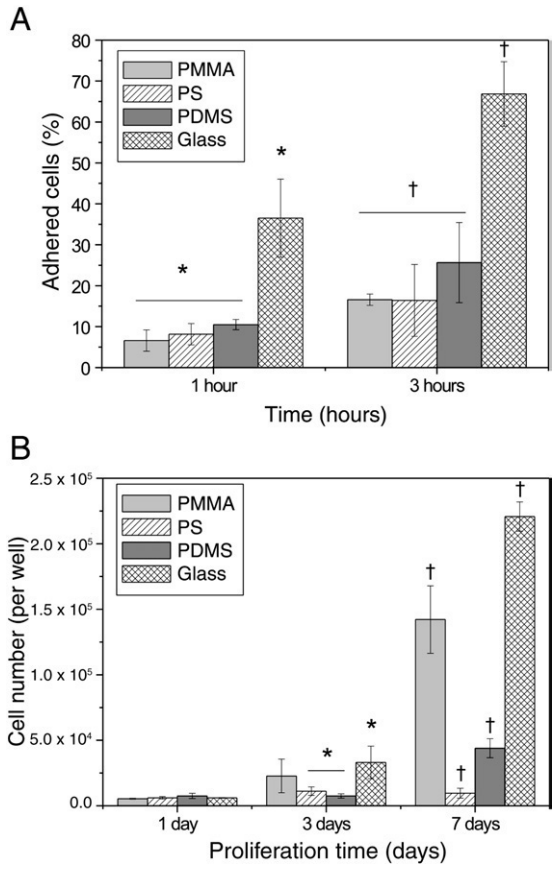

Figure 5. **(A)** Adhesion of MG-63 cells on the studied surfaces based on the number of cells seeded (10,000) at 1 and 3 hours, expressed as a percentage. **(B)** Proliferation curves for the different substrates. Symbols ∗ and † indicate statistically significant differences ($P < .05$) between the signaled substrates.

cell proliferation on biomaterials.[20,21,27,28] The role of surface topography in protein adsorption and cell behavior has also been widely studied[19,29,30]; in general, it is difficult to differentiate between the relative effect of topography and chemistry on protein adsorption and cell behavior.[20] In the present study, measurements for surface roughness revealed that differences for surface roughness values between the studied substrates were significant ($P < .05$) in all cases, except when comparing glass and PDMS. Moreover, when the evaluated substrates were studied in detail using spectral analysis, it was observed that their surface roughness was very similar at the protein length scale (Figure 1). Therefore, although topography could alter other physicochemical properties such as surface energy, we consider that differences in topography between the substrates are negligible in this study. Subsequently, the analysis of substrate behavior should focus on the role of surface energy in protein adsorption and thus, in cell behavior.

Consistent with other research,[12,21,31,32] we observed that protein adsorption was higher on hydrophobic substrates. However, this trend was not as apparent when comparing results for protein adsorption obtained from PS and glass. When the results for both wettability (typically hydrophobicity/hydrophilicity) and the computed values of the surface energies are considered, the discrepancy with PS and glass protein adsorption values disappears. In fact, a linear trend ($r = 0.98$) is observed in Figure 6, *A* which shows the amount of adsorbed protein versus the substrate surface energy values, therefore, highlighting that serum proteins adsorb preferentially on low-energy surfaces. However, when considering the different components of the surface energy, no correlation was found. This result could be explained taking into account that some proteins, such as albumin, preferentially adsorb on substrates with surface energies that have a high polar component, whereas other ones, such as fibronectin, do not share this trend.[20,33] Therefore, when working with protein mixtures (such as serum) it is difficult to determine such protein-dependent effects.

The correlation of adhesion and proliferation experiments with both the amount of adsorbed proteins (Figure 6, *D*) and the surface energy values (Figure 6, *C*) was not straightforward. However, consistent with Redey et al,[34] our results showed low cell adhesion on surfaces on which the polar component of the surface energy was low (polymeric substrates). In contrast, cell adhesion was high on substrates with a high polar component (glass slides) (Figure 6, *B*). This could be explained in two ways: first, contrary to most research found in the literature,[8,33] our experimental set-up incorporated the measurements of protein adsorption on cell culture conditions, which implied the use of serum proteins without purification. Although this makes conclusions more difficult to extract, it replicates what happens with the material in vitro. Serum contains many kinds of proteins (adhesive and nonadhesive) of varying sizes, all of which compete for surface adsorption. This could explain why the adsorbed proteins do not directly correlate with cell adhesion.

Second, some authors have reported conformational changes during protein adsorption on hydrophobic surfaces,[12,35-37] which could influence protein functionality. In aqueous environments most proteins, either in solution or linked to a surface, present their polar groups to their surroundings and their apolar groups to their cores. In the adsorption process, both the adsorbate and the surface adsorbent must dehydrate (at least partially). This process is energetically favorable when both the substrate and the adsorbate are hydrophobic, because it increases water entropy.[35] Close to a hydrophobic surface, some proteins may undergo conformational changes, which expose their apolar groups to the surface, thus making them hydrophobic at that particular interface, while the polar groups are exposed at the protein–water interface.[12] This leads to an energetically favorable adsorption process but causes the adsorbed protein to show a denatured conformation.

AFM images of PMMA and PDMS surfaces obtained after the serum proteins were added to the medium (Figure 4, *B* and *D*) showed globular structures ranging in diameter from 50 to 100 nm, which can be related to aggregates of denatured proteins as described in the literature.[12] AFM images obtained for glass under the same conditions did not reveal any large structures (Figure 4, *H*). Moreover, glass surface roughness after serum addition was lower than it had been in the whole length scale (25 nm up to 25 μm). These data indicate differences in the protein adsorption process between polymeric substrates and glass. The denatured conformation of adsorbed proteins is often related to the loss of their bioactivity. Adsorption studies of fibronectin (a high-molecular weight extracellular matrix glycoprotein that

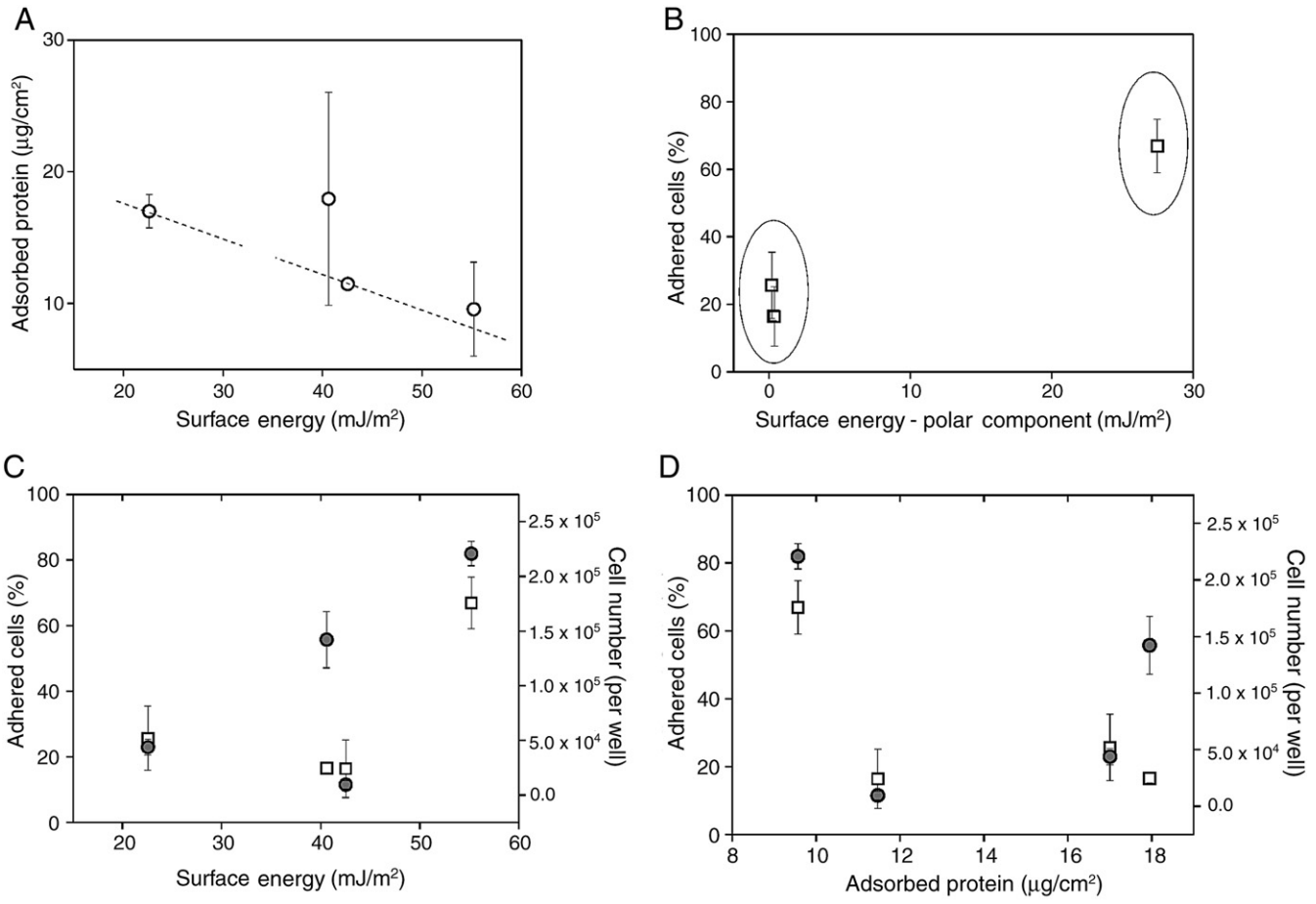

Figure 6. **(A)** The amount of protein adsorbed as a function of the surface energy values. Data were fitted with a linear relation ($r = 0.98$). **(B)** Percentage of adhered cells as a function of the polar component value of the surface energy. **(C)** Percentage of adhered cells at 3 hours (□) and cell number at 7 days (●) as a function of the surface energy. **(D)** Percentage of adhered cells at 3 hours (□) and cell number at 7 days (●) as a function of the amount of protein adsorbed.

binds to membrane receptor proteins) have shown that fibronectin adsorbed on hydrophobic surfaces loses its ability to bind cell membrane proteins, even though adsorption is a requirement for the protein to become active.[35]

In the present study results showed that PMMA performed better than PDMS and PS in osteoblast proliferation assays, although PMMA is also a hydrophobic substrate. It was also observed that the number of cells on PMMA after 1 week of culture was similar to that observed for glass, whereas proliferation rates for PDMS and PS were much lower. These results are consistent with other biocompatibility studies of polymeric surfaces. Peterson et al found that hydrophobic PDMS was inhospitable to cell growth.[9] Studies of fibronectin adsorption on PDMS have shown that although protein adsorption was high on hydrophobic PDMS, poor cell attachment and proliferation were reported. These results can therefore be related to the results for fibronectin conformation.[8] The behavior of PMMA reported in the present study also corresponds with the work of Stangegaard et al, in which the whole-genome expression of human carcinoma cells was analyzed, showing that cells grown on PMMA presented no change in gene expression compared to cells grown in cell culture flasks.[38] PMMA proliferation results could be explained not only in terms of the surface energy of the substrate, but also by the amount of adsorbed protein and its conformation.

In conclusion, our measurements have shown that the sterilization process carried out in this research does not significantly modify surface parameters such as wettability and surface energy for the polymers tested. Moreover, the contribution of surface roughness to protein adsorption has not proved to be a relevant parameter for the substrates tested; because, at the size range of the proteins, the spectral analysis performed did not reveal any significant differences among the substrates. However, the results of this study have revealed a correlation between the amount of protein adsorbed and the total surface energy of the substrate, in that adsorption is higher on low energy substrates. Cell adhesion and proliferation tests revealed that although the amount of protein adsorbed cannot be directly correlated to cell behavior, a relationship between cell adhesion and the polar component of the surface energy could exist. In fact, AFM images revealed differences in serum absorbed proteins together with the presence of large globular structures on the PMMA and PDMS surfaces. Overall, the analysis of the different parameters led us to conclude that PMMA performs better than PDMS and PS in cell proliferation experiments. This fact, together with the previously developed methods for PMMA

moulding, milling, and micro- and nanofabrication, makes PMMA an excellent choice for the fabrication of lab-on-chip, biosensors, and tissue engineering applications.

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