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Biocompatibility Evaluation of Glycolide-Containing Polyesters in Contact with Osteoblasts and Fibroblasts

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ABSTRACT: Biodegradable aliphatic polyesters have numerous biomedical applications and their capacity to degrade in biological fluids provides the significant advantage of their removal. Three glycolide-containing aliphatic polyesters: a copolymer of glycolide and L-lactide (PGLA), a terpolymer of glycolide, L-lactide and ε-caprolactone (PGLCap) and a copolymer of glycolide, and ε-caprolactone (PGCap) were tested to evaluate their biocompatibility towards osteoblasts and fibroblasts. Each of the polymer units was previously reported to have acceptable biological properties and good biodegradability, and PGLA is already used for biomedical applications. Here we report that both PGLCap and PGCap affected cell adherence, and compromised cell viability as estimated by flow cytometric analyses of apoptotic and necrotic cells. The two polymers enhanced also production of numerous inflammation-related factors: nitric oxide, matrix metalloproteinases (MMP-2 and MMP-9), and cytokines, including pro-inflammatory TNF-α, IL-1β, IL-6, and chemokines (IL-8 or MCP-1) attracting leukocytes. The effects of PGLCap and PGCap were similar despite the fact that they possess different characteristics: amorphous/smooth surface and semicristalline/rough surface, respectively. However, their common feature, distinctive from PGLA, is a presence of ε-caprolactone units in their structure. This compound is considered to be acceptably biocompatible but our data suggest that its copolymerization with glycolide and L-lactide does not provide satisfactory biocompatibility towards osteoblasts and fibroblasts. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: poly(glycolide-*co*-L-lactide); poly(glycolide-*co*-ε-caprolactone); poly(glycolide-*co*-L-lactide-*co*-ε-caprolactone); MG-63; L-9292; adhesion/proliferation; cytokines; MMP-9

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INTRODUCTION

Age or injury-dependent tissue loss requires implant materials to fill the gap. One of the most affected tissues, which functioning must be regained in such situations for normal limb performance is the bone. One possibility is an autogenous bone grafting when a bone fragments are translocated within the body of a patient.[1,2] However, this is not always possible and a degree of complication can reach up to 30%.[3] Alternatively, synthetic bone scaffolds can be used but their grafting is not meant to permanently replace the bone, instead the biomaterials are intended to provide temporary structural three-dimensional (3D) support for tissues, and foremost to stimulate natural bone growth.[3] This demands certain characteristics of the implants such as good mechanical properties but the materials should also fulfill physico–biological requirements: be porous to allow for bone tissue ingrowth, biodegrade with time, and not be toxic for the body cells (biocompatible).[4]

Among several types of polymeric biomaterials that were produced and tested till now polyesters are probably the most common and they also include biodegradable aliphatic (linear) polyesters.[5–7] For example, the widely known aliphatic polyester, namely poly-glycolide was used to develop the first synthetic absorbable suture, which entered the medical market in 1962 under the trade name Dexon.[5] In our studies, we synthesized three glycolide-based materials i.e., a copolymer of glycolide and L-lactide (PGLA) (15/85; PGLA), a terpolymer of glycolide, L-lactide and ε-caprolactone (10/70/20; PGLCap), and a copolymer of glycolide and ε-caprolactone (10/90; PGCap).[8,9] Each of their components was tested before and was shown to possess

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acceptable biodegradability and biocompatibility.[3,5] Poly(L-lactide) is derived from the ring-opening polymerization of L-lactide (cyclic di-ester of 2-hydroxypropionic acid) and is one of the most commonly tested and used components of polymers while glycolide is a thermoplastic polymer and the simplest of aliphatic polyesters.[5] Polymers issued from L-lactide and glycolide are used worldwide as bioresorbable devices in surgery and in pharmacology.[10] The last component, ε-caprolactone, is a semicrystalline polymer having a very low glass transition temperature.[11]

We synthesized PGLA, PGLCap, and PGCap and described previously in detail their physicochemical characteristics[8,9,12] but our current aim was to test their mechanical properties and biocompatibility towards cells, which would be in their closest proximity upon *in vivo* implantation. These include the bone cells–osteoblasts, and fibroblast, one of the most abounded cells in the body. There are three major cell populations of the bone: osteoblasts, osteocytes, and osteoclasts.[13] Extracellular matrix (ECM)-producing osteoblasts originate from mesenchymal stem cells and subsequently differentiate into osteocytes, the cells located within the bone matrix, composing majority of the cells in the adult bone.[13] To maintain bone homeostasis, a part from the above bone forming cells, there are also bone resorbing osteoclasts originating from a monocyte/macrophage precursor.[14]

Fibroblasts are a class of cells sharing surface similarities with regard to their morphologies and biosynthetic activities. There are multiple subsets/phenotypic types of fibroblasts because of connective tissue specialization and anatomical localization.[15] Moreover, fibroblast-like cells of mesenchymal origin were identified among circulating mononuclear blood cells and termed fibrocytes.[16] The ECM molecules synthesized by fibroblasts form stroma but the role of fibroblasts extends their structural functions as they participate in the initiation of the body response to injury or foreign body incursion. Additionally, fibroblasts participate in this process by proliferating within injured sites contributing to scar formation and the long-term remodeling of damaged tissue.[17]

The aim of the current study was to evaluate effects of the three polymers composed of a common pool of monomeric units (glycolide, L-lactide, and ε-caprolactone) in different configurations on two different types of cells—the bone forming osteoblasts and the ECM forming fibroblasts. Building these monomeric units into larger macromolecular frameworks might provide further control over polymer characteristics and launch new applications. A PGLA was previously tested for its biocompatibility on different types of cells and in most cases it was found to have satisfactory tolerability (e.g., as given in Ref. [18]). Thus, our main aim was to test if its modifications, PGLCap and PGCap would have improved properties in comparison to PGLA.

For this we evaluated the cell adhesion/proliferation to material surface, activation of free radical nitric oxide synthesis, production of pro-inflammatory mediators: cytokines (pro-inflammatory TNF-α, IL-1β, IFN-γ, IL-6, IL-12p70, and anti-inflammatory IL-10), chemokines (IL-8 or MCP-1), and matrix metalloproteinases (MMP-2 and MMP-9) degrading ECM. Our results revealed that the least cytotoxic and significantly biocompatible turned out to be a PGLA while PGLCap (a terpolymer of glycolide, L-lactide, and ε-caprolactone) and PGCap (a copolymer of glycolide and ε-caprolactone) activated both fibroblasts and osteoblasts in a proinflammatory manner and significantly up-regulated a rate of their apoptotic death.

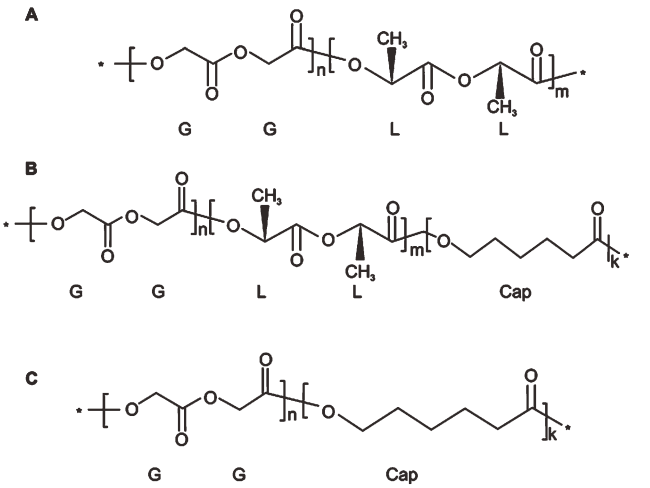

Figure 1. Chemical structure of (A) poly(glycolide-*co*-L-lactide) (PGLA), (B) poly(glycolide-*co*-L-lactide-*co*-ε-caprolactone) (PGLCap), and (C) poly(glycolide-*co*-ε-caprolactone) (PGCap).

EXPERIMENTAL

Synthesis, Processing, and Characterization of Polymers

A PGLA (15 : 85), a terpolymer of glycolide, L-lactide, and ε-caprolactone (PGLCap 10 : 70 : 20), and a copolymer of glycolide, and ε-caprolactone (PGCap, 10 : 90) were synthesized with a low-toxic zirconium (IV) acetylacetonate ($Zr(acac)_4$) as an initiator. Chemical structure of the polymers is presented in Figure 1. Synthesis of the polymers was previously described in detail.[8,12] Briefly, it was performed in bulk by ring opening polymerization of corresponding cyclic monomers, e.g., L-lactide (Purasorb L, Purac, The Netherlands), glycolide (Purasorb G, Purac, The Netherlands), or ε-caprolactone (Fluka, Germany) in the presence of the Zr(acac)4 initiator (Sigma-Aldrich, Germany)—at a molar ratio of 1.25×10^{-3} at 100°C by a conventional method using a vacuum line for degassing and sealing of the ampoules.

The foils were cast from 10% (w/v) polymer solution in methylene chloride (POCh, Gliwice, Poland) on glass petri dishes, followed by air drying for 24 h and vacuum drying for the next 72 h. Then, the foils were rinsed with ultra high purity water (UHQ-water of the resistivity of 18.2 MΩcm, produced by Purelab UHQ, Elga, UK) for 12 h. UHQ-water was exchanged for six times. Afterwards, the foils were air and vacuum dried for 24 and 72 h, respectively. The resulting foils had a thickness of 0.18 mm. For all experiments the bottom surface of the foils, e.g., contacting glass during preparation process was used.

Tensile testing of the polymeric foils was conducted with universal testing machine (Zwick 1435, Germany). The test speed

was 100 mm/min with 0.1*N* preload, specimen length of 40 mm, and specimen width of 5 mm. Six samples originating from each type of the polymeric foils were tested.

The detailed characteristics of the materials were published previously and can be found in Ref. 8. They included: composition of copolymers, molecular weights, thermal properties [glass-transition temperature (T_g), and melting temperature (T_m)], surface chemical composition, contact angles, surface free energies (SFEs), as well as topography, and average roughness (R_a) evaluations by atomic force microscopy.

Cell Cultures

The murine fibroblast L-929 and human osteoblast MG-63 cell lines were used in the studies. The cells were cultured in 75 mL plastic bottles (Nunc, Denmark) in DMEM culture medium enriched with glucose, L-Glutamine (PAA, Austria), 10% fetal bovine serum (PAA, Austria) and 5% antibiotic solution containing penicillin 10 UI/mL and streptomycin 10 ηg/mL (PAA, Austria). The cells were cultured in the incubator (Nuaire, MN) at 37°C and 5% of CO_2. Every 2–3 days, when the cells were forming high confluence monolayers, the cell cultures were passaged by trypsinization (0.25% solution of trypsin; Sigma-Aldrich, Germany).

In Vitro Cell-Biomaterial Studies

For cell culture studies the polymeric foils were washed in 70% ethanol, sterilized with UV irradiation (45 min for each side) and placed at the bottom of 24-well dishes (Nunc, Denmark). The cells that were harvested after 7–10 passages were counted in Bürker's hemocytometer, diluted to 3×10^4 cell/mL, and placed in the wells of 24-well culture dishes (Nunc, Denmark) containing discs of the tested biomaterials. A PGLA was used as a control material (CTR). However, in some studies we also used TCPS (tissue culture polystyrene) as a control for PGLA biocompatibility (Figure 3). In such conditions the cells were cultured for 3 or 5 days. Subsequently, morphology of cells adhering to the polymeric foils was observed under an inverted microscope (Jenamed, Germany) and it was further verified by the crystal violet (CV) staining test. Alternatively, the ratio of apoptotic and/or necrotic, cells was estimated. The supernatants were collected and frozen at −20°C prior to further analyze the inflammatory mediator content.

Cell Adherence and Proliferation

The ability of the cells to adhere to polymeric surfaces was tested using the CV test. The cells adhering to the foils or CTR were fixed with 2% paraformaldehyde for 1 h, and then stained with CV (CV 0.5% in 20% methanol for 5 min). After that time wells were washed with water and their content was transferred to a new 24-well culture plate. After drying, the absorbed dye was extracted by addition of 1 mL of 100% methanol (POCh, Gliwice, Poland). The optical density (OD) was measured at 570 nm with the Expert Plus spectrophotometer (Asys Hitach, Austria). Since, the polymers absorb some CV, additional controls containing polymeric foils, and cell-free medium were run. The OD results from these controls were subtracted from the experimental data.

Cell Apoptosis and Necrosis

Apoptotic cells were identified quantitatively by Annexin V-PE Apotosis Detection Kit I (BD Pharmingen, CA) that enables cell staining with Annexin V and 7-Amino-actinomycin (7-AAD). Annexin V binds to phosphatidylserine exposed on the outer leaflet of the plasma membrane of apoptotically dying cells, while 7-AAD is a vital dye that enters any dead cells. The cells detached by trypsinization from polymers were stained with the kit according to the established protocol.[8,19] Briefly, the cells were washed twice with cold PBS and resuspended in binding buffer (0.1*M* HEPES/NaOH (pH 7.4), 1.4*M* NaCl, 25 m*M* $CaCl_2$). Then 2.5 μL of Annexin V-PE and 2.5 μL of 7-AAD was added to the cells (10^5 cells/100 μL binding buffer) and incubated for 15 min at room temperature in the dark. Measurements were performed by a flow cytometer using CellQuest software (FACS Caliber; Becton Dickinson). The Annexin V-PE signal was measured in FL-2 and the 7-AAD in FL-3 channels. Dying cells were distinguished on the basis of the FL-2/FL-3 signals: early apoptotic Annexin $V^+/7\text{-}AAD^-$; late apoptotic Annexin $V^+/7\text{-}AAD^+$; necrotic cells Annexin $V^-/7\text{-}AAD^+$; alive cells Annexin $V^-/7\text{-}AAD^-$.

Inflammatory Mediator Synthesis/Release

Determination of Nitrite/Nitrate. The total amount of NO (NO_2^- and NO_3^-) was measured as described previously.[20] Briefly, nitrate was reduced to nitrite by addition of nitrate reductase, FAD, and NADPH (all from Sigma-Aldrich, Germany) and then NADPH was oxidized by lactate dehydrogenase in presence of sodium pyruvate (both are from Sigma-Aldrich, Germany). Finally, nitrite concentration in the samples was measured by the Griess reaction, by adding Griess reagents (0.1% naphthalethylenediamine dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_3PO_4; 1 : 1 vol/vol) in a ratio 1 : 1 to samples and standards. Sodium nitrite solution was used as an internal control for the Griess assay and $NaNO_3$ (both from Sigma-Aldrich, Germany) as controls for reduction step. The NO levels were measured at 540 nm with a Expert Plus spectrophotometer (Asys Hitech, Eugendorf, Austria).

Determination of Protein Concentration. Protein concentration in the supernatants collected from cell cultures was measured by the colorimetric BCA method. A mixture of copper (II) sulfate solution (CS, Sigma-Aldrich, Germany) and bicinchoninic acid solution (BCA; Sigma, Germany) in ratio 1 : 50 was firstly prepared. Subsequently, 10 μL of each tested sample was transferred to wells of a 96-well plate and then 200 μL of the CS/BCA mixture was added. The plates were incubated for 30 min in the dark. The OD was measured at 570 nm with Expert Plus spectrophotometer (Asys Hitach, Austria).

MMP-9 and MMP-2 Gelatinolytic Activity: Gelatin Zymography. Zymography was performed as described earlier.[21] Briefly, samples of supernatant were normalized for protein concentration. Then the exudates were electrophoresed in 10% SDS-polyacrylamide gels, containing 1% porcine gelatin, (Sigma-Aldrich, Germany) with nonreducing conditions. The gels were washed twice in 2.5% Triton X-100 (15 min each) and developed overnight at 37°C in incubation buffer (50 m*M* Tris-HCl, pH 8.0, 5 m*M* $CaCl_2$, 0.02% NaN_3, 1 μM $ZnCl_2$). The gels

were fixed and stained with 0.5% Coomassie brilliant blue (Sigma-Aldrich, Germany) in acetic acid/isopropanol/distilled water 1 : 3 : 6, and then washed in equilibrating solution with 40% methanol, 10% acetic acid, and 3% glycerol (all from Sigma-Aldrich, Germany). Protein bands with gelatinolytic activity appeared as clear lysis zones within the blue background of the gelatin gel. The degradation of gelatin was visualized under long wave UV light. A prestained broad range molecular weight standard (Bio-Rad, CA) was used. Densitometric analysis of protein bands was performed through use of the UVISoft-UVIMap program (UVItec, UK).

Determination of Cytokines/Chemokines by Cytometric Bead Array (CBA). Cytometric Bead Array sets (Mouse Inflammation Kit and Human Inflammatory Cytokines Kit, CBA; BD Biosciences, CA) were used to study cytokines and chemokines in supernatants as described earlier.[20] A mouse inflammation kit simultaneously detects mouse IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 and the human kit IL-8, IL-1β, IL-6, IL-10, TNF-α, IL-12p70. Both kits were used according to the manufacturer's instructions. Briefly, a mixture of six capture bead populations (50 μL) with distinct fluorescence intensities (detected in FL3) coated with antibodies specific for the above cytokines/chemokines was mixed with each sample/standard (50 μL). Additionally, PE-conjugated detection antibodies (detected in FL-2; 50 μL) were added to form sandwich complexes. After 3 h incubation (Human Inflammatory Cytokines Kit, CBA; BD Biosciences) or 2 h incubation (Mouse Inflammation Kit CBA; BD Biosciences) in dark the samples were washed once (200 g, 5 min) and resuspended in 300 μL of wash buffer before acquisition on a FACScan cytometer (FACSCalibur flow cytometer, Becton Dickinson, NJ). Following acquisition of data by two-color cytometric analysis, the sample results were analyzed using CBA software (BD Biosciences). Standard curves were generated for each cytokine using the mixed cytokine/chemokine standard provided by the kit. The concentration for each cytokine in cell supernatants was determined by interpolation from the corresponding standard curve.

Statistical Analysis

Results are expressed as mean ± SE. Statistical significance was determined by one way analysis of variance (ANOVA) followed by a *post hoc T*-Tukey test and the differences were regarded as significant at $P < 0.05$. At the beginning of each experiment the cell numbers were the same (3×10^4/mL) in each well. However, upon incubation of cells with some of the tested polymers their numbers significantly changed in time and this is reflected by changes in numbers of adhering/proliferating cells [Figure 4(A,B)] as fibroblasts and osteoblasts must adhere in order to survive.[22] Therefore, the levels of released inflammatory mediators might be a direct consequence of changes in the cell numbers, thus all data were recalculated to cell adherence results (parameter/cell adherence). On the figures the letter-code is used to show statistically significant differences according to ANOVA. The letters (a, b, c etc.) present the symbolic way to express statistical significance and are randomly chosen. The letter-code should be understood as follow: the values that are significantly different according to ANOVA are marked with different letters (e.g., "a" and "b") while the values sharing the same letter (e.g., "a" and "ab", "a" and "a") are similar i.e., not statistically different. Differences between day 3 and 5 were estimated by the student *t*-test were $P < 0.05$.

RESULTS AND DISCUSSION

Significant advantages have been made in the development, characteristics, and testing of biodegradable polymers in the past three decades. As the result, the polymers were designated as preferred biomaterials for developing devices for temporary implants, 3D porous structures (scaffolds) for tissue engineering but also drug delivery vehicles.[23] The biodegradable polymers were shown not only to provide 3D frame for cell growth and ECM formation but also offer a wide range of physical properties and degradation rates that make them suitable as biocompatible and noncytotoxic biomaterials.[24]

Physico-Chemical and Mechanical Characteristics of the Tested Polymers

The polymers used in our studies are composed of either two or three homopolymer units of L-lactide, glycolide, and ε-caprolactone. Poly-L-lactide is a naturally occurring crystalline (~37% of crystallinity) isomer of the lactic acid with a glass transition temperature of 60–65°C and a melting temperature of ~175°C, and with a good tensile strength, low extension, and a high modulus (4.8 GPa).[25] Moreover, poly(L-lactide) is a slowly degrading polymer (2–6.5 years *in vivo*), which degradation occurs through the bulk erosion into lactic acid, a natural metabolite, via the Krebs cycle.[25,26]

Poly-glycolide is a highly crystalline polymer (44–55% of crystallinity) thus with a high tensile modulus leading to excellent fiber forming ability but very low solubility in organic solvents.[23] Its glass transition temperature ranges from 35 to 40°C and a melting temperature is greater than 200°C. The high crystallinity assures its good initial mechanical qualities, e.g., it is stiffer than any other degradable biomedical polymer and has a modulus of ~12.5 GPa.[27] Similarly to poly(L-lactide), poly-glycolide is a bulk degrading polymer but its degradation (to glycolic acid removed via the Krebs cycle) is much quicker: the polymer losses its strength in 1–2 months and its mass within 6–12 months.[23] Therefore, together with its poor solubility the above features somehow limit its biomedical application. However, copolymers of glycolide and L-lactide might overcome the problems. In fact, PGLA has already been tested in multiple systems and it was shown that in the composition rate of 85/15 (the same as used in our studies) the polymer's rate of degradation is 5–6 months.[25]

In contrast to the above polymers, poly-ε-caprolactone is a semicrystalline polyester, soluble in a wide range of organic solvents, with a low melting point (55–60°C) and a glass-transition temperature (−60°C).[23] Poly-ε-caprolactone has a low tensile strength (~23 MPa) but an extremely high elongation at breakage (−700%).[28] Its degradation is rather slow and takes about 2–3 years and for this is rather used as a drug delivery vehicle.

Each of the homopolymers possesses unique valuable characteristics as well as disadvantages. This led us to synthesize their co- and terpolymers, which detailed physical, chemical, and surface properties are described elsewhere.[8,9] Briefly, although the

Table I. Mechanical Properties of Poly(glycolide-*co*-L-lactide) (PGLA), poly(glycolide-*co*-L-lactide-*co*-ε-caprolactone) (PGLCap), and poly(glycolide-*co*-ε-caprolactone) (PGCap)

Polymer	σ (MPa)	*E* (MPa)	ε (%)
PGLA	74.5 ± 2.7	2910 ± 100	3.2 ± 0.1
PGLCap	22.8 ± 1.4	560 ± 40	275 ± 21
PGCap	10.7 ± 0.2	285 ± 7	14.5 ± 1.1

σ: tensile strength; *E*: Young's modulus; ε: total elongation at break; $n = 6$, means ± SE; statistically significant differences in σ, *E*, and ε were found for all polymers at $P < 0.05$.

polymers have different chemical structure (Figure 1) they have similar number-average molecular weight (PGLA: 105 kDa, PGCap: 78 kDa, and PGLCap: 91 kDa) and polydispersion index (1.9–2.1). PGLA and PGLCap turned out to be amorphous with glass transition temperatures (T_g) at 57.7°C and 29.0°C, respectively. On the other hand, PGCap is semicrystalline with $T_g = -60$°C and $T_m = 54.0$°C. Analysis of surface composition revealed that the highest molar ratio of oxygen to carbon was detected on PGLA (0.72) and PGLCap (0.61), while the lowest on PGCap (0.37). It corresponds with the values of surface free-energy and water contact angle: PGCap has the lowest polar part of surface free energy (1.8 mJ/m^2) and the highest water contact angle (81.5°) among all polymers used in this experiment. The atomic force microscopy evaluations revealed that the apparently amorphous polymers (PGLA and PGLCap) are quite smooth with the average roughness R_a ~10 nm, while semicrystalline PGCap is more rough (R_a ~100 nm) and more textured.[8,9]

In this study, mechanical properties of the polymeric foils in tensile test were evaluated. The results show that PGLA foil has the best mechanical properties, the highest strength, and Young's modulus, but the lowest elongation at break (Table I). Strength–strain curve shows that PLGA is an elastic-brittle material (Figure 2). PGLCap, because of its lower T_g, has viscoelastic properties and its elongation at break is very high (up to 300%); it has also much lower tensile strength and Young's modulus as compared to PGLA (Figure 2, Table I). PGCap has the lowest strength and Young's modulus and intermediate elongation at break, which result from presence in its structure of both amorphous and crystalline regions.

Biological Impact of the Tested Polymers

Among the three polymers tested by us, PGLA biocompatibility was examined previously in numerous studies. In general PGLA is considered as a noncytotoxic and low-immunogenic polymer (for details see Refs. 18, 29, and 30). However, we wanted to confirm the biocompatibility of PGLA in our system and for this we performed analyses comparing all tested parameters between PGLA and TCPS (tissue culture polystyrene) that is a standard and most commonly used *in vitro* cell culture surface (representative results are shown in Figure 3). The data clearly show that all the parameters, but one, were either unaltered (e.g., TNF-α and MMP-9 levels on day 3) or even improved (e.g., better adhesion/increased proliferation, and chemokine release by either cell population) in the presence of PGLA (Figure 3). The only parameter that was affected by this polymer was a ratio of apoptosis as more osteoblasts and fibroblasts were entering the apoptotic pathway (early apoptosis) (Figure 3); however, at the same time the numbers of necrotic cells were not increased (not shown). This suggests that PGLA does not significantly activate the cells since production/release of proinflammatory mediators is not enhanced. And thus, the main aim of the current study was to test if PGLA modifications, PGLCap, and PGCap would have improved biological properties as compared to PGLA.

The first tested parameter was cell adhesion/proliferation because it is an important aspect of cell interaction with a polymeric material and the implant must integrate into the adjacent tissue(s).[22] Moreover, the adhesion of anchorage-dependent cells such as osteoblasts and fibroblasts, is a prerequisite for the subsequent successful proliferation of cells. There are two major phases of cell adhesion to the biomaterial surface (i) the attachment phase that is short-term and involves physico–chemical bonds (e.g., ionic and van der Walls forces), and (ii) the adhesion phase, longer, that requires biological molecules: ECM elements, and adhesion and cytoskeleton molecules etc.[22] In our studies, we observed that the presence of PGLCap and PGCap very strongly limited cell (osteoblasts and fibroblasts) adherence/proliferation in comparison to PGLA on either day of incubation [Figure 4(A,B)]. Evaluation of the cause of the inadequate cell adherence to any biomaterial is challenging as it is difficult to assess empirically, which comes first, the inappropriate cell attachment that leads to cell mortality or the lack of attachment results from cell death. However, in the case of PGCap we also detected higher ratio of osteoblast apoptosis on day 5 of culture [Figure 4(C)]. In contrast, PGLCap had only minor effect of fibroblast apoptosis [more "late" than "early" apoptotic cells were detected on day 5; Figure 4(D)], though the polymer increased numbers of necrotic cells on day 3 [Figure 4(H)]. Therefore, both PGCap and PGLCap, showed some degree of cytotoxicity. The apoptotic, genetically controlled, cell death is an important component of tissue morphogenesis and a cell life-span regulator, and e.g., *in vivo* 50–70% of osteoblasts undergo apoptosis.[31] However, if the ratio of apoptotic cells

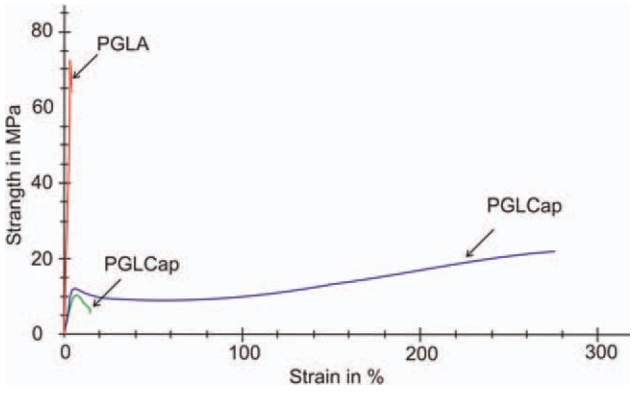

Figure 2. Representative tensile test curves of (A) poly(glycolide-*co*-L-lactide) (PGLA), (B) poly(glycolide-*co*-L-lactide-*co*-ε-caprolactone) (PGLCap), and (C) poly(glycolide-*co*-ε-caprolactone) (PGCap). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.].

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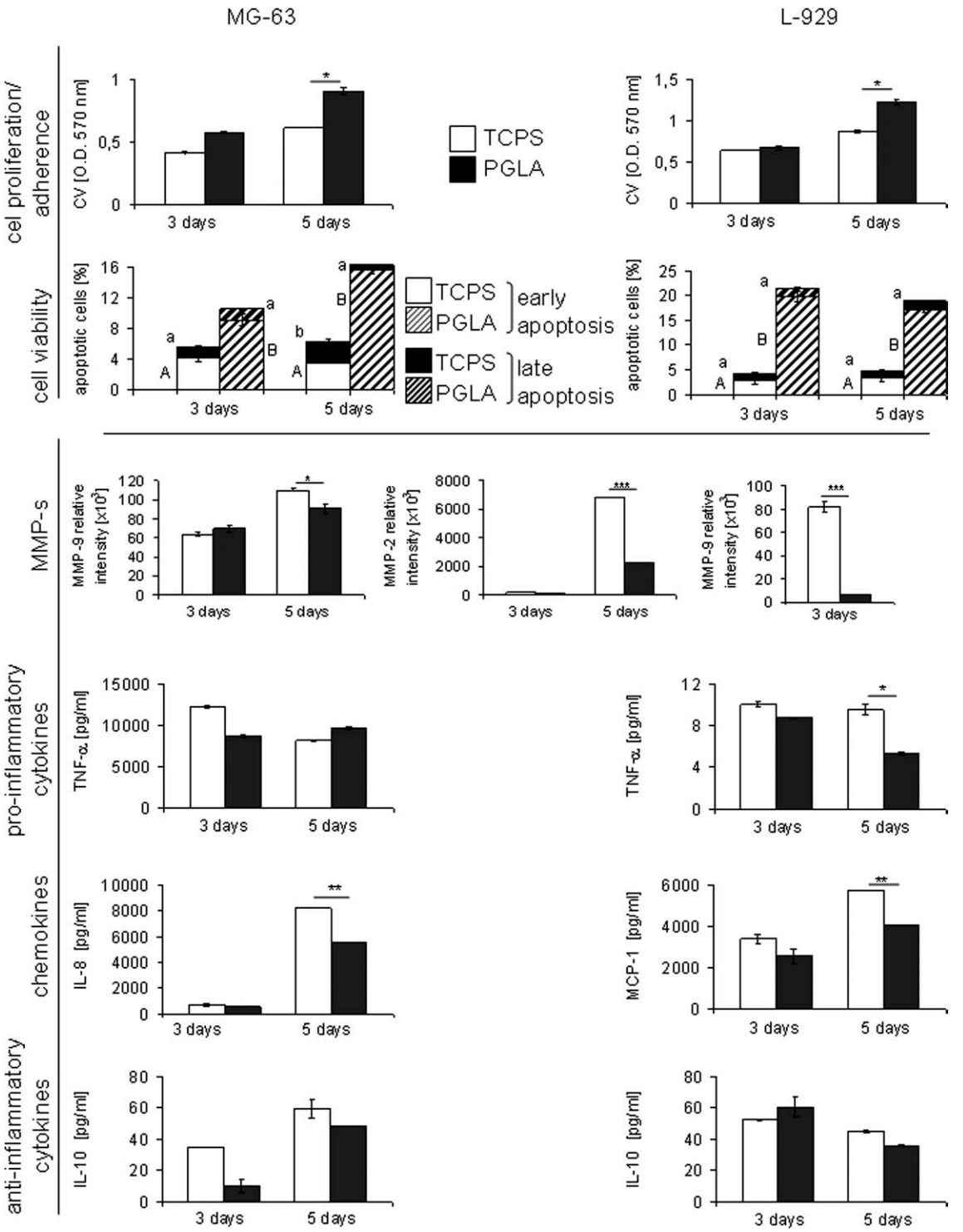

Figure 3. Effects of TCPS (tissue culture polystyrene) and PGLA poly(glycolide-*co*-L-lactide) (PGLA) on cytological/immunological parameters (cell proliferation/adherence, cell viability, MMPs, pro- and anti-inflammatory cytokines, and chemokines) of osteoblasts MG-63 and fibroblasts L-929 cells.

increases this might imply that the homeostasis of the cell/tissue is unbalanced e.g., by the lack/abundance of survival-promoting factors such as growth factors.[32]

The CV test applied in the studies detects adhering cells (at the given time unit) and when two or more time units are compared an information on the cell proliferation might be obtained. Accordingly, the data from the CV test might also be interpreted as decreased proliferation of osteoblasts and fibroblasts in the presence of PGLCap and PGCap in time [Figure 4(A,B)]. This would be confirmed by the morphological evaluation (representative pictures from day 3 are presented in Figure

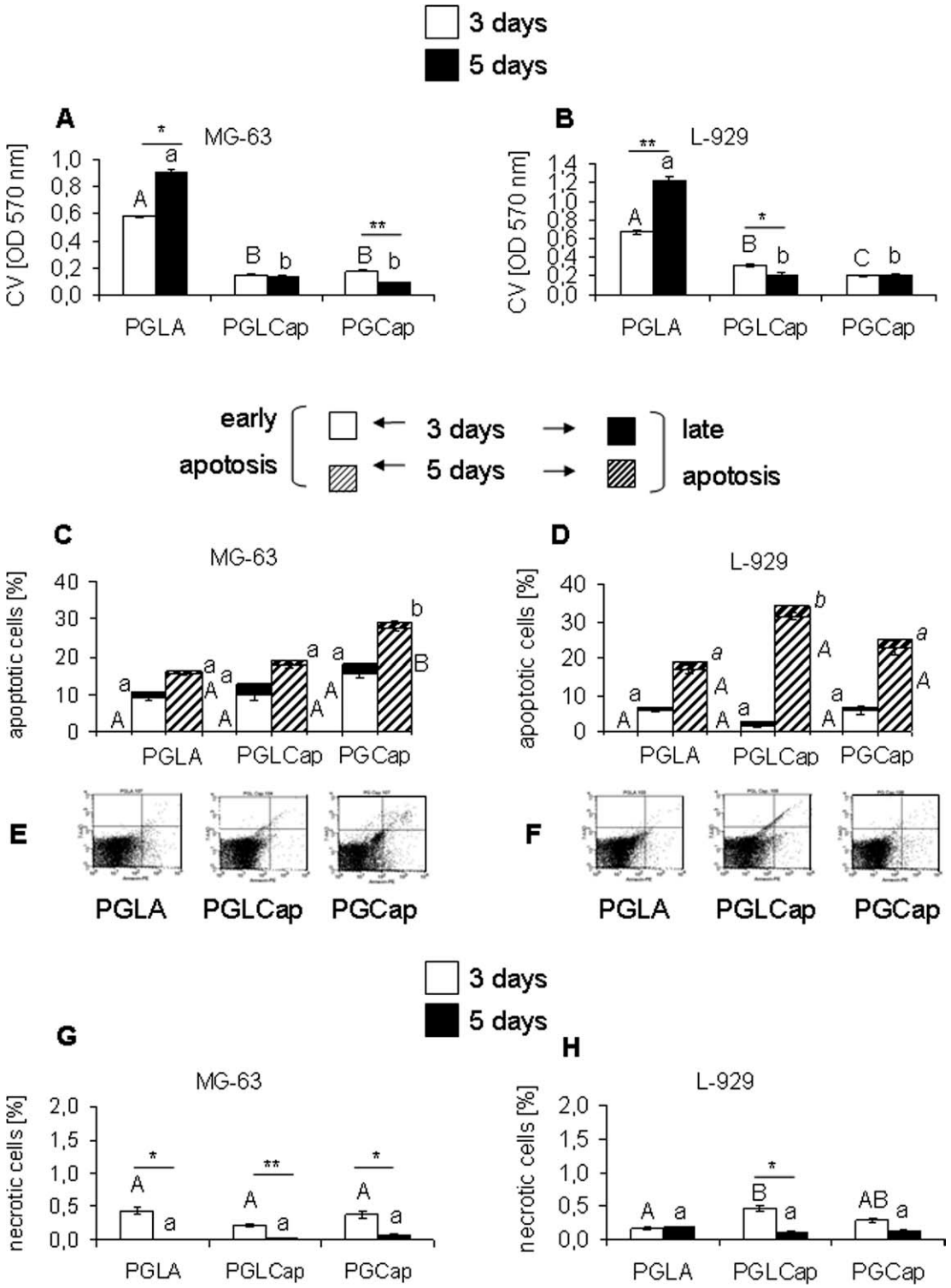

Figure 4. Effects of polymers on survival and proliferation of osteoblasts MG-63 and fibroblasts L-929. (A) Cell proliferation/adherence of MG-63 and (B) L-929 in the presence of tested polymers. Percentage of dead cells was assessed by flow cytometry, (C) apoptotic MG-63 cells, (D) apoptotic L-929 cells, (G) necrotic MG-63 cells, and (H) necrotic L-929. The cells were cultured on polymers: poly(glycolide-*co*-L-lactide) (PGLA), poly(glycolide-*co*-L-lactide-*co*-ε-caprolactone) (PGLCap), and poly(glycolide-*co*-ε-caprolactone) (PGCap) for either 3 or 5 days. Representative dot blots from cytometric analyses of viability of MG-63 osteoblasts (E) and L-929 fibroblasts (F) for day 5 are presented: early apoptotic Annexin V^+/7-AAD^-; late apoptotic Annexin V^+/7-AAD^+; necrotic cells Annexin V^-/7-AAD^+; alive cells Annexin V^-/7-AAD^-. The results are presented as means ± SE (data from three independent experiments). Different letters (e.g., A vs. B or a vs. b) indicate statistically significant differences between the groups according to ANOVA. Asterisks indicate statistical differences between day 3 and 5: $^*P < 0.05$; $^{**}P < 0.01$.

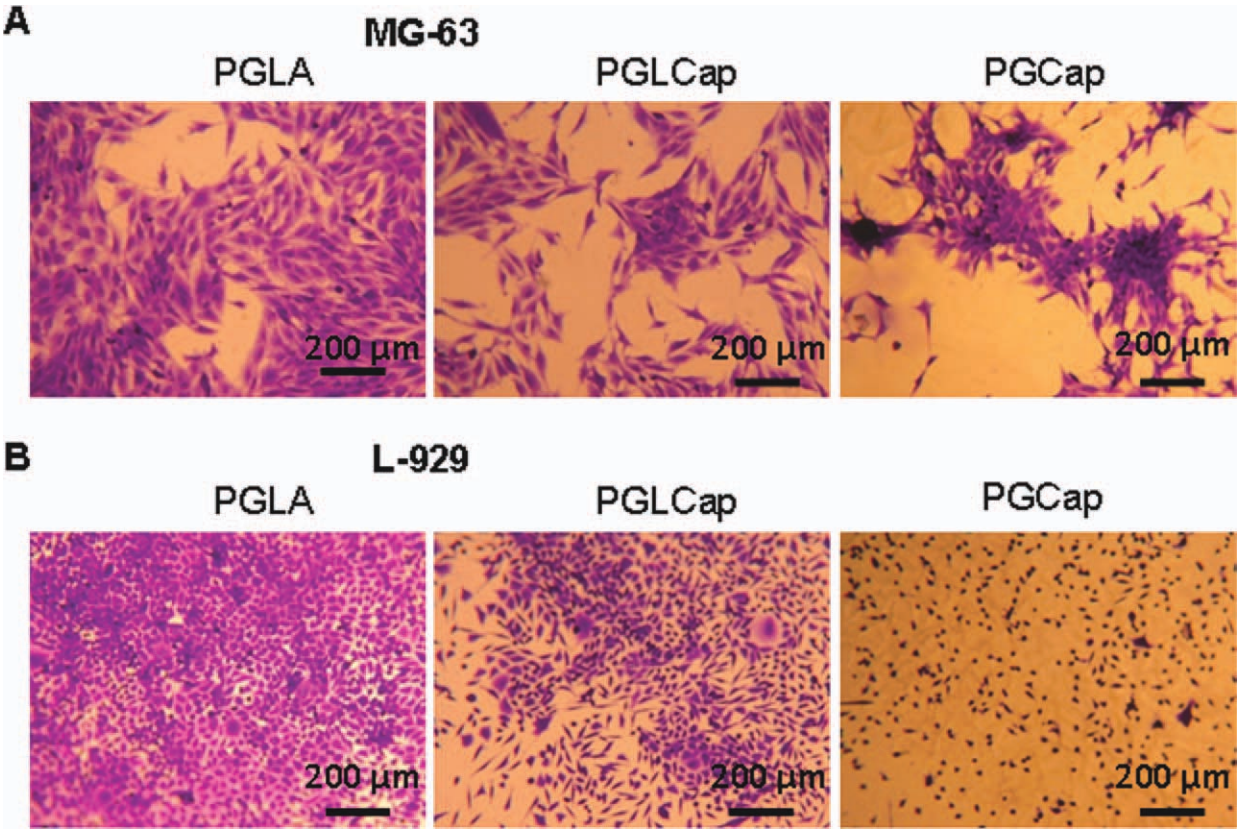

Figure 5. Morphology of MG-63 osteoblasts and L-929 fibroblasts cultured on PGLA, PGLCap, and PGCap for 3 days. The pictures were taken under the inverted microscope with 10× objective after staining with crystal violet. Bar 200 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.].

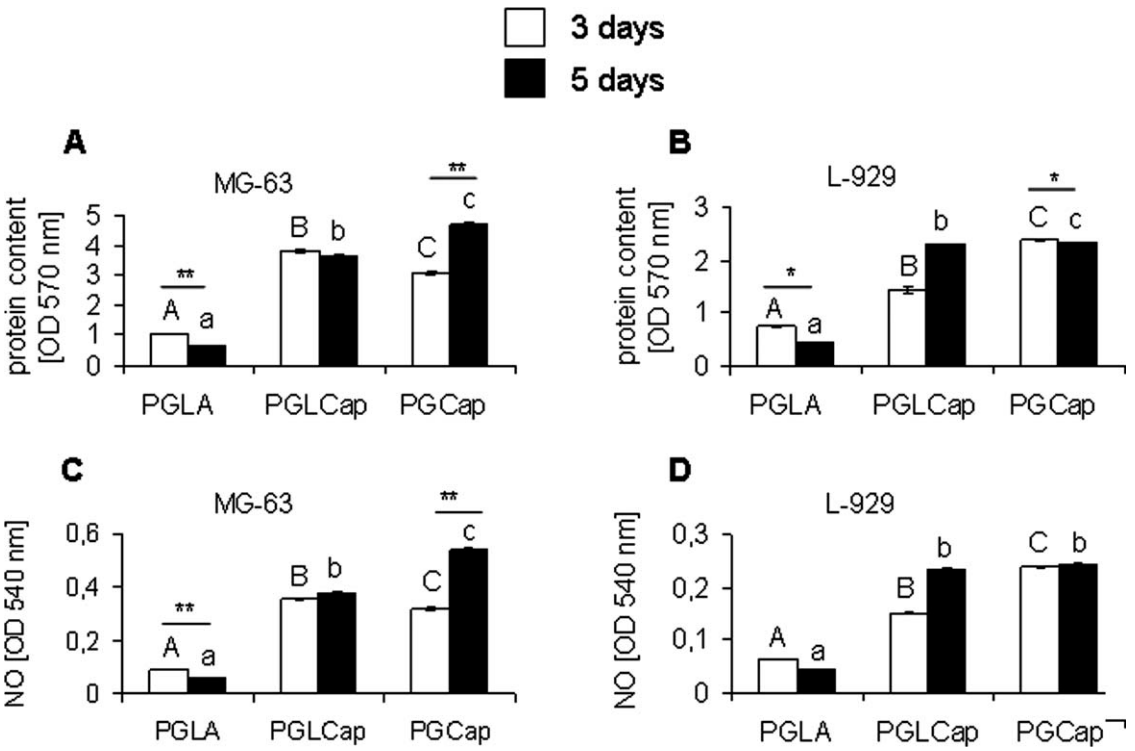

Figure 6. Production/release of proteins and nitric oxide by MG-63 osteoblasts and L-929 fibroblasts in the presence of the tested polymers. The cells were incubated with the polymers: poly(glycolide-*co*-L-lactide) (PGLA), poly(glycolide-*co*-L-lactide-*co*-ε-caprolactone) (PGLCap), and poly(glycolide-*co*-ε-caprolactone) (PGCap) either 3 days or 5 days. (A) Total protein content in supernatants from MG-63 and (B) L-929 cells. (C) Levels of nitric oxide in supernatants from MG-63, and (D) L-929. The results are presented as means ± SE (data from three independent experiments). Different letters (e.g., a vs. b) indicate statistically significant differences between the groups according to ANOVA. Asterisk indicates statistical differences between day 3 and 5: $^{*}P < 0.05$; $^{**}P < 0.01$.

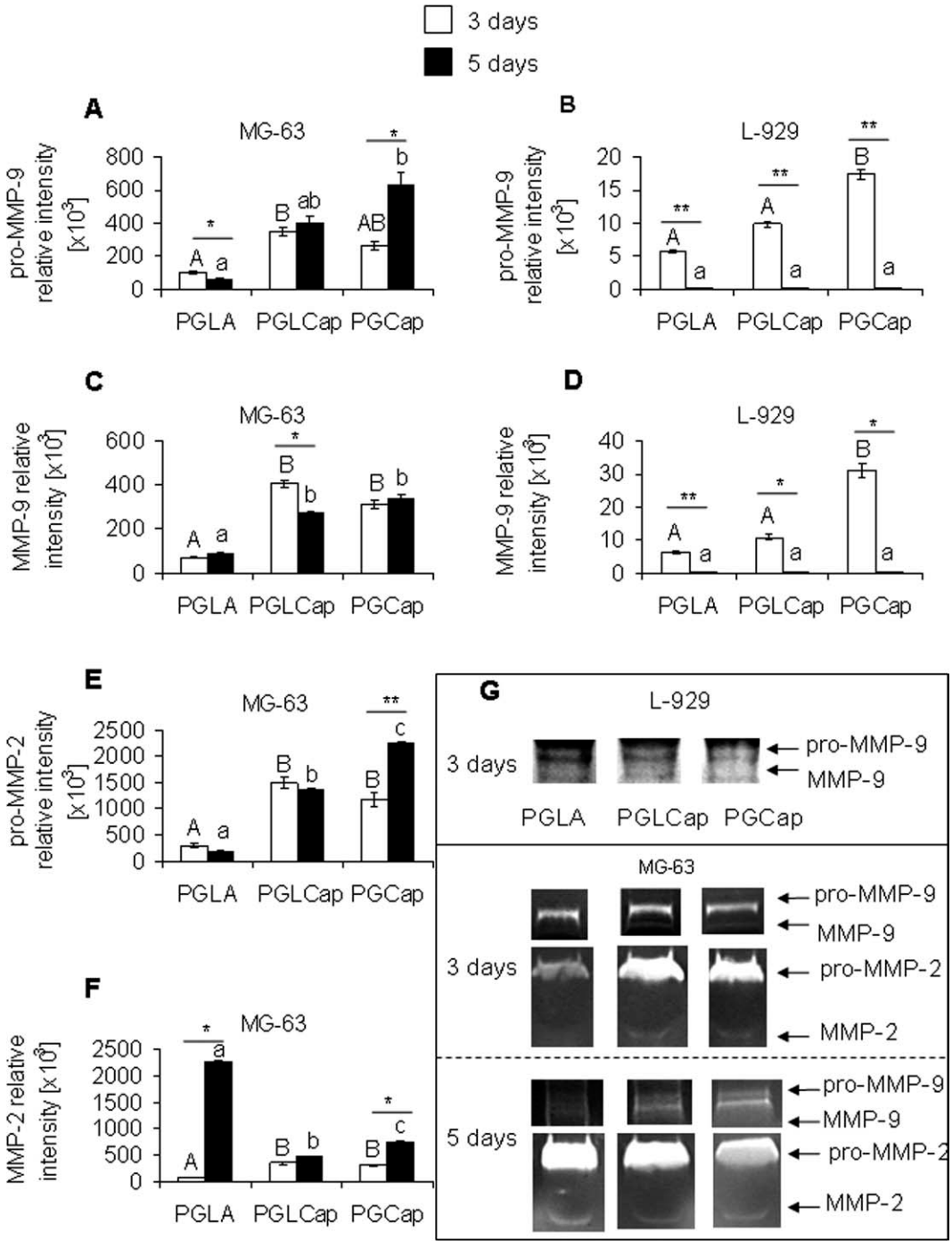

Figure 7. Release of MMP-9 and MMP-2 by osteoblasts MG-63 and fibroblasts L-929 in the presence of the tested polymers. Relative expression of MMPs was assed by densitometric analysis of zymographic gels. Levels of (A, B) pro-MMP-9, (C, D) MMP-9, (E) pro-MMP-2, and (F) MMP-2. Levels of MMPs were estimated in supernatants from cells cultured on polymers poly(glycolide-*co*-L-lactide) (PGLA), poly(glycolide-*co*-L-lactide-*co*-ε-caprolactone) (PGLCap), and poly(glycolide-*co*-ε-caprolactone) (PGCap) for 3 or 5 days. Representative zymographic gels are presented (G). The results are presented as means ± SE (data from three independent experiments). Different letters (e.g. a vs. b or A vs. B) indicate statistically significant differences between the groups according to ANOVA.

5). Namely, on day 3 both MG-63 osteoblasts and L-929 fibroblasts were more numerous on PGLA than on PGLCap and PGCap (Figure 5), and this pattern did not change during the next 2 days (data not shown). Nevertheless, morphology of osteoblasts was not changed in the presence of PGLCap and PGCap but the cells, especially the ones grown on PGCap, were forming clusters instead of spread monolyers [Figure 5(A)]. On the other hand, the morphology of L-929 cultured on PGCap,

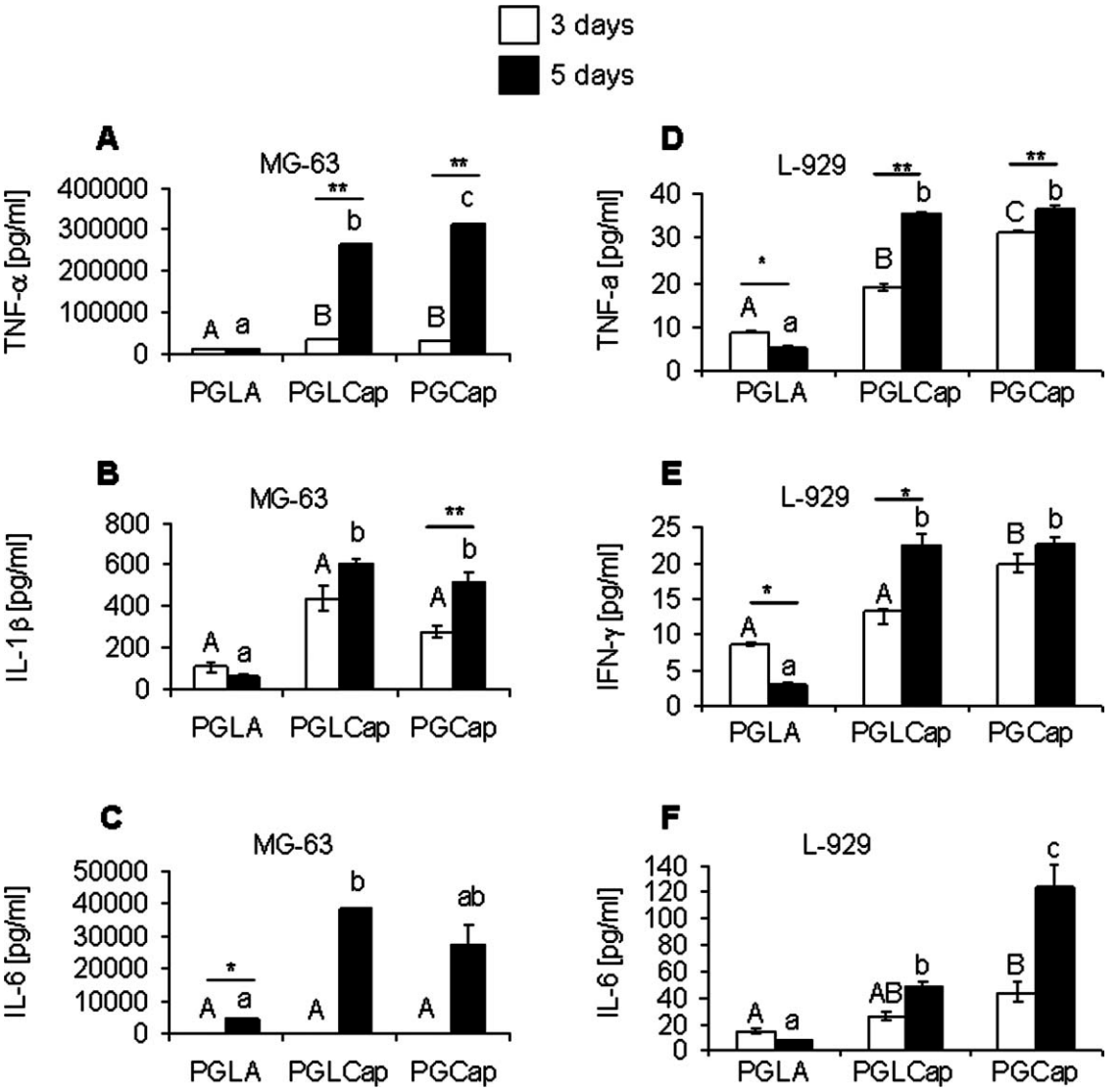

Figure 8. Synthesis of pro-inflammatory cytokines by MG-63 osteoblasts and L-929 fibroblasts in the presence of the tested polymers. The levels pro-inflammatory cytokines produced by osteoblasts (**A**) TNF-α, (B) IL-1β, (C) IL-6, and fibroblasts (D) TNF-α, (E) IFN-γ, and (F) IL-6 were measured in supernatants collected from the cell cultures 3 or 5 days in the presence of poly(glycolide-*co*-L-lactide) (PGLA), poly(glycolide-*co*-L-lactide-*co*-ε-caprolactone) (PGLCap), and poly(glycolide-*co*-ε-caprolactone) (PGCap). The results are presented as means ± SE. Different letters (e.g. A vs. B or a vs. b) indicate statistically significant differences between the groups according to ANOVA. Asterisk indicates statistical differences between day 3 and 5: $^{*}P < 0.05$; $^{**}P < 0.01$.

but not PGLCap, was altered [Figure 5(B)]. Specifically, most of the cells incubated on PGCap did not exhibit a fibroblast-like characteristic elongated shape but instead were rounded giving impression as if their numbers were even fewer [Figure 5(B)]. However, they did not form aggregates as in the case of osteoblasts, and remained evenly distributed on the material surface [Figure 5(A) vs. (B) for PGCap].

One possible explanation of impaired cell adherence/proliferation to the implant surface (putatively leading to cell death) is its smoothness since it was shown that rough surfaces support cell adherence.[33] However, our results on the cell adherence/proliferation and death cannot be simply attributed to the surface properties of PGCap and PGLCap as the surface topography of the former is rough and that of PGLCap is quite smooth, similarly as that of PGLA.[8] Moreover, surface chemistry and surface free energy seem to be not critical factors either, because both PGLCap and PLGA are more polar than PGCap.[8] The most probable reason of weaker adhesion/proliferation of osteoblasts and fibroblasts on PGCap and PGLCap are mechanical properties of the substrate, i.e., higher flexibility and deformability and lower Young's modulus (Table I). It was reported in the literature that soft and deformable substrates cannot resist tractional forces generated by cells at the initial stage of contact with the material, and as a result cell adhesion, spreading, and survival are impaired.[34,35]

All foreign material implanted into living tissues initiates a host response that is the first step of tissue repair.[36] A degree of this inflammatory response is critical as the strong and prolonged activation might lead to chronic inflammation and thus loss of

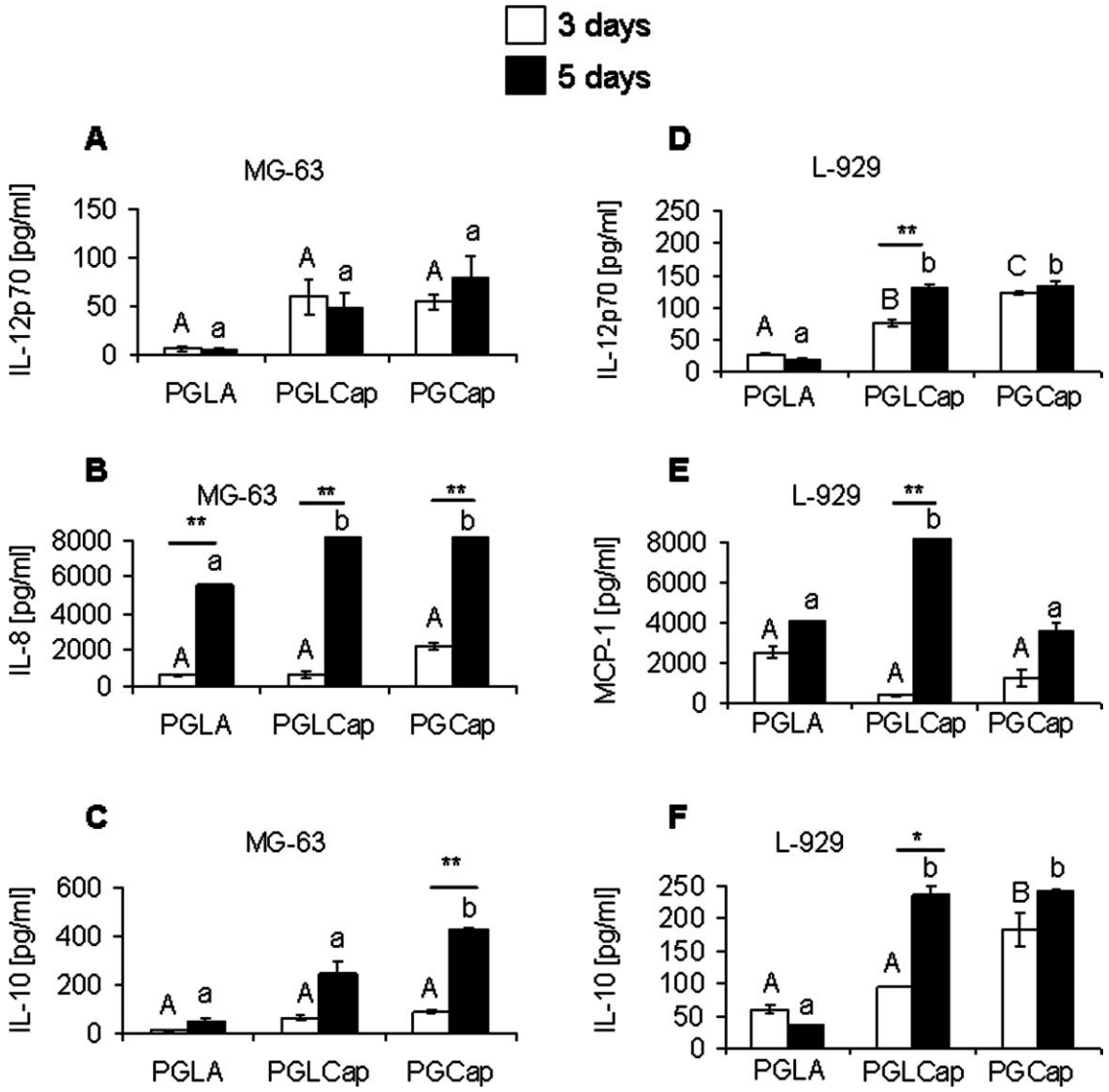

Figure 9. Synthesis of pro- and anti-inflammatory cytokines and chemokines by MG-63 osteoblasts and L-929 fibroblasts in the presence of the tested polymers. The levels of pro-inflammatory cytokines produced by osteoblasts (A) IL-12, (B) IL-8, (C) IL-10, and fibroblasts (D) IL-12p70, (E) MCP-1, and (F) IL-10 were measured in supernatants collected from the cell cultures after 3 or 5 days. The cells were cultured in the presence of poly(glycolide-*co*-L-lactide) (PGLA) or on poly(glycolide-*co*-L-lactide-*co*-ε-caprolactone) (PGLCap), or poly(glycolide-*co*-ε-caprolactone) (PGCap). The results are presented as means ± SE. Different letters (e.g., A vs. B or a vs. b) indicate statistically significant differences between the groups according to ANOVA. Asterisk indicates statistical differences between day 3 and 5: $^{*}P < 0.05$; $^{**}P < 0.01$.

the intended implant function.[37] For this we had evaluated induction of some inflammatory mediators/parameters and we have observed that PGCap and PGLCap, in comparison to PGLA, increased release of proteins [Figure 6(A,B)] and cytotoxic nitric oxide [Figure 6(C,D)] on either day and by either cell population. To evaluate which protein release was enhanced we analyzed synthesis of several proinflammatory factors including matrix metalloproteinases (MMPs) and cytokines. MMPs, and among them especially MMP-9, are endoproteases known for their capacity to degrade ECM molecules and facilitate leukocyte influx to the site of inflammation.[38] In fibroblast cultures with the polymers, the release of pro-MMP-9 and its activation into MMP-9 were enhanced by PGCap [Figure 7(B,D)] while in osteoblasts cultures also PGLCap increased the two parameters [Figure 7(A,C)]. This effect implies that the two polymers might initiate the proinflammatory events. However, fibroblasts did not continue production of MMP-9 during the longer incubation [Figure 7(B,D)]. In the case of osteoblast we also evaluated levels of another MMP, namely MMP-2, which cooperates with MMP-9 in guarding physiological bone remodeling.[39] It turned out that PGCap and PGLCap increased generation of pro-MMP-2 on both investigated days [Figure 7(E)]; however, the formation of active MMP-2 was at first (day 3) up-regulated while later (day 5) it was decreased [Figure 7(F)]. Thus overall these results suggest that both polymers might potentially impair the proper bone remodeling. Impairment of this process might be fatal for osteoblast–osteoclast communication and therefore proper bone formation and mineralization.[40]

The analyses of cytokine data revealed that PGCap and PGLCap stimulated their synthesis. Osteoblasts were activated to release increased amounts of potent pro-inflammatory TNF-α, IL-1β, and IL-8 [Figures 8(A,B) and 9(B), respectively] by both polymers and additionally PGLCap enhanced synthesis of IL-6 [Figure 8(C)]. And PGCap increased production of antiinflammatory IL-10 [Figure 9(C)], which also indirectly signals that the inflammatory response took place.[41] The above effects were particularly apparent during the long-term 5-day incubation with the cells. Similarly, fibroblasts were stimulated to release higher amounts of TNF-α [Figure 8(D)] and IL-12p70 [Figure 9(D)] in the presence of PGCap and PGLCap on both investigated days. And moreover, PGCap enhanced synthesis of IFN-γ, IL-6, and IL-10 [Figures 8(E,F), and 9(F), respectively] on either day of incubation. The levels of chemotactic MCP-1 were increased only by PGLCap [day 5; Figure 9(E)]. In our previous studies we investigated impact of PGLA, PGLCap, and PGCap on activation of macrophages,[8] the leukocytes that along with fibroblasts participate in the immune response, and the biomaterial–host interaction is believed to be primarily mediated by the macrophage.[42] Also in this case PGLCap and PGCap significantly up-regulated multiple pro-inflammatory parameters of those cells.[8] Therefore, all these data suggest that PGCap and PGLCap stimulated the bone-related osteoblasts, stroma-related fibroblasts, and macrophages of the immune system to react strongly on them within just first 5 days of incubation. Considering that the nature of the early cell-material interactions can strongly influence the long-time behavior of cells within the implant the results suggest rather unsolicited outcome.

The common characteristic of PGLCap and PGCap is present in their structure of ε-caprolactone and glycolide units. However, glycolide is also present in the structure of PGLA, which was shown to be biocompatible by others[18,29,30] and our group,[8,9] as well as this notion is sustained by the current data. Therefore, we postulate that this is a presence of poly-ε-caprolactone units that—due to impairing mechanical properties—decides about the reduced cell adherence and low biocompatibility of PGLCap and PGCap described in the current article. Poly-ε-caprolactone is considered to be generally biocompatible and due to its remarkable slow (years) degradation rate suggested for long-term implants.[43] Moreover, compared to other biodegradable polymers poly-ε-caprolactone has advantageous properties such as high permeability to small drug molecules and maintenance of neutral pH upon degradation.[23,44,45] On the other hand, however, its rather high crystallinity lowers its degradability and makes it less compatible with soft tissues. The latter problem, although foreseen, was believed to be solvable by ε-caprolactone polymerization with other monomers.[44,45]

CONCLUSIONS

The obtained data show that the presence of PGCap and PGLCap compromises cell adhesion/proliferation, viability and leads to release of pro-inflammatory factors by fibroblasts and osteoblasts. Specifically, we demonstrate that ε-caprolactone copolymerization with either glycolide (as in PGCap) or glycolide and L-lactide (as in PGLCap) does not provide materials that fulfill the expectation of satisfactory biocompatibility for bone tissue applications. In the more general aspect, our study postulates a caution, the fact that single monomeric units result in polymers of confirmed biocompatibility does not prejudice biocompatibility of the new co- and terpolymers which, in principle, require their full testing.

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