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Platelet Lysate-Modified Porous Silicon Microparticles for Enhanced Cell Proliferation in Wound Healing Applications

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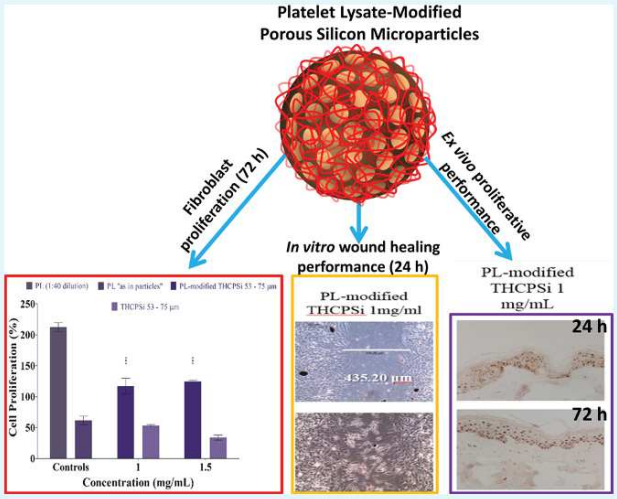
Supporting Information

ABSTRACT: The new frontier in the treatment of chronic nonhealing wounds is the use of micro- and nanoparticles to deliver drugs or growth factors into the wound. Here, we used platelet lysate (PL), a hemoderivative of platelets, consisting of a multifactorial cocktail of growth factors, to modify porous silicon (PSi) microparticles and assessed both *in vitro* and *ex vivo* the properties of the developed microsystem. PL-modified PSi was assessed for its potential to induce proliferation of fibroblasts. The wound closure-promoting properties of the microsystem were then assessed in an *in vitro* wound healing assay. Finally, the PL-modified PSi microparticles were evaluated in an *ex vivo* experiment over human skin. It was shown that PL-modified PSi microparticles were cytocompatible and enhanced the cell proliferation in different experimental settings. In addition, this microsystem promoted the closure of the gap between the fibroblast cells in the wound healing assay, in periods of time comparable with the positive control, and induced a proliferation and regeneration process onto the human skin in an *ex vivo* experiment. Overall, our results show that PL-modified PSi microparticles are suitable microsystems for further development toward applications in the treatment of chronic nonhealing wounds.



KEYWORDS: *porous silicon, platelet lysate, wound healing, fibroblast, microparticles*

1. INTRODUCTION

The effects of aging in the world's population are continuously increasing the healthcare costs. Chronic wounds (e.g., decubitus ulcers, diabetic sores, and leg ulcers) represent one of the most common diseases affecting the elderly during or after hospitalization, and thus, intensive research has been focused on finding new therapeutic options for the treatment of chronic nonhealing wounds.[1−3] Wound healing is the collective name comprehensive of the different, consequent, and sometimes overlapping stages a wound should face before reaching complete healing (i.e., a restoration of the anatomic continuity and function).[4,5] The healing of wounds is based upon complex interactions between signaling molecules and growth factors.[6,7]

The innovative therapeutics in wound treatments are based on this network of signals and aim to provide the wound with the necessary growth factors.[8,9] Several recent studies proposed the development of formulations containing growth factors or hemoderivatives in therapeutic applications.[10,11] Platelet lysate (PL) represents a unique compound due to its multifactorial composition and its proved efficacy in promoting wound healing.[12] For example, Del Fante et al. loaded PL into mucoadhesive gels for patients suffering from graft-versus-host disease. In a preliminary clinical study, they showed a good-to-complete response in 5 out of 7 patients treated.[13] Sandri et al. developed thermosensitive eye-drops containing PL for the treatment of corneal lesions.[14] In addition, topical formulations constituted of freeze-dried systems based on biopolymers of solid lipid nanoparticles coloaded with PL and silver sulfadiazine, and of alginate microparticles coloading PL with vancomycin, showed an enhanced proliferation of fibroblasts *in*

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Table 1. Physical Properties of the PSi Microparticles Tested in This Study[a]

properties	THCPSi	THCPSi	THCPSi	TOPSi	UnTHCPSi	UnTHCPSi
size (μm)	< 25	25−53	53−75	53−75	<25	25−53
surface area (m^2/g)	109	273	298	324	292	255
pore volume (cm^3/g)	1.09	0.94	0.63	0.71	1.01	0.82
average pore diameter (nm)	14.1	10.8	8.4	8.7	13.8	12.8

[a]THCPSi = thermally hydrocarbonized PSi; TOPSi = thermally oxidized PSi; UnTHCPSi = undecylenic-modified THCPSi.

vitro and the possibility to effectively coload antibiotics with growth factors.[15−17] Moreover, careful choices and studies in the systems' composition led to the production of dressings with characteristics suitable for different kinds of wounds.[15] PL has also been effectively loaded in 3D systems and membranes designed for hard and soft tissues regeneration. These studies showed that PL-based systems are suitable for inducing the directed differentiation of human mesenchymal stem cells into osteoclasts or chondrocytes.[18−21]

Mesoporous micro- and nanoparticles are currently studied as drug delivery systems for topical applications.[22,23] In particular, porous silicon (PSi) microparticles have been extensively studied in different fields[24,25] and are excellent materials for controlled drug delivery applications due to the pore size of the particles and the easiness in the functionalization of their surface with biomolecules.[26−30] The toxicity of PSi microparticles has also been assessed during the last years, both *in* vitro in different cell lines and *in vivo*. In *in vitro* assays, the particles' size and the surface chemistry determined the PSi's toxicity.[31,32] Overall, the *in vivo* studies on the cytotoxicity of PSi-based particles did not show significant immunogenic or toxicity issues.[33−37]

Recently, we have investigated PSi microparticles coated with biopolymers (chitosan and hyaluronic acid) for potential wound healing applications, and we have demonstrated the successful loading of vancomycin and resveratrol in PSi, with a controlled release of the drugs.[38] Following our previous works and to improve the stability and applicability of PL on cutaneous wounds, the aims of this study were to assess the PSi microparticles' toxicity in fibroblasts cells, to load PL onto the PSi microparticles, and to evaluate the proliferative effect of the formulation both *in vitro* in a wound healing assay and also *ex vivo* over human skin, in order to fully characterize the formulation for potential future applications in the treatment of chronic nonhealing wounds.

2. EXPERIMENTAL SECTION

2.1. Porous Silicon (PSi) Microparticles. The PSi microparticles tested in this study and their physicochemical characterization are presented in Table 1 (see the Results and Discussion section). The synthesis of the PSi microparticles was performed as described elsewhere.[27,38,39]

2.2. Compounds. PL was kindly provided by the Aphaeresis Service of Immunohematology and Transfusion Service Center for Transplant Immunology of Fondazione IRCCS, Policlinico S. Matteo, Pavia, Italy, from healthy donors, as described elsewhere.[13−16] Briefly, platelet rich plasma was separated by centrifugation in the presence of heparin as anticoagulant. It was then frozen and thawed until the platelets broke and released the growth factors. PL was stored at −80 °C.

2.3. Scanning Electron Microscopy. SEM pictures of THCPSi 53−75 μm particles before and after modification with PL were taken with a FEI Quanta 250 Field Emission Gun Scanning Electron Microscope (FEG, USA).

2.4. Cell Culturing. An immortalized fibroblast cell line, derived from human newborn foreskin (CCD 1112Sk, ATTC CRL-2429TM), was used in the *in vitro* studies. Cells were stored in liquid nitrogen (−196 °C) and were used at passages #11−28. Cells were fed with heat inactivated fetal bovine serum (10%, HIFBS, HyClone, USA)−Dulbecco's Modified Eagle's Medium (DMEM, HyClone, USA) medium, supplemented with 1% nonessential amino acids (HyClone, USA), 1% L-glutamine (EuroClone SpA, Italy), 1% (100×) penicillin-streptomycin-L-glutamin (PEST; HyClone, USA), and 1% (100 mM) sodium pyruvate (Lonza, Switzerland). A standard incubator (16 BB gas, Heraeus Instruments GmbH) at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity was used for maintaining the conditions for culturing the cells. The harvesting of the cells prior to each experiment was performed with a 0.25% (v/v) trypsin-PBS−EDTA solution.

2.5. Cell Viability. To evaluate the cytotoxicity of the PSi microparticles in fibroblast cells, the cell viability was assessed by measuring their ATP activity, as described elsewhere.[38] About 2×10^4 cells were seeded in each well in a 96-well plate (Corning Incorporated, USA) and allowed to attach overnight. Then, 100 μL of the samples solution was added to each well, and the plates were incubated for up to 48 h. Particle suspensions of thermally hydrocarbonized PSi (THCPSi), thermally oxidized PSi (TOPSi), and undecylenic acid-conjugated THCPSi of concentrations of 0.5, 1, and 1.5 mg/mL were suspended in 1 mL of DMEM medium without phenol red and serum (M w/serum). 100 μL of each sample solution was added to the cells. The positive and negative controls were M w/serum and Triton X-100 (1%, Merck KGaA, Germany), respectively. After incubation, the wells of the plates were washed twice with Hank's balanced salt solution (HBSS)−4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4). Then, 50 μL of HBSS−HEPES (pH 7.4) and 50 μL of CellTiter-Glo reagent (Promega Corporation, USA) were added to each well. After mixing with an orbital shaker for 2 min to induce cell lysis, the plates were left for 15 min at room temperature before measuring the luminescence with a Varioskan Flash reader (Thermo Fisher Scientific Inc., USA).

2.6. Cell Proliferation Studies. About 2×10^4 cells/well in DMEM without serum were seeded in each well of a 96-well plate and left to attach for 2 h. Then, 50 μL of the samples solution was added to each well, and the plates were incubated, up to 24 h, at 37 °C, 5% CO_2, and 95% relative humidity. M w/serum and PL (1:40 dilution) were used as controls. After 24 h, the medium was removed, and the plates were washed three times with HBSS−HEPES (pH 7.4). We added 50 μL of HBSS (pH 7.4) and 50 μL of CellTiter-Glo reagent (Promega Corporation, USA) to each well, as previously described.[38]

2.7. Drug Loading of PSi Microparticles. About 250 μL of PL solution and 250 μL of 0.9% NaCl physiological solution were added to different PSi microparticle concentrations (0.5, 1, and 1.5 mg/mL). The particles underwent continuous stirring at approximately 200−300 rpm at room temperature for 90 min. Then, the particles were collected and centrifuged (Eppendorf Microcentrifuge 5415, Eppendorf AG, Germany) at 8000 rpm for 4 min; the supernatant was removed, and the resulting PL-modified particles were stored at +4 °C until further use.

2.8. Bicinchoninic Acid Assay (BCA). The quantification of the protein content was done using a BCA assay. Briefly, a calibration curve in the range 5−250 μg/mL was made at a wavelength of 562 nm, according to Pierce BCA Protein Assay Kit (Pierce Biotecnology, USA) instructions. The PL-modified PSi microparticles (THCPSi 53−75 μm), at the concentration of 1 mg/mL, were suspended in 1 mL of 0.9% NaCl physiological solution. PL (1:40 dilution) was used as a control, in order for its concentration to fit within the range of the

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calibration curve of the BCA assay. The absorbance of all the samples was measured with an UV spectrophotometer within 10 min.

2.9. Assessment of PDGF-AB Content in the Formulation. The amount of platelet derived growth factor (PDGF-AB) present in the loading supernatant of the PSi particles and in the PL (1:40 dilution) control sample was determined using an ELISA kit (Human PDGF AB Quantikine PharmPak, R&D Systems, Minneapolis, MN, USA; assay range: 31.2−2000 pg/mL), as described elsewhere.[15] This allowed the estimation of the total amount of PL present in the PSi particles. Three replicates were performed for each sample.

2.10. Transport Studies. Transport experiments were conducted to evaluate the release of PL from the particles and the effect on the cell proliferation of the released growth factors. The first assay intended to quantify the total amount of proteins released from the system by means of a BCA assay and to specifically quantify PDGF-AB only. The PL-modified THCPSi microparticles at the concentration of 1 mg/mL (53−75 μm) were centrifuged and suspended in 1 mL of 0.9% NaCl physiological solution. PL (1:40 dilution) in 0.9% NaCl physiological solution was used as a control. Then, 600 μL of 0.9% NaCl physiological solution was added to the basolateral chamber of 24-well plates (HTS Transwell, 0.4 μm pore size, Costar, Corning Inc., USA), and 200 μL of the sample solution was added to the apical chamber. The plates were left at room temperature for up to 72 h. At each time point (8, 16, and 24 h), about 100 μL from both the apical and basolateral chambers was collected to perform the BCA assay. The concentration of PL released in the transport experiments was determined by preparing a standard curve plotting the average absorbance (blank-corrected) for each PL standard vs its concentration in μg/mL. The amount of PDGF-AB released from the particles was determined for particle concentrations of 1 and 1.5 mg/mL. After centrifugation, the supernatant was carefully collected in Eppendorf tubes and stored at +4 °C until further use. This supernatant was used both as such and diluted 1:40 in 0.9% NaCl solution. PL (1:40 dilution in physiological solution) and PL "as in particles" (PL control undergone to all the treatments as in the PL-modified particles' preparation) were used as controls.

We also performed a transport study in the presence of fibroblasts to evaluate their proliferation at different time points (24 and 72 h). Concentrations of 1 and 1.5 mg/mL of THCPSi 53−75 μm microparticles (Table 1) were modified with PL. After centrifugation, each sample was suspended in 1 mL of M w/serum. The cell suspension (1.5 mL), in M w/serum, was seeded in the basolateral chambers of a 12-well plate with a final concentration of 1×10^5 cells/well. The samples solutions (500 μL) were added in the apical chamber. The plate was then incubated at +37 °C, 5% CO_2, and 95% relative humidity in an incubator (Heraeus Instruments, Germany). At each time point the plate was removed from the incubator, and the cell proliferation was determined with the 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay. The MTT test was performed here because PSi particles were not in direct contact with the cells, thereby avoiding an interaction between MTT and PSi, as reported elsewhere.[39]

2.11. In Vitro Wound Healing Assay. In this assay, μ-dishes (Ibidi, Giardini, Italy) were used to assess the wound closure properties of the developed system. The dishes have an enclosed insert consisting of two chambers, with a total growth area of 0.22 cm^2, divided by a septum which will create a cell-free gap of 500 μm. Fibroblasts were seeded at a density of 1×10^5 cells/cm^2 and left to grow to confluence for 24 h. The inner insert was removed, displaying two cells' leaflets divided by a gap with prefixed dimensions. PL-modified PSi microparticles (THCPSi 53−75 μm) of concentrations of 1 and 1.5 mg/mL were centrifuged, and each sample was suspended again in 1 mL of DMEM without serum. About 400 μL of the particle's concentrations were put in contact with the cells. The μ-dishes were then incubated at 37 °C, in 5% CO_2, and 95% relative humidity. The wound healing test was also performed with unmodified PSi microparticles, using the same settings as for PL-modified PSi microparticles. At fixed times (24, 48, and 72 h), microphotographs were taken with a Leica DMI 3000 B microscope (Leica Microsystems, Germany) equipped with a Leica LAS Ez software to evaluate the cell migration and growth in the gap area.

2.12. Assessment of Cell Proliferation by Bromodeoxyuridine Incorporation in the DNA. To verify the proliferative status of the cells in the wound healing assay after treatment with the samples (1 and 1.5 mg/mL of bare or after PL modification THCPSi 53−75 μm microparticles−the same samples used for the *in vitro* wound healing assay), DNA synthesis was analyzed by measuring the incorporation of 5-bromo-2′-deoxyuridine (BrdU). During the last hour in culture, cells were labeled by adding 30 μM BrdU (Sigma-Aldrich, Saint Louis, MO, USA) to the medium. The samples were then washed with PBS and fixed in 70% ethanol. The incorporated BrdU was detected by an immunostaining reaction with Amersham monoclonal anti-BrdU antibody (GE Healthcare UK Ltd., Amersham Place, Buckinghamshire, England). Briefly, the dishes were washed with PBS and incubated with HCl 2 N for 30 min at room temperature. 0.1 M sodium tetraborate (pH 8.5) was used to neutralize the solution for 15 min, and then the cells were washed twice for 5 min in PBS and incubated for 20 min in the blocking solution (1% w/v BSA and 0.02% w/v Tween 20 in PBS Tween Albumin (PTA)). Cells were then incubated for 1 h with mouse anti-BrdU antibody diluted 1:100 in PTA. The cells were washed three times (10 min each) in PTA and then incubated again for 30 min in PTA containing antimouse IgG FITC-antibody (Sigma-Aldrich, Saint Louis, MO, USA) diluted 1:100. The slides were extensively washed in PBS, counterstained for DNA with 0.5 μg/mL Hoechst 33258 (Sigma-Aldrich, Saint Louis, MO, USA), and mounted in Mowiol (Sigma-Aldrich, Saint Louis, MO, USA). Cells were scored for BrdU immunofluorescence positivity with a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Oberkochen, Germany). At least 500 cells were counted for each condition, and each experiment was repeated at least three times.

2.13. Ex Vivo Test on Human Skin. Human skin was obtained from biopsies of a mastectomy in the plastic surgery of a 37-year old healthy patient (kindly provided by the University of Pavia, Plastic Surgery Department − IRCCS Fondazione "S. Maugeri", Pavia, Italy). Skin was cut in circular scraps using a punch of 6 mm diameter, and each one was incised with a circular sterile punch (3 mm diameter), removing the partial thickness of the central portion of the epidermis and dermis. The scraps were put in 24-well Transwell inserts (membrane pores 0.40 μm, insert area 0.33 cm^2 Corning Costar Inc., Life Sciences, MA, USA). About 100 μL of bare or after modification with PL THCPSi 53−75 μm microparticles in PBS was added to the wounded skin as such. The two control scraps were treated one with PL "as in particles" and the other with PBS. About 700 μL of 10% HIFBS DMEM was added in the basolateral chamber to keep the skin alive providing correct hydration and nutritive substances by diffusion from hypoderma. At different time points the biopsies were fixed and prepared for the histological analysis (immunohistochemistry for proliferating cell nuclear antigen (PCNA) and BrdU expression), as described in the Supporting Information and elsewhere.[40]

2.14. Statistical Analysis. In all the experiments, the obtained values are reported as mean ± standard deviation (SD) from at least three independent measurements. One-way analysis of variance (ANOVA), followed by the Bonferroni post-test, was used to statistically evaluate the obtained values with the level of significance set at probabilities of $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$. ANOVA followed by the Tukey post-test was used in the statistic evaluation of the data obtained in the quantification of PCNA positive cells in the *ex vivo* assay. GraphPad Prism 5 software (GraphPad Software Inc., USA) was used for the statistical analyses.

3. RESULTS AND DISCUSSION

3.1. Microparticles Preparation and Characterization. One of the aims of this work was the evaluation of PSi microparticles prepared in different sizes (nominal sieve mesh limits of <25 μm, 25−53 μm, and 53−75 μm) and with different surface chemistries (according to the preparation

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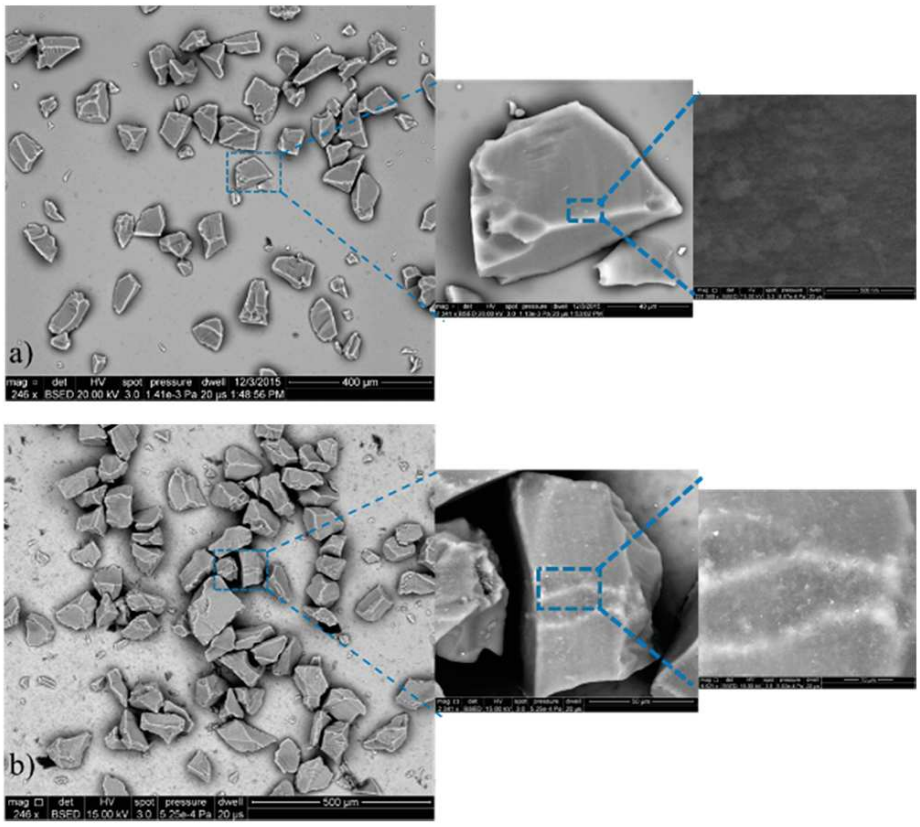

Figure 1. SEM pictures of THCPSi 53−75 μm particles (a) and PL-modified THCPSi 53−75 μm particles (b). Scale bars: 400 μm, 40 μm, and 500 nm for a) and 500 μm, 50 μm, and 10 μm for b).

methods).[31] Surface area, pore volume, mean pore diameter, and zeta potential were determined for each particle, as shown in Table 1 and Figure S1. The surface area was similar between all the particles, except for THCPSi < 25 μm that presented lower surface area value. On the contrary, the same particles, together with UnTHCPSi (<25 μm), were the ones displaying the highest pore volume, while THCPSi 53−75 μm showed the lowest pore volume. The average pore diameter was similar among all the particles tested, with THCPSi 53−75 μm presenting also the lowest value.

SEM pictures of THCPSi 53−75 μm particles before and after modification with PL did not display differences in the surface of the particles (Figure 1).

3.2. Cytocompatibility Studies. Cell viability experiments were conducted to assess the cytocompatibility of the PSi microparticles when incubated with skin fibroblast cells. The particles with different sizes and surface chemistries were tested at concentrations of 0.5, 1, and 1.5 mg/mL (Figure 2).

In Figure 2a, at the lowest PSi microparticle concentration (0.5 mg/mL) there was no statistical significant difference among the samples tested and the negative control (M w/ serum). However, at the concentration of 1 mg/mL, a statistically significant difference was found between all the particles assessed and the control. After 48 h of incubation with the particles, even at the highest concentration, there was no evident cytotoxicity in fibroblasts. The cell viability values did not drop below 80% in the lowest and highest concentrations tested (except for the sample UnTHCPSi 25−53 μm at the concentration of 1.5 mg/mL). In addition, for the THCPSi particles, the cell viability was size independent, with no significant differences in cell viability observed for the particles with different sizes. On the contrary, UnTHCPSi particles of the two different sizes displayed significant differences in the cytotoxicity behavior for the three concentrations tested (data not shown; 0.5 mg/mL, $p < 0.05$; 1 mg/mL, $p < 0.01$; and 1.5 mg/mL, $p < 0.001$).

Next, the effect of the surface chemistry of the particles on the fibroblasts' toxicity was assessed. THCPSi microparticles (53−75 μm) were the particles presenting cell viability values comparable to those of the control (M w/serum) at the lowest and highest concentrations tested, while there was a significant difference after the incubation with the intermediate concentration (Figure 2b). Regarding the particles characterized by different surfaces, TOPSi displayed values of cell viability statistically different from the control (M w/serum) over all the range of the concentrations tested (Figure 2b). Moreover, THCPSi microparticles proved to be better tolerated by the fibroblast cells than the other particles when assessed at the lowest ($p < 0.01$; TOPSi) and highest concentrations ($p < 0.001$; TOPSi) (data not shown). THCPSi 53−75 μm particles, despite presenting higher cell−particle interactions, as previously reported,[31] were less cytotoxic than the TOPSi onto fibroblast cells compared to TOPSi. Following the results obtained in Figure 2, THCPSi 53−75 μm particles were chosen to be further tested. In addition, since the lowest (0.5 mg/mL) concentration was not discriminative between the different PSi microparticles, further studies assessed the particle's concentrations of 1 and 1.5 mg/mL only.

3.3. Loading and Quantification of the Total Protein Content. First, the total protein content of the PL-modified

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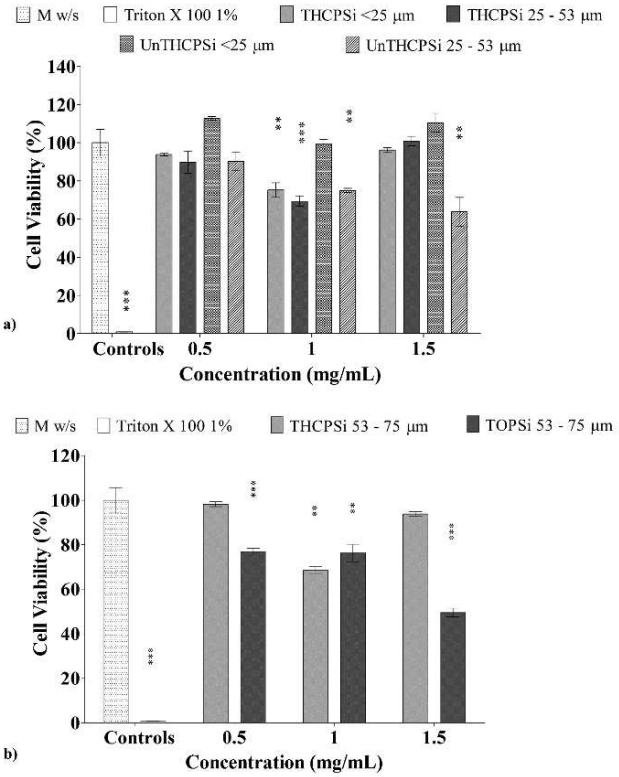

Figure 2. Cell viability (%) of fibroblasts after 48 h incubation with the PSi microparticles. The particles were tested (a) in two different sizes (<25 μm and 25−53 μm) and (b) in two different surface chemistries for concentrations of 0.5, 1, and 1.5 mg/mL. DMEM without serum (M w/s) and Triton X-100 (1%) were used as controls. The level of significance for the cell viability after incubation of the cells with the samples was measured against the levels of the control M w/s; this level was set at probabilities of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Error bars represent mean ± SD ($n > 3$).

PSi microparticles was quantified by a BCA assay. The amount measured for PL-modified THCPSi 53−75 μm (1 mg/mL) was 48.0 ± 0.5 μg/mL. Then, the amount of PDGF-AB adsorbed onto the PL-modified PSi particles was determined by a PDGF-AB ELISA kit. PDGF-AB was selected as a growth factor representative of the pool of growth factors (GFs) contained in PL, as suggested elsewhere.[15] However, PL "as in particles" displayed a reduction of 49.5% in the content of PDGF-AB in comparison with a sample of fresh PL. Therefore, PL "as in particles" was used as the positive control in the quantification of PDGF-AB. Thereby, the percentage of PDGF-AB adsorbed onto the THCPSi microparticles was 86.7% ± 4.9 (1 mg/mL) and 89.0% ± 5.7 (1.5 mg/mL), calculated against the control PL "as in particles".

3.4. Proliferative Studies. We next assessed the proliferative effect of the PL-modified THCPSi microparticles in the presence of GFs in skin fibroblast cells (Figure 3).

Based on the results of Figure 3, over short incubation times (24 h), PL-modified THCPSi microparticles induced a proliferative effect comparable with the one promoted by the control fresh PL (1:40 dilution) solution, in particular at the highest concentration tested (1.5 mg/mL), without any statistical significant difference. PL (1:40 dilution) was chosen as control in order to fit its concentration within the range of the calibration curve in the BCA assay. In addition, PL-modified

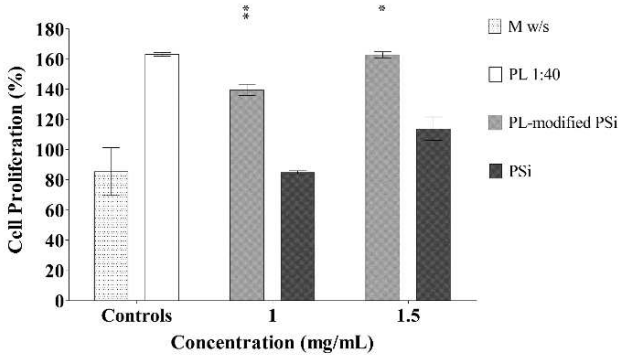

Figure 3. Fibroblast proliferation (%) after 24 h of incubation with PL-modified and unmodified THCPSi microparticles using an ATP-based luminescence assay. Two microparticle concentrations were tested, 1 and 1.5 mg/mL. DMEM without serum (M w/s) and PL (1:40 dilution) were used as controls. The level of significance of the cell proliferation after incubation with the PL-modified PSi microparticles was measured against the unmodified particles. The level of significance for cell proliferation after incubation of the cells with the samples was measured against the levels of the control M w/s; this level was set at probabilities of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Error bars represent mean ± SD ($n > 3$).

THCPSi microparticles induced a statistically enhanced proliferation of the fibroblasts ($p < 0.001$ at 1 mg/mL and $p < 0.05$ at 1.5 mg/mL) compared to the unmodified particles. The proposed microsystem (PL-modified THCPSi microparticles) induced cell proliferation probably due to the GFs contained in the PL. According to this data, we selected PL-modified THCPSi microparticles for further *in vitro* and *ex vivo* studies, as shown and discussed below.

3.5. Transport and Fibroblast Proliferation Experiments. The incorporation of bromodeoxyuridine (BrdU) into the DNA of cells in the proliferative phase S provides information about the cells proliferation process (Figure 4). The proliferative status of the cells treated with the developed microparticulate system was assessed and imaged in the *in vitro* wound healing assay (see sections 3.6 and 3.7 for details).

These results show that the PL-modified THCPSi microparticles, at the concentration of 1.5 mg/mL, can induce a significant proliferative effect compared to those observed for

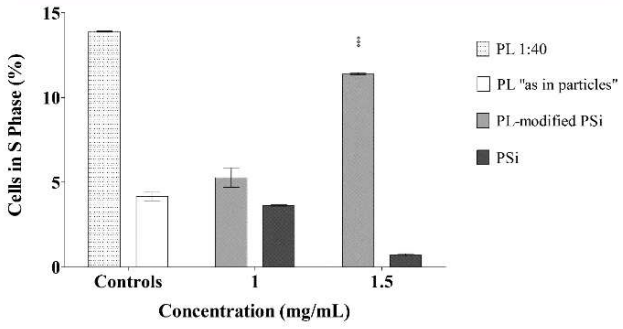

Figure 4. Percentage of cells in proliferative phase (S). Bare or after PL-modification THCPSi particles at the concentrations of 1 and 1.5 mg/mL were tested. Fresh PL (1:40 dilution) and PL "as in particles" served as positive controls. The significance level of cells in S phase after incubation with the PL-modified THCPSi particles was measured against the control PL "as in particles" and was set at a probability of $***p < 0.001$. Error bars indicate mean ± SD ($n = 3$).

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the positive control PL "as in particles" and for THCPSi microparticles alone (Figure 4), which further support the cell proliferative studies in Figure 3. In addition, these results show that THCPSi particles may protect the GFs contained in PL during and after the loading, when compared with the control PL "as in particles".

It is also of the highest importance to quantify the amount of GFs released from the PL-modified PSi microparticles over time in order to understand their mode of action. First, we determined the total amount of protein (GFs) released from the particles using the BCA assay. The total amount of PL released from THCPSi was 2.90 μg/mL (16.4% of the loaded PL) already after 8 h (Figure 5); the released amount increased slightly at 16 h (3.47 μg/mL, 20.0% of the loaded PL), before assessing onto 3.18 μg/mL at 24 h (18.4% of the loaded PL).

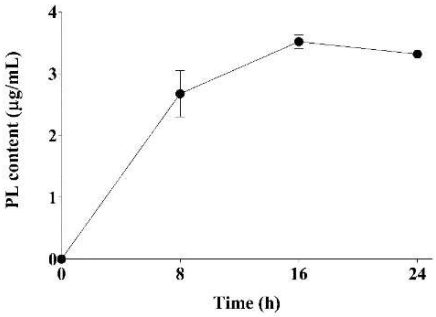

Figure 5. Amount of proteins released from the PL-modified THCPSi microparticles (1 mg/mL) at different time points (8, 16, and 24 h). Error bars represent mean ± SD (n = 3).

Next, we measured the amount of PDGF-AB released after 24 h from the PL-modified THCPSi microparticles, at the concentrations of 1 and 1.5 mg/mL, using an ELISA assay (Figure S2). The results showed that the total amount of PDGF-AB released from the particles at that time point was 0.14% and 0.42% of the adsorbed amount, for the concentrations of 1 and 1.5 mg/mL, respectively.

The proliferative effect caused by the GF released from the microparticles was further assessed in fibroblasts cells (Figure 6a). The cells were seeded in the basolateral chamber of a Transwell system, while the particles suspended in DMEM without serum were added to the apical chamber. Over short periods of time (24 h), the proliferative effect induced by GFs released from the THCPSi microparticles was statistically different from the fresh PL (1:40 dilution) control ($p < 0.001$). In addition, there were significant differences between the bare and the PL-modified THCPSi microparticles. No significant statistical differences in the proliferative effect were also observed between the two concentrations of the THCPSi microparticles tested.

The proliferative effect of the PL-modified PSi microparticles was however not improved over time (72 h) in comparison with the control fresh PL (1:40 dilution) (Figure 6b). Nevertheless, the PL-modified THCPSi microparticles induced statistically higher levels of proliferation when compared to the THCPSi microparticles alone or the control PL "as in particles". Overall, the GFs released from the PSi particles seem to be the responsible for the proliferative effect observed in the fibroblast cells.

3.6. In Vitro Wound Healing Assay. Next, we performed a wound healing assay (see Figure 7) using fibroblasts to

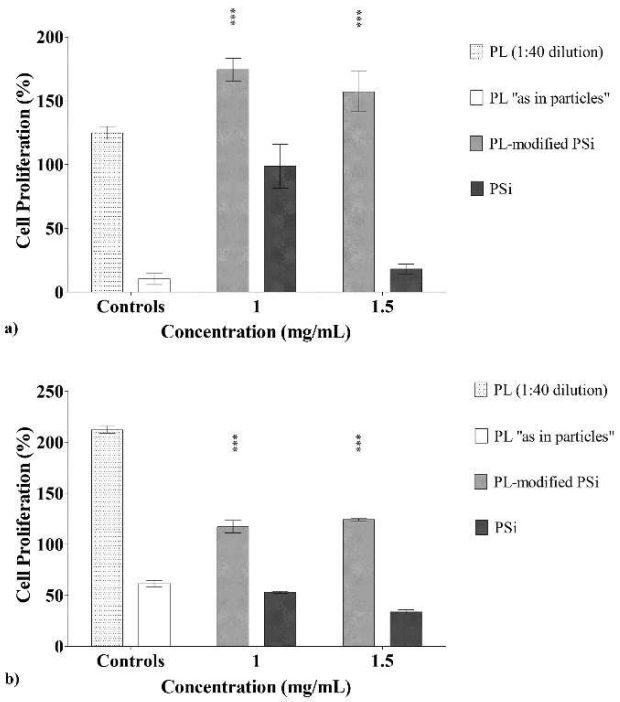

Figure 6. Fibroblast proliferation (%) after (a) 24 h and (b) 72 h of incubation with bare and PL-modified PSi microparticles at the concentrations of 1 and 1.5 mg/mL. Fresh PL (1:40 dilution) and "PL as in particles" were used as controls. The level of significance of cell proliferation after incubation with PL-modified THCPSi was measured vs unmodified particles. The level of significance was set at a probability of ***$p < 0.001$. Error bars represent mean ± SD ($n > 5$).

highlight the wound closure-promoting properties of the developed microparticulate formulation.

The cells' gaps were completely closed after 24 h of incubation with PL-modified THCPSi microparticles at both concentrations tested (1 and 1.5 mg/mL), as well as in the positive control (PL 1:40 dilution) (Figure 7). On the contrary, the controls PL "as in particles" and M w/serum, as well as the unmodified PSi microparticles, did not reach a closure of the gap during the time of the experiments (24 h). With this assay, it can be concluded that the developed PSi microparticulate system induces the complete closure of the gap between the fibroblast cells within 24 h similarly to the positive control (PL 1:40 dilution), under the experimental conditions tested.

3.7. Ex Vivo Assessment of the Proliferative Properties of PL-Modified PSi Microparticles. Finally, we evaluated the wound healing potential of PL-modified PSi microparticles in an *ex vivo* setup. Human skin was obtained from a cutaneous biopsy and divided into small pieces, which were lesioned with a punch. The samples were then applied over the lesions, and the tissues were fixed, at two different time points (24 and 72 h), before the immunostaining.

The morphological analysis of the tissues with the H&E analysis showed that both the samples (PL-modified THCPSi particles and unmodified ones) and the positive control (PL "as in particles") enhanced the acidophilia of the collagen fibers at 24 h (Figure 8a). This effect persisted over time in the positive control (PL "as in particles"), as an index of an ongoing regenerative process. In contrast, the tissue samples treated with bare and PL-modified THCPSi particles showed a faded staining, indicative of a resolved regeneration process.[40]

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ACS Appl. Mater. Interfaces 2016, 8, 988−996

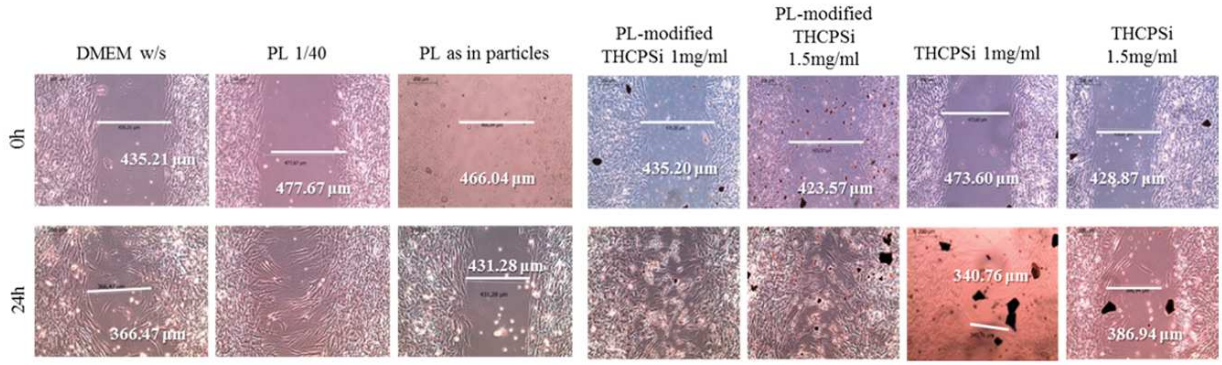

Figure 7. Photographs of the cells' gaps at 0 and 24 h after incubation with the samples at 37 °C in 5% CO_2 and 95% relative humidity. The cells were seeded into the two chambers of the inserts and left in the incubator overnight in order to attach to the μ-dish. On the following day, the insert was removed, and the samples were seeded into the appropriate dish. The white lines measure the width of the gaps in μm. Samples: DMEM without serum (M w/s); PL (1:40 dilution; PL 1/40); PL "as in particles"; PL-modified THCPSi 1 and 1.5 mg/mL; and THCPSi 1 and 1.5 mg/mL.

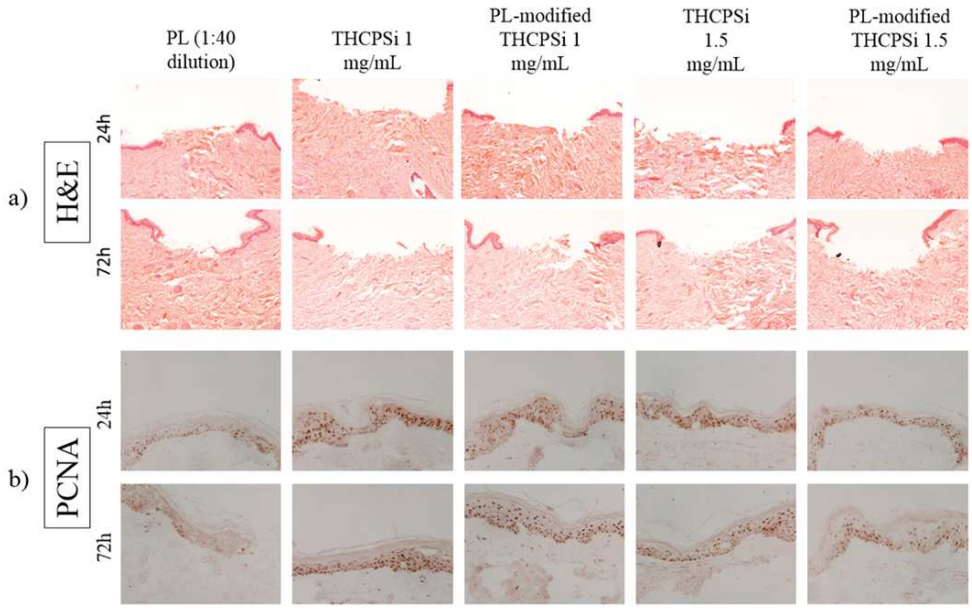

Figure 8. (a) Photographs of the tissues stained with H&E after fixation at two different time points (24 and 72 h). Tissues were treated both with bare and PL-modified THCPSi microparticles at the concentrations of 1 and 1.5 mg/mL. PL "as in particles" was used as positive control. (b) Photographs of the tissues immunostained with mouse antiproliferating cell nuclear antigen (PCNA) antibody after fixation at 24 and 72 h. Tissues were treated with bare and PL-modified THCPSi particles. PL "as in particles" was used as positive control. All microphotographs were taken at the same magnification with Zeiss Plan-Apochromat 10× /0.32 objective.

The immunostaining for the proliferative activity (antiproliferating cell nuclear antigen (PCNA) antibody) reveals an epithelial positivity, expressed as a marked nuclear staining of the epidermal keratinocytes, in particular those from the deep layers. The immunoreactivity to PCNA and BrdU (data not shown) was higher after 24 h of treatment with the PL-modified THCPSi particles, compared to the control PL "as in particles" and lasted until 72 h (see Figure 9). A statistically significant difference could be found between the three samples: between PL-modified THCPSi and the control PL "as in particles" with a level of probability $p < 0.01$, while among the PL-modified PSi particles and the microparticles as such with a level of probability of $p < 0.05$.

4. CONCLUSIONS

We successfully modified the PSi microparticles with PL, as shown both by the BCA and ELISA results. The transport experiments showed that the amount of GLs released from the PL-modified PSi microparticles was 3.18 μg/mL in 24 h, according to the BSA assay. THCPSi 53−75 μm showed the best cytocompatibility values among all the tested particles when exposed to fibroblast cells and was chosen for the following experiments. PL-modified THCPSi induced, over 24 h, a proliferative effect comparable to the positive control (PL 1:40 dilution). In addition, the transport experiments demonstrated that PL-modified THCPSi can induce a proliferative effect even higher than the one produced by the positive control. This was also confirmed by the assay evaluating the incorporation of BrdU in the DNA. However, for longer periods of time (72 h), the effect due to the proposed system faded in comparison to the positive control (PL 1:40 dilution). Nevertheless, the PL-modified PSi microparticles retained a significant difference compared to the negative controls (particles as such). Furthermore, the

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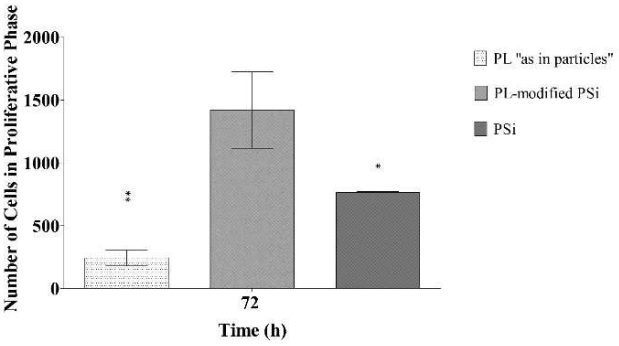

Figure 9. Number of cells in proliferation (anti-PCNA antibody) in the lesion after 72 h. PL "as in particles" was used as control. Bare and PL-modified THCPSi microparticles were assessed at the concentration of 1.5 mg/mL. The level of significance was measured against the sample PL-modified THCPSi and was set at levels of probability of $*p < 0.05$ and $**p < 0.01$. Error bars represent mean ± SD ($n \geq 2$).

assessment of the wound-closure promoting properties of the PL-modified THCPSi microparticles using an *in vitro* wound healing assay showed that this microparticulate system can close the gap in periods of time comparable to the positive control (PL 1:40 dilution). Finally, the *ex vivo* assay demonstrated that the developed microparticulate system induced an effective, although short-lasting, acidophilia of the collagen fibers, sign of ongoing regeneration. In addition, the immunostaining against PCNA and BrdU showed a massive proliferative effect of the PL-modified THCPSi compared to the positive control (PL "as in particles"). Overall, PL-modified THCPSi microparticles show promising properties for future applications in the treatment of chronic nonhealing wounds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b10950.

Experimental details and protocols on the immunostaining of the *ex vivo* samples, and results of the assessment of the particles' surface charge and of the quantification of PDGF-AB released from the system (PDF)

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Notes

The authors declare no competing financial interest.

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