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Oxygen Tolerant and Cytocompatible Iron(0)-Mediated ATRP Enables the Controlled Growth of Polymer Brushes from Mammalian Cell Cultures

Amine Layadi,‡ Benjamin Kessel,† Wenqing Yan,‡ Matteo Romio,‡,¥ Nicholas D. Spencer,‡ Marcy Zenobi-Wong,† Krzysztof Matyjaszewski,# and Edmondo M. Benetti*,‡,¥

‡ Laboratory for Surface Science and Technology, Department of Materials, ETH Zürich; Vladimir-Prelog-Weg 5, 8093 Zürich,

† Tissue Engineering + Biofabrication Laboratory, Department of Health Sciences and Technology, ETH Zürich, 8093 Zürich, Switzerland

Department of Chemistry, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, PA 15213, USA.

¥ Biointerfaces, Swiss Federal Laboratories for Materials Science and Technology (Empa), Lerchenfeldstrasse 5, CH-9014, St. Gallen, Switzerland

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ABSTRACT: The use of zerovalent iron (Fe^0)-coated plates, which act both as a source of catalyst and as a reducing agent during surface-initiated atom transfer radical polymerization (SI-ATRP), enables the controlled growth of a wide range of polymer brushes under ambient conditions, and utilizing either organic or aqueous reaction media. Thanks to its cytocompatibility, Fe^0 SI-ATRP can be applied within cell cultures, providing a tool that can broadly and dynamically modify the substrate's affinity towards cells, without influencing their viability. Upon systematically assessing the application of Fe-based catalytic systems in the controlled grafting of polymers, Fe^0 SI-ATRP emerges as an extremely versatile technique that could be applied to tune the physicochemical properties of cell's microenvironments on biomaterials or within tissue engineering constructs.

INTRODUCTION

Surface-modification strategies involving the controlled growth of polymer brushes by exploiting reversible deactivation radical polymerizations (RDRP) that are tolerant to ambient conditions have been a subject of increasing interest in the materials-science community.[1] The most prominent examples of such processes have involved the application of oxygen scavengers, in the form of photosensitizers,[2,3] reducing agents,[4] enzymes,[5] or zerovalent metal surfaces.[6-13]

While the evolution of oxygen-tolerant, surface-initiated RDRP (SI-RDRP) is paving the way for the translation of "grafted-from" polymers from fundamental studies into technology, the development of controlled polymerizations that are additionally compatible with physiological environments would substantially broaden the applicability of SI-RDRP in materials design. In particular, an RDRP process capable of modifying the physicochemical properties of cellular microenvironments through polymer grafting, without altering cell viability, would allow one to adjust the biological affinity of scaffolds towards a particular cell type during culturing, or alternatively to tune the presentation of biochemical cues triggering a defined cell behavior. More generally, the controlled growth of polymer brushes from cell-loaded supports would enable the fabrication of active biomaterials, capable of re-shaping themselves in a dynamic fashion following biocompatible processes.

Focusing on surface-initiated atom transfer radical polymerization (SI-ATRP), the most widely applied SI-RDRP method, we demonstrate here that the application of Fe^0 plates, acting as a source of both biocompatible catalyst and reducing agent, can enable the controlled, rapid growth of polymer brushes within organic as well as in aqueous and cell-culture media.

While introducing and systematically assessing the use of Fe-based catalytic systems in the fabrication of polymer brushes,[14] we show that Fe^0-mediated SI-ATRP (Fe^0 SI-ATRP) can be performed in the presence of primary articular chondrocytes (ACs) that had been previously cultured on initiator-bearing substrates, generating in situ polymer brushes that substantially alter the settlement of cells without affecting their viability.

RESULTS AND DISCUSSION

When a monomer and ligand (L) mixture is sandwiched between a Fe^0-coated plate and an ATRP-bearing substrate, the metallic surface rapidly consumes oxygen through the formation of Fe_xO_y species, which act as a source of Fe^{II}/Fe^{III} catalysts, diffusing towards the initiating sites (Figure 1). The growth of polymer brushes then proceeds according to the ATRP equilibrium, and can be efficiently performed without the need for prior deoxygenation of monomer mixtures, or an inert atmosphere.

To illustrate the mechanism of Fe^0 SI-ATRP and its main features, the synthesis of polymer brushes in organic solvents was first examined. Similarly to what was previously reported in the case of Cu^0-mediated SI-ATRP,[10] in the absence of externally added deactivator ($Fe^{III}Br_3$) the growth of poly(methyl methacrylate) (PMMA) brushes using anisole as solvent and 20 mM of weakly coordinating halide ligands (in the form of tetra-n-butylammonium bromide, TBABr)[15-17] proceeded slowly, reaching just a few nm of dry thickness in several hours of reaction (Figure 2a), as measured ex situ by variable-angle spectroscopic ellipsometry (VASE). In contrast, when 10 mM $Fe^{III}Br_3$ was added to the mixture, the polymerization proceeded rapidly, leading to a progressive growth of PMMA brushes, which reached ~ 20 nm after 5 hours of reaction. It is important to emphasize that an excess of TBABr with respect to $Fe^{III}Br_3$ was necessary to guarantee the complete dissolution of Fe species, especially in 50:50 anisole:MMA mixtures (v/v), where their solubility in the absence of ligand would be limited.[15]

In both these cases, an induction time necessary to oxygen consumption was never observed, suggesting that oxidation of Fe^0 surface quickly takes place just at the very early stages of the reaction.

An analogous result was obtained in the case of Fe^0 SI-ATRP of oligo(ethylene glycol) methacrylate (OEGMA) performed in N,N-dimethylformamide (DMF), where a comparable amount of $Fe^{III}Br_3$ was required to attain a significant growth of POEGMA brushes, reaching nearly 30 nm of thickness in 5 hours (Figure 2b).

The role played by added $Fe^{III}Br_3$ was elucidated while monitoring the variation of POEGMA-brush thickness as a function of $Fe^{III}Br_3$ concentration for fixed polymerization times (Figure 2c). The thickness of POEGMA brushes increased with [$Fe^{III}Br_3$], reaching a maximum between 5 and 10 mM. Under these conditions, Fe^{III} species acted both as a deactivator, suppressing irreversible termination by radical recombination between growing chains, and contributed to generating Fe^{II}-based activators through comproportionation with Fe^0 (Figure 1), in this way accelerating the grafting process.[15]

A further addition of $Fe^{III}Br_3$ beyond 10 mM led to a decrease in brush thickness, suggesting a significant deactivation and a simultaneous slowing down of the polymerization.

It is important to emphasize that the balance between the above-mentioned effects determined the position of the maximum of polymer-brush thickness as a function of [$Fe^{III}Br_3$], which might shift during the progression of the grafting process. An increment in the content of activators through comproportionation led to an increase in brush-growth rate, especially during the early stages of polymerization. Simultaneously, an increase in deactivation by added Fe^{III} species slowed down the grafting process, although it additionally suppressed irreversible termination by radical recombination between propagating grafts, which is especially relevant after relatively long reaction times (for more flexible, high-molecular-weight grafted chains).[18]

Fe^0 SI-ATRP was subsequently investigated in aqueous environments, and using ethylenediaminetetraacetic acid (EDTA) as a ligand. EDTA strongly binds Fe species to form cytocompatible complexes[19] to efficiently catalyze Fe-based ATRP in solution and heterogeneous systems, as previously demonstrated.[20,21]

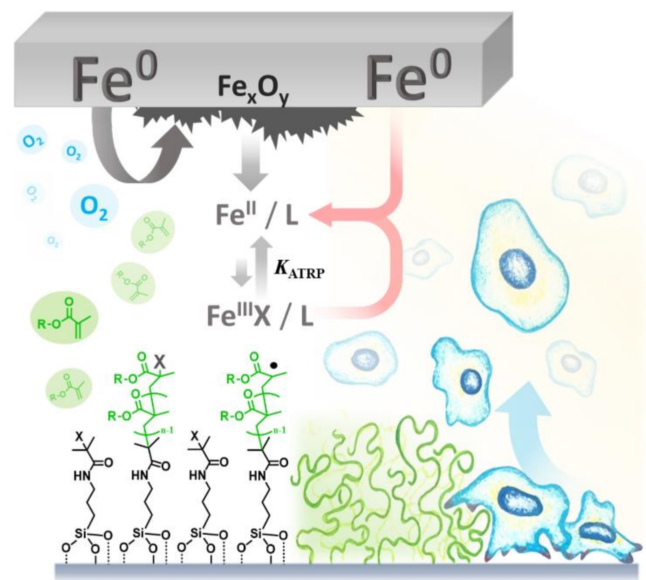

Figure 1. Schematic depicting the mechanism of Fe^0 SI-ATRP.

In the exemplary case of Fe^0 SI-ATRP of OEGMA performed in 50% water mixtures (v/v), the growth of brushes followed much faster kinetics compared to those recorded in organic media, as expected from the significantly larger ATRP equilibrium constant (K_{ATRP}) that is characteristic for aqueous systems.[22-24] More than 150 nm-thick POEGMA brushes could be grown in just one hour of polymerization when 2.5-5 mM $Fe^{III}Cl_3$-EDTA were added to the aqueous reaction mixture (Figure 2d and Figure S1), whereas just ~ 5 nm-thick brushes were grafted after the same time using DMF as solvent, and no appreciable brush growth could be recorded in anisole.

The presence of 5 mM EDTA alone enabled the synthesis of significantly thinner films, which initially grew following

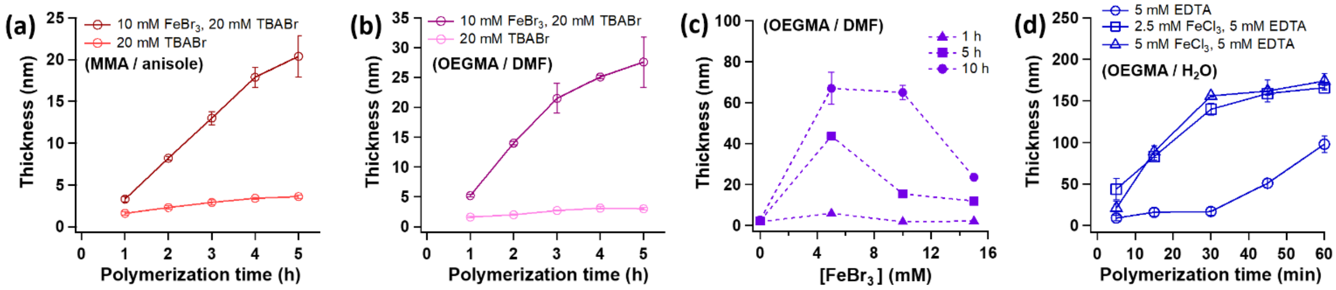

Figure 2. (a) Dry thickness of PMMA brushes measured ex situ by VASE during Fe° SI-ATRP of MMA performed in 50% (v/v) anisole solutions with 20 mM TBABr and with or without 10 mM $Fe^{III}Br_3$. (b) Fe° SI-ATRP of OEGMA performed in 50% (v/v) DMF solutions with 20 mM TBABr and with or without 10 mM $Fe^{III}Br_3$. (c) Variation of POEGMA-brush thickness measured by VASE, as a function of $Fe^{III}Br_3$ concentration, while keeping the concentration of TBABr fixed at 20 mM. (d) Dry thickness of POEGMA brushes measured by VASE during Fe° SI-ATRP of OEGMA performed in 50% (v/v) water solutions containing 5 mM EDTA, 10 mM NaCl and variable concentrations of $Fe^{III}Cl_3$.

relatively slow kinetics. This was presumably due to the time required by Fe^{II}/Fe^{III}-EDTA adducts to diffuse from the Fe plate to the initiator-bearing substrate.[10]

Using 5 mM $Fe^{III}Cl_3$-EDTA, a range of bio-relevant polymer brushes could be grafted by polymerizing 2-methacryloyloxyethyl phosphorylcholine (MPC, 3 M), N-isopropylacrylamide (NIPAM, 2 M) and 2-hydroxyethyl methacrylate (HEMA, 4 M), obtaining extremely thick PMPC (250 ± 7 nm), PNIPAM (219 ± 12 nm) and PHEMA (137 ± 5 nm) films just after 45 min of reaction (Figure 3a). Aqueous Fe° SI-ATRP could be further applied for growing similar brush films from large substrates, as demonstrated in the case of PMPC brushes, which could be grown from 4-inch silicon wafers, forming ~ 200 nm-thick films with homogeneous thickness and morphology after 30 min of polymerization (Figure 3c and Figure S3). Detachment of PMPC grafts[25-27] enabled their characterization by size-exclusion chromatography (SEC), yielding a M_n of 600 kDa and a $Đ$ of 1.34 (corresponding to a surface coverage of 0.23 chains nm^{-2}),[28] and suggesting a well-controlled polymerization process (inset in Figure 3c).

In order to test the applicability of Fe° SI-ATRP within cell cultures, we subsequently analyzed the growth of PMPC brushes using Dulbecco's modified Eagle's medium (DMEM) as a solvent, and simultaneously tested the cytocompatibility of the polymerization conditions employed towards ACs.

PMPC brushes are especially relevant in the design of biointerfaces due to their biocompatibility and inertness towards unspecific protein and cell contamination.[29-31] As a result of their pronounced hydration, the growth of PMPC grafts from substrates previously cultured with cells would directly translate into an evident variation in the strength of cell attachment, leading to the progressive detachment of ACs when the thickness and density of the underlying brushes reached sufficiently high values.[32]

Fe° SI-ATRP of a diluted, 0.3 M MPC solution in DMEM using 5 mM $Fe^{III}Cl_3$-EDTA resulted in a slow but notable growth of brushes, which reached ~ 15 nm after 60 min of reaction (Figure 3b). Relevantly, the slower polymer-brush thickening rate compared to those recorded using pure water as solvent (Figure 3a) was mainly due to the relatively low monomer concentration employed (Figure S4). When ACs were cultured for 24 h on tissue culture polystyrene wells (TCPS), and subsequently exposed for 1

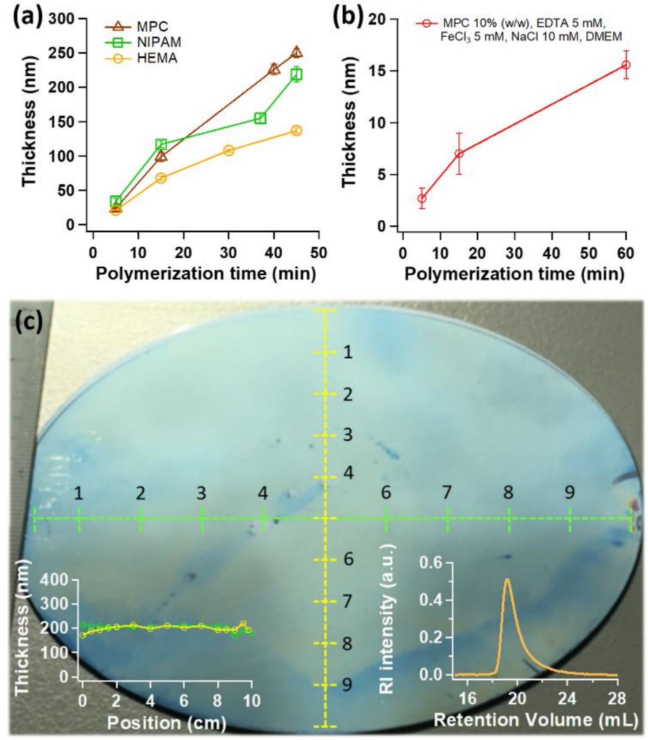

Figure 3. (a) Evolution of brush thickness measured by VASE during Fe° SI-ATRP of MPC, NIPAM and HEMA. (b) PMPC-brush thickness measured by VASE during Fe° SI-ATRP performed in 0.3 M MPC DMEM solutions. (c) PMPC brushes grafted from an entire, 4-inch silicon wafer by Fe° SI-ATRP. The brush thickness measured by VASE across the surface is reported in the inset. A SEC trace from detached PMPC brushes is also shown.

hour to a 0.3 M DMEM solution of MPC, the cell density did not significantly vary (from 45000 ± 12700 to 43200 ± 7500 cells cm^{-2}, $p = 0.99$) (Figure 4a), while cell viability was higher than 90% just after exposure to monomer solution, and increased to 98% after 48 h of additional culture in standard DMEM-based medium (Figure 4b). Incubation for 1 h in 5 mM DMEM solution of $Fe^{III}Cl_3$-EDTA resulted in a significant reduction in cell density (17500 ± 5300 cells cm^{-2}) both with respect to the control ($p = 0.03$), and compared to the substrates treated with just 0.3 M MPC ($p = 0.05$) (Figure 4a).

Partial cell detachment after treatment with $Fe^{III}Cl_3$-EDTA was presumably due to complexation of Ca^{II} within membrane proteins by residual amounts of unbound ligand, which influenced integrin-mediated ACs

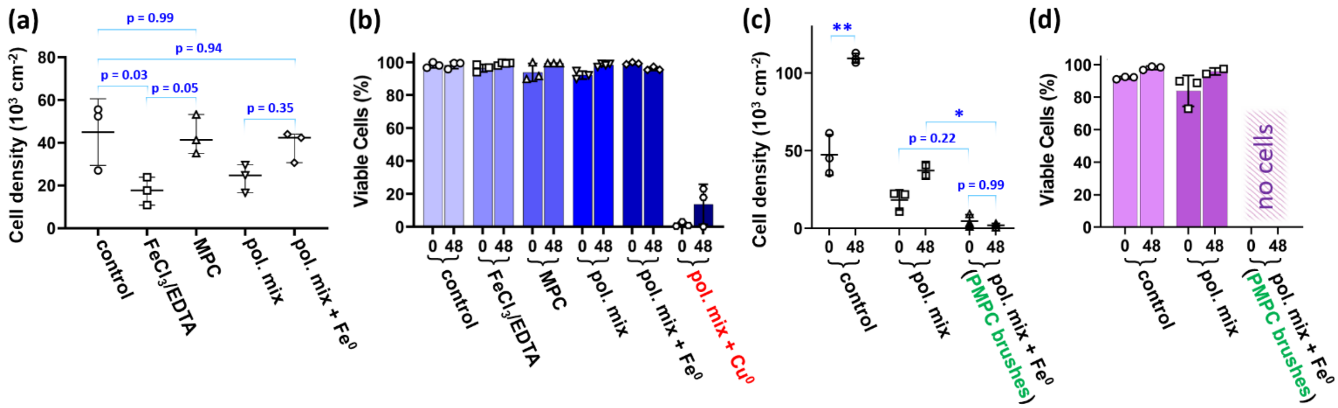

Figure 4. (a) Density of ACs estimated after 24 h of culture on TCPS, followed by exposure for 1 h to different media, including DMEM (indicated as control), 5 mM EDTA with 5 mM $Fe^{III}Cl_3$ in DMEM (indicated as EDTA/$FeCl_3$), 0.3 M MPC in DMEM (indicated as MPC), 0.3 M MPC with 5 mM $Fe^{III}Cl_3$-EDTA and 10 mM NaCl (indicated as pol. mix), and the latter mixture covered by a Fe^0 plate (indicated as pol. mix + Fe^0). Statistical analysis was conducted by one-way analysis of variance (ANOVA) with a Dunnett's post-hoc-Test; * indicates $p < 0.05$. (b) Cell viability estimated by live-dead staining on AC cultures subjected for 1 h to the solutions indicated above, and a polymerization mixture including 0.3 M MPC, 5 mM $CuBr_2$-tris(2-pyridylmethyl)amine (TPMA), 10 mM NaBr, and covered with a Cu^0 plate.[12] (c) Density of ACs estimated after 24 h of culture on ATRP initiator-functionalized SiO_x substrates and subjected to 1 h of exposure to different mixtures, and after the growth of PMPC brushes (pol. mix + Fe^0). Statistical analysis was conducted by two-way ANOVA with Tukey's multiple comparisons test; * indicates $p < 0.05$, ** indicates $p < 0.001$. (d) Cell viability estimated by live-dead staining for AC cultures on ATRP initiator-bearing SiO_x surfaces subjected for 1 h to different mixtures.

adhesion.[33,34] A comparable result was observed when ACs were incubated in the complete polymerization mixture (0.3 M MPC + 5 mM $Fe^{III}Cl_3$-EDTA), while a slight increment in cell density was recorded when Fe^0-coated plates were additionally placed in contact with the cultures, reproducing the reaction setup typically employed during Fe^0 SI-ATRP of MPC ($p = 0.35$, Figure 4a). In these samples, additional Fe^{II}/Fe^{III} species leaking from the iron plates were probably complexed by the residual, unbound EDTA, finally preventing complexation of Ca^{II} from cell proteins.

It is important to emphasize that, despite the observed variations in cell density after exposure to the different monomer/ligand mixtures, AC viability was in all cases higher than 90%, and comparable to that measured for the controls. Even when the oxygen dissolved in the culture media was rapidly consumed by exposure to Fe^0, the tolerance of chondrocytes towards anaerobic conditions[35] guaranteed their remarkably high viability, while AC densities were similar to those recorded for the control samples (39000 ± 6000 and 45000 ± 12700 cells cm^{-2}, respectively, in Figure 4a, $p = 0.94$). The cytocompatibility of the polymerization mixtures employed during Fe^0 SI-ATRP was further highlighted by comparing the viability of ACs exposed to a corresponding Cu-based catalytic system, which was previously reported to enable the rapid growth of polymer brushes by Cu^0 SI-ATRP.[6-13] In this latter case, AC viability was nearly 0% after 1 h of exposure, and increased just to ~ 10% following 48 h of further culture in standard medium (Figure 4b and S5), confirming the toxicity towards mammalian cells of mixtures typically employed during Cu-based ATRP.

When TCPS substrates were replaced by ATRP-initiator-bearing SiO_x surfaces, the effect of PMPC brush growth on AC adhesion was analyzed.

Also in these experiments, ACs were initially cultured on the substrates for 24 h, followed by exposure to different mixtures for 1h, followed by additional 48 h of culture.

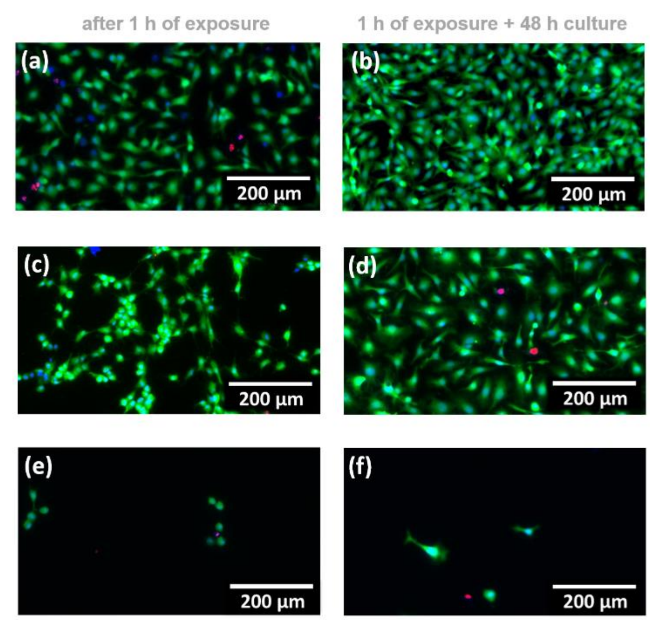

Figure 5. Immunofluorescence micrographs displaying ACs stained with calcein AM (green), Hoechst 33342 (blue), and propidium iodide (red) after 1 h of exposure to the different polymerization mixtures (a, c, e) and following 48 h of further culture (b, d, f).

Cell density significantly decreased after 1 h of exposure to the polymerization mixture (0.3 M MPC, 5 mM $Fe^{III}Cl_3$-EDTA) compared to that measured when ACs were simply incubated in DMEM-based medium, from 47400 ± 11000 to 18300 ± 12700 cells cm^{-2} ($p < 0.05$). Nevertheless, cell viability remained relatively high (83 ± 15%). As highlighted in the immunofluorescence micrographs reported in Figure 5c, ACs exhibited a more rounded morphology as a result of partial inhibition of cell-cell and cell-substrate adhesion through Ca^{II} complexation by EDTA. However, it is remarkable that following a further 48 h of culture in standard medium an increment in cell density from 18300 ± 12700 to 37300 ± 6900 cells cm^{-2} ($p = 0.08$) was observed, due to AC proliferation, basically attaining cell densities similar to those recorded before exposure to the polymerization medium. This result confirmed the cytocompatibility of the polymerization conditions applied during Fe^0 SI-ATRP. In addition, a well-spread cell morphology was completely recovered (Figure 5d), indicating that ACs strongly adhered to the substrate after an additional 48 h of culture.

When Fe^0-coated plates were applied together with the polymerization mixtures on AC cultures, the rapid growth of PMPC brushes led to the formation of a highly hydrated, and cell-repellent interface, which substantially weakened AC attachment and lead to a nearly complete detachment of cells (4700 ± 4300 cells cm^{-2}, after 1 h of exposure). Relatively few, rounded cells remained on the substrates, adhering to surface defects where polymer grafting did not occur due to the adventitious, mechanical removal of ATRP initiator, and were surrounded by a uniform film of PMPC brushes, as evidenced by atomic force microscopy (AFM) (Figure 6). Selective deposition of ATRP initiator on just one side of a silicon substrate additionally proved that Fe^0 SI-ATRP could be easily applied to spatially control the growth of biopassive polymer brushes, and trigger cell detachment specifically on the areas of the substrate where polymer grafting could be initiated (Figure S6).

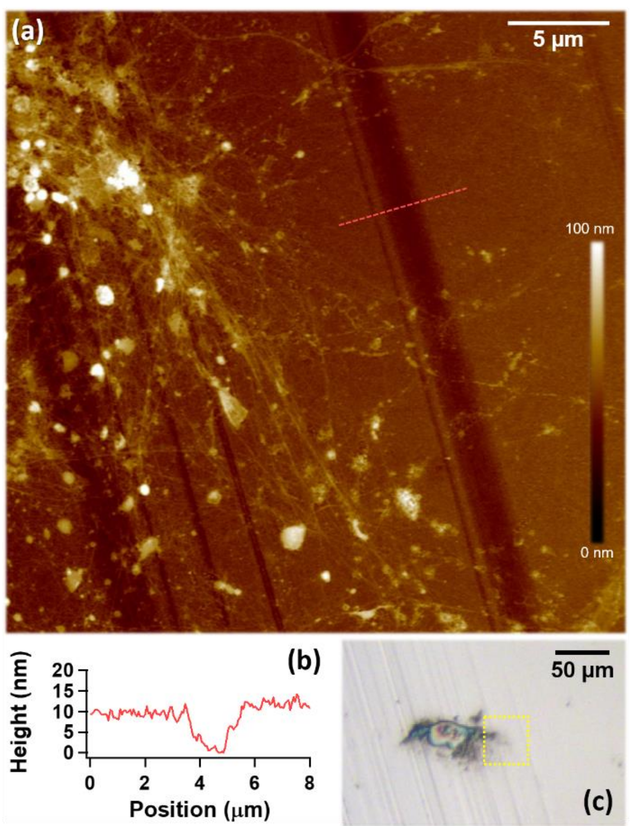

Figure 6. (a) AFM tapping mode micrographs displaying cellular components of ACs extending on surface defects where PMPC brushes did not grow following Fe^0 SI-ATRP, due to mechanical removal of ATRP initiator layer. In (b) a cross section highlighting the height of PMPC brushes around a mechanically scratched area on the substrate is provided. The optical micrograph (c) displays how ACs remained attached on areas where PMPC brushes could not be grown due to the removal of ATRP initiator layer by mechanical scratching.

These results collectively indicated that PMPC-brush growth by Fe^0 SI-ATRP could be performed from AC cultures, substantially altering the interfacial physicochemical properties of the supporting substrates, and thus strongly influencing cell attachment. The observed release of cells was clearly not due to the polymerization conditions applied during culturing, as confirmed by the tolerance of ACs towards the reaction mixtures, their markedly high viability and tendency to proliferate in such media.

The growth of bio-repellent brushes, mediated by Fe^0, emerges as the main phenomenon leading to a critical shift in the characteristics of the surrounding environment, to which ACs respond by weakening their affinity towards the substrates, ultimately being released from a surface that is no longer bio-adhesive.

It is noteworthy to mention that ATRP methods were already demonstrated to be applicable within cell cultures while maintaining viable cells.[36] However, polymer growth was tested in the presence of yeast cells, which are known to be extremely resilient, and which, similarly to bacteria, are protected by strong cell walls and can survive greater stresses than mammalian cells.[37]

CONCLUSIONS

Due to its tolerance to ambient conditions and scalability, Fe^0 SI-ATRP emerges as a versatile method to synthesize chemically diverse polymer brushes for a variety of materials formulations. Its highly confined nature enables the generation of uniform brush films over very large areas, without the need of previous deoxygenation of the reaction mixtures or the presence of an inert atmosphere, and by employing just few microliters of monomer/catalyst solutions. These unique features, which are highlighted through a comprehensive analysis of its mechanistic aspects, make Fe^0 SI-ATRP a very promising technique for translating SI-RDRP processes into technologically relevant surface fabrications.

Thanks to its cytocompatibility towards mammalian cells, Fe^0 SI-ATRP could be easily applied to biomaterials and within tissue engineering constructs, dynamically tuning the physicochemical properties of cell microenvironments.

ASSOCIATED CONTENT

Experimental details and further characterization are included in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

* Edmondo M. Benetti, Laboratory for Surface Science and Technology, Department of Materials, ETH Zürich, Vladimir-Prelog-Weg 5, 8093 Zürich; Biointerfaces, Swiss Federal Laboratories for Materials Science and Technology (Empa), Lerchenfeldstrasse 5, CH-9014, St. Gallen, Switzerland.
EMail: edmondo.benetti@mat.ethz.ch

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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