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Developing a Continuous Bioprocessing Approach to Stromal Cell Manufacture

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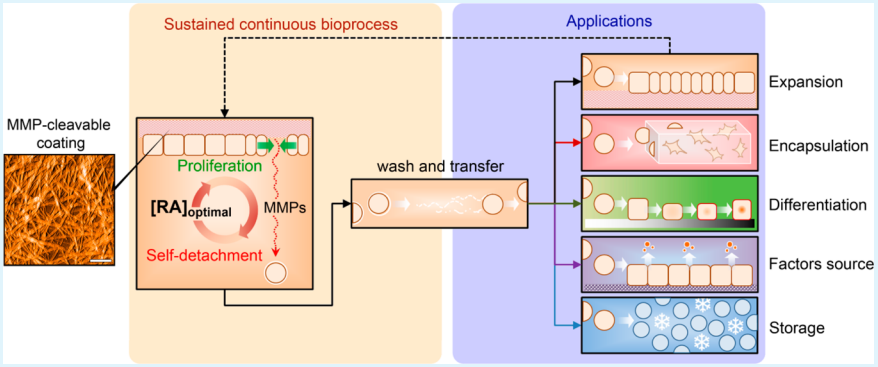
Supporting Information



ABSTRACT: To this day, the concept of continuous bioprocessing has been applied mostly to the manufacture of molecular biologics such as proteins, growth factors, and secondary metabolites with biopharmaceutical uses. The present work now sets to explore the potential application of continuous bioprocess methods to source large numbers of human adherent cells with potential therapeutic value. To this purpose, we developed a smart multifunctional surface coating capable of controlling the attachment, proliferation, and subsequent self-detachment of human corneal stromal cells. This system allowed the maintenance of cell cultures under steady-state growth conditions, where self-detaching cells were continuously replenished by the proliferation of those remaining attached. This facilitated a closed, continuous bioprocessing platform with recovery of approximately 1% of the total adherent cells per hour, a yield rate that was maintained for 1 month. Moreover, both attached and self-detached cells were shown to retain their original phenotype. Together, these results represent the proof-of-concept for a new high-throughput, high-standard, and low-cost biomanufacturing strategy with multiple potentials and important downstream applications.

KEYWORDS: *continuous bioprocessing, peptide amphiphiles, retinoic acid, cell self-release, smart materials, corneal stromal cells*

1. INTRODUCTION

It is well-known that regenerative medicine, and in particular cell-therapy techniques, has been expanding its repertoire in terms of both purposeful cell types and applications, raising the potential to cure a wide range of diseases. However, the high number of therapeutic cells required for some treatments (this can be up to 1 billion) presents, in terms of cell manufacture, a major challenge. Furthermore, as the final product is represented by the cells themselves, numerous requirements have to be met under the current regulatory framework for cellular therapy products. In particular, the system used for cell expansion has to be compliant with the current good manufacturing practices (cGMP); the fluid path should be closed and sterile while minimizing human intervention; the culture media should be chemically defined and preferably not contain serum or other xenobiotics; and the process should be standardized, reproducible, and in general possessing characteristics that maintain the desired cell phenotype throughout the entire process while ensuring reasonable costs of goods are maintained.[1] Thus, implementing and adapting industrial processes, previously developed for large-scale mammalian cell culture for the production of biologics, has been attempted in order to sustain such a great demand for cells and to meet the required conditions.[2] Of these processes, one of the most advanced methods for obtaining large numbers of adherent cells is through automation[3] or microcarrier-based expansion using perfusion bioreactors.[4] While substantial increases in cell yield have been achieved by improving microcarrier function, some other critical production steps are still undergoing optimization. For instance, improved cell harvesting methods for easier and more effective recovery

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of the expanded cells from their microcarrier culture substrates are required.[5] For these reasons and others such as cost, microcarrier preparation, and the time/resources required to translate established 2D flask-based protocols to 3D microcarrier cultures, the uptake of microcarrier-based systems by commercial organizations has been slow.

We suggest that the development of a continuous bioprocessing system could meet all the required criteria for therapeutic cell expansion, in terms of both efficiency and regulatory compliance while avoiding the pitfalls of microcarrier-based systems and the limitations of conventional 2D cell culture.[6] The general concept of continuous bioprocessing, introduced more than 30 years ago,[7] denotes a closed system that operates unremittingly, processing the constantly flowing raw material into the intermediate or final products. There are several advantages to such a system, including reduction in manufacturing costs and in the size of manufacturing facilities, as well as the improvements in quality, reproducibility, and standardization of the final product.[8] Continuous bioprocessing is currently being applied in biotechnology (e.g., for the production of biopharmaceuticals, recombinant proteins, and monoclonal antibodies).[9] However, this methodology is not currently being implemented for the manufacture of cells with therapeutic potential.

Therefore, in this study we developed a proof-of-concept strategy for the continuous manufacture of human primary cells. Specifically, we designed a culture system that controlled the adhesion/detachment of human primary corneal stromal cells (CSCs) to a bioactive substrate while maintaining a proliferative steady-state condition, i.e., where the number of cells detached/produced for downstream applications was matched by their proliferation, ensuring the maintenance of a closed, continuous bioprocessing platform. The technology underpinning this is a smart enzyme-sensitive coating comprising a fully synthetic, multifunctional peptide amphiphile (PA). This PA resulted from a mix of two different self-assembling molecules, a biofunctional one containing a matrix metalloprotease (MMP)-cleavable site contiguous to an RGDS cell adhesion motif (MMP/RGDS), and a second PA (ETTES) used as a diluent.[10] Thus, the number of self-detached cells from the PA coating was modulated by the extent of the specific MMP/RGDS PA cleavage, which in turn was regulated by the degree of endogenous MMP expression, a factor controlled by the addition of all-*trans* retinoic acid (RA) in serum-free medium (SFM) conditions.[11] This preliminary study puts forward a nascent technology on which the future development of a continuous bioprocess system for cell manufacture could be built.

2. EXPERIMENTAL METHODS

2.1. Human Corneal Stromal Cell Isolation and Culture. Corneal stromal cells (CSCs) were isolated (as previously described[11]) from cadaverous human corneal tissue (donors' ages between 39 and 76; average ± SD = 61 ± 12 years, male−female donor ratio of 2:3, with no prior history of corneal diseases or ocular trauma). All corneas were obtained from NHS Blood and Transplant (NHSBT) through a service level agreement with Newcastle-upon-Tyne Hospitals NHS Foundation Trust, U.K. All donors gave consent for their corneas to be used in research, in full accordance with our regional ethics committee approval and research agreement. Briefly, the epithelia-depleted corneal tissues were minced using a scalpel, transferred to DMEM/F12 medium (Thermo Scientific, Waltham, MA) supplemented with 5% fetal bovine serum (FBS; BioSera), 2 g L^{-1} (450 units mL^{-1}) collagenase type-1 (Thermo Scientific) and incubated at 37 °C under continuous rotation for 5 h, followed by incubation with 0.25% trypsin-EDTA (Thermo Scientific) for 10 min. The isolated CSCs were plated onto tissue culture flasks (Greiner Bio-One) and maintained using DMEM/F12 medium supplemented with 5% FBS and 1% penicillin/streptomycin (Thermo Scientific). Media were changed every 2−3 days, and cultures were maintained until reaching 70−80% confluence. Cells were then maintained for 3 days in serum-free medium (SFM) comprised of DMEM/F12 with 1×10^{-3} M ascorbic acid (Sigma-Aldrich, St. Louis, MO), 1× ITS (Sigma-Aldrich), and 1% penicillin/streptomycin to induce CSCs quiescence, after which cells were passaged and used for the different assays. Each experiment was performed three times using CSCs at passage 3−5 from three different donors.

2.2. Preparation and Stability of PA Coatings. PAs were custom-synthesized (CS Bio, Menlo Park, CA) as >95% pure trifluoroacetic acid salts and their molecular weight (MW) confirmed by electrospray−mass spectrometry. Lyophilized C_{16}-TPGPQG↓IAGQRGDS (↓ indicates cleavage site for MMP1; MW, 1578.89) and C_{16}-ETTES (MW, 803.60) were weighed and dissolved in ultrapure double-distilled water as binary component solutions to the final concentration of 0.125 mM at the molar ratio of 15:85 and stored at 4 °C. For the production of thin, dry film coatings, the PA solution was drop-spotted onto the surface of UV-sterilized glass coverslips 12 mm Ø (Menzel Glaäser) or low-attachment tissue culture plates (Corning Inc., Corning, NY) at 50 μL cm^{-2} and dried overnight at room temperature inside an aseptic Class II cell culture cabinet. Wettability and contact angle of PA solutions were determined using a static sessile drop method. Photographs of PA drops were taken using a D90 digital camera (Nikon). The MMP/RGDS−ETTES PA was analyzed for its sensitivity to matrix metalloprotease (MMP) cleavage. Briefly, PA-coated glass coverslips were transferred to 12-well plates (Greiner Bio-One) and incubated with SFM for 15 days, together with 4×10^4 CSCs seeded in 12 mm Ø Transwell cell culture inserts (Corning) to serve as source of MMPs. The amount of MMPs expressed by CSCs was regulated by supplementing the medium with 0.01, 1, or 10 μM RA, with increasing RA levels resulting in decreasing MMP concentrations in the medium, and corresponding lower capacity to cleave the cocultured PA coatings. At day 15, PA-coated coverslips were recovered, dried, and analyzed for PA topography and structure using atomic force microscopy (AFM). PA coatings before and after incubation with medium only were similarly analyzed, to assess coating stability in aqueous environment.

2.3. Atomic Force Microscopy (AFM). Analysis of PA-coated surface topography was performed using an Easyscan 2 atomic force microscope (Nanosurf) equipped with ContAI-G soft contact mode cantilevers (BudgetSensors) with a resonant frequency of 13 kHz and nominal spring constant of 0.2 N m^{-1}. Briefly, the different PAs (single-system PAs MMP/RGDS and ETTES, and binary-system PA MMP/RGDS−ETTES) were used to coat glass slides, and surface topography was analyzed from three separate regions in each sample, with 512× two-direction lines scanned at 10 μm s^{-1}, at 1 nV, and with P- and I-gains of 1. Topographic data was processed for line-wise and tilt correction using the Scanning Probe Image Processor software package (Image Metrology A/S). Data was analyzed using the OrientationJ plugin from ImageJ v1.46 software for measuring dimensions and distribution of 100 individual PA nanotapes. All experiments were performed on three individual areas in each coating (n = 3).

2.4. Static Cell Culture. Each PA-coated coverslip was seeded with 3.5×10^4 stromal cells cm^{-2} and cultured in SFM. RA stock solutions were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a 0.01−25 mM concentration range, and stored at −80 °C. Prior to use, each stock solution was diluted 1:1000 in SFM to reach final concentrations of 0.01, 0.1, 0.25, 0.5, 0.75, 1, 5, 10, and 25 μM (the latter is known to be toxic). For experimental control, DMSO only was diluted 1:1000 in SFM and used to culture cells in both coated and uncoated wells. The RA treatment was started 24 h postseeding, and cells were cultured for a further 6 days. Every 48 h, 1:10 of the media volume from each condition was removed and the same amount of fresh media (SFM+RA; 10× concentration) was added back into the well in order to maintain the RA concentration in

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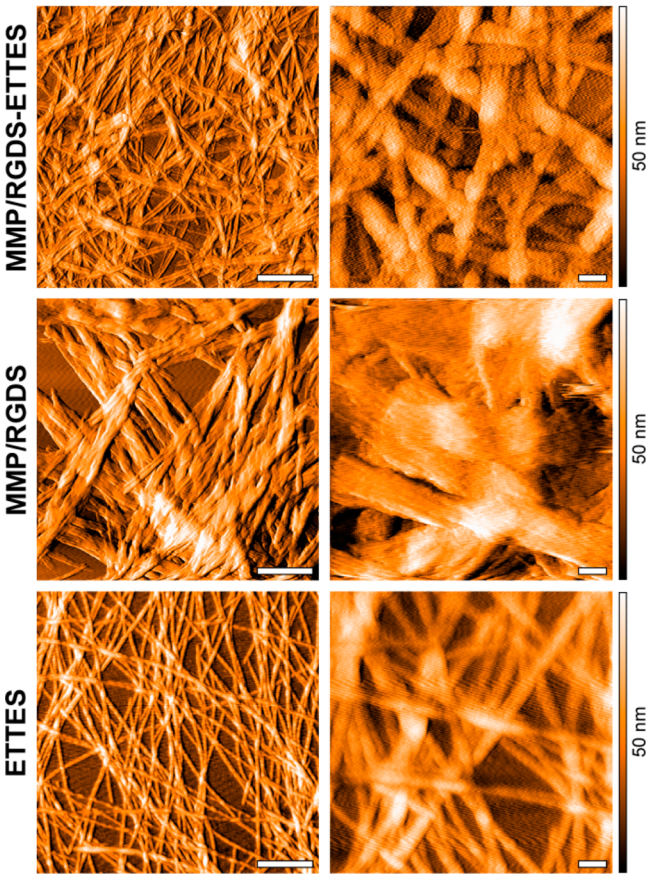

Figure 1. Topography and nanostructure of PAs used as coatings. False color images of forward deflection scans performed by atomic force microscopy (AFM) of multifunctional MMP/RGDS−ETTES (upper panels), and of each of its comprising PAs, MMP/RGDS (central) or ETTES (lower panels) as single-system nanotapes. The overall topography and distribution (left panels) and the detailed dimensions of PA nanotapes forming the coatings (right panels) can be observed. False color scale, 50 nm. Scale bars, 1 μm (left) and 200 nm (right panels).

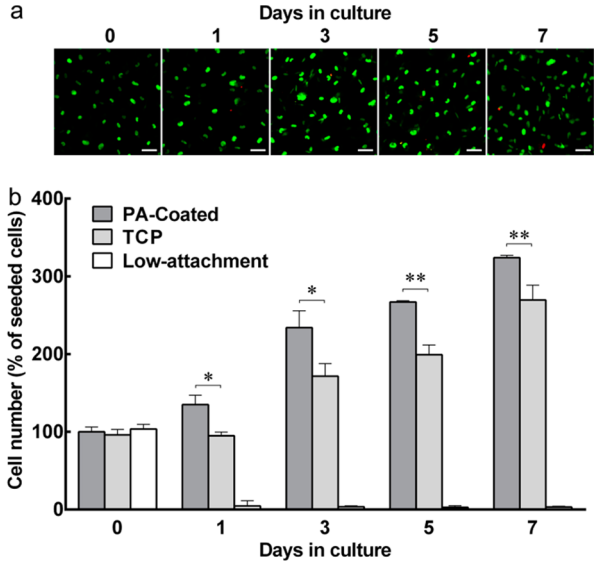

Figure 2. Effect of PA coating on cell viability and proliferation. (a) CSCs grown up to 7 days on PA-coated low-attachment surfaces were shown to remain highly viable throughout the period in culture, as indicated by the live/dead double cell staining (Calcein-AM, green; propidium iodide, red). Scale bars: 100 μm. (b) CSCs seeded onto PA-coated (dark gray) or uncoated low-attachment (negative control; white bars) surfaces, as well as on normal uncoated TCP (positive control; light gray bars), were quantified using the AlamarBlue assay throughout the period in culture. Data (mean ± SD), represented as percentage of initial cell seeding number, was obtained from three independent experiments (n = 3), and analyzed statistically using one-way ANOVA with Bonferroni's *post hoc* test (* and ** corresponded to $p < 0.05$ and 0.01, respectively).

each condition. For evaluation of cell proliferation, AlamarBlue assay was performed on cells attached to the coating. Briefly, cells were incubated with resazurin reagent (Sigma-Aldrich), prepared in 1:10 dilution using fresh culture medium, for 3 h, after which 100 μL of culture supernatants (in triplicate) were sampled for fluorescence emission analysis at 590 nm using a Fluoroskan Ascent fluorescent spectrophotometer (Thermo Scientific). The supernatant from each culture condition was collected at day 7, with cells detached via MMP-induced cleavage of the PA coating being retrieved following centrifugation at 1500 rpm for 5 min. Such cells were then seeded onto a new 6-well TCP plate (Greiner Bio-One) and allowed to grow in DMEM/F12 supplemented with 5% FBS. This approach allowed us to evaluate the viability and the capacity of reattachment of the self-detached cells. An AlamarBlue assay was performed on these detached/reattached cells, as previously described, following 3 days of culture. Subsequently, cell viability of both attached and self-detached/reattached cells was analyzed using the calcein-AM/propidium iodide (PI) double staining assay (Sigma-Aldrich) according to the manufacturer's instructions. After 30 min of incubation and three PBS washes, cells were visualized under a Zeiss Axiovert 1 fluorescence microscope (Zeiss Microscopy) using the appropriate filters. Calcein-AM/PI images were merged using ImageJ v1.46 software.

2.5. Dynamic Cell Culture. Each PA-coated coverslip was seeded with 3.5×10^4 stromal cells cm^{-2}. Cells were left to attach for 2 h in SFM at 37 °C. Subsequently each coverslip was placed upside-down on top of a Transwell cell culture insert (Corning) with a hole cut into it having a diameter slightly smaller than the coverslip. The constructs were then placed in 6-well plates and cultured in SFM containing either 0 or 0.1 μM RA for up to 30 days, renewing the RA treatments every 2 days. The number and viability of cells remaining attached to the coverslips and of those reattached to the bottom surface of the TCP plates were measured at day 15 and day 30, as described for the static cell culture.

2.6. Quantitative Polymerase Chain Reaction (qPCR) Analysis. At the end of the 7 day experiment in static culture, all CSCs from different RA concentrations including the controls were harvested, and RNA was isolated using standard Trizol (Thermo Scientific) extraction. The assessment of RNA quality was performed using a Nanodrop 2000 spectrophotometer (Thermo Scientific) to ensure the 260/280 ratio was within the range 1.7−2.0. Synthesis of cDNA from isolated total RNA was done using the RT2 First Strand kit (Qiagen) according to the manufacturer's protocol, in a TcPlus thermocycler (Techne). The polymerase chain reaction (PCR) was carried out using the default thermal profile of the Eco Real-Time System (Illumina, San Diego, CA), with the following 40× three-step cycle: 10 s of denaturation, 95 °C; 30 s of annealing, 60 °C; 15 s of elongation, 72 °C. The relative expressions of *MMP1* from three independent experiments from three different donors were calculated by the comparative threshold cycle (CT; Eco Software v3.1; Illumina) and normalized to the expression of the *POLR2A* housekeeping gene. Results from each group were normalized relative to the expression from CSCs cultured using control medium.

2.7. In-Gel Western Detection. The expression of MMP1 protein was analyzed using concentrated supernatants from all culture conditions collected after 7 days in static culture. All supernatants (2 mL from each group) were centrifuged using Vivaspin 500 centrifugal concentrators (GE Healthcare, Chicago, IL) until they were 85 times concentrated to produce 25 μL lysates. These lysates were run by nonreducing SDS-PAGE using 4−15% Mini-Protean precast gels (Bio-Rad, Hercules, CA), followed by gel incubation in 50% isopropanol

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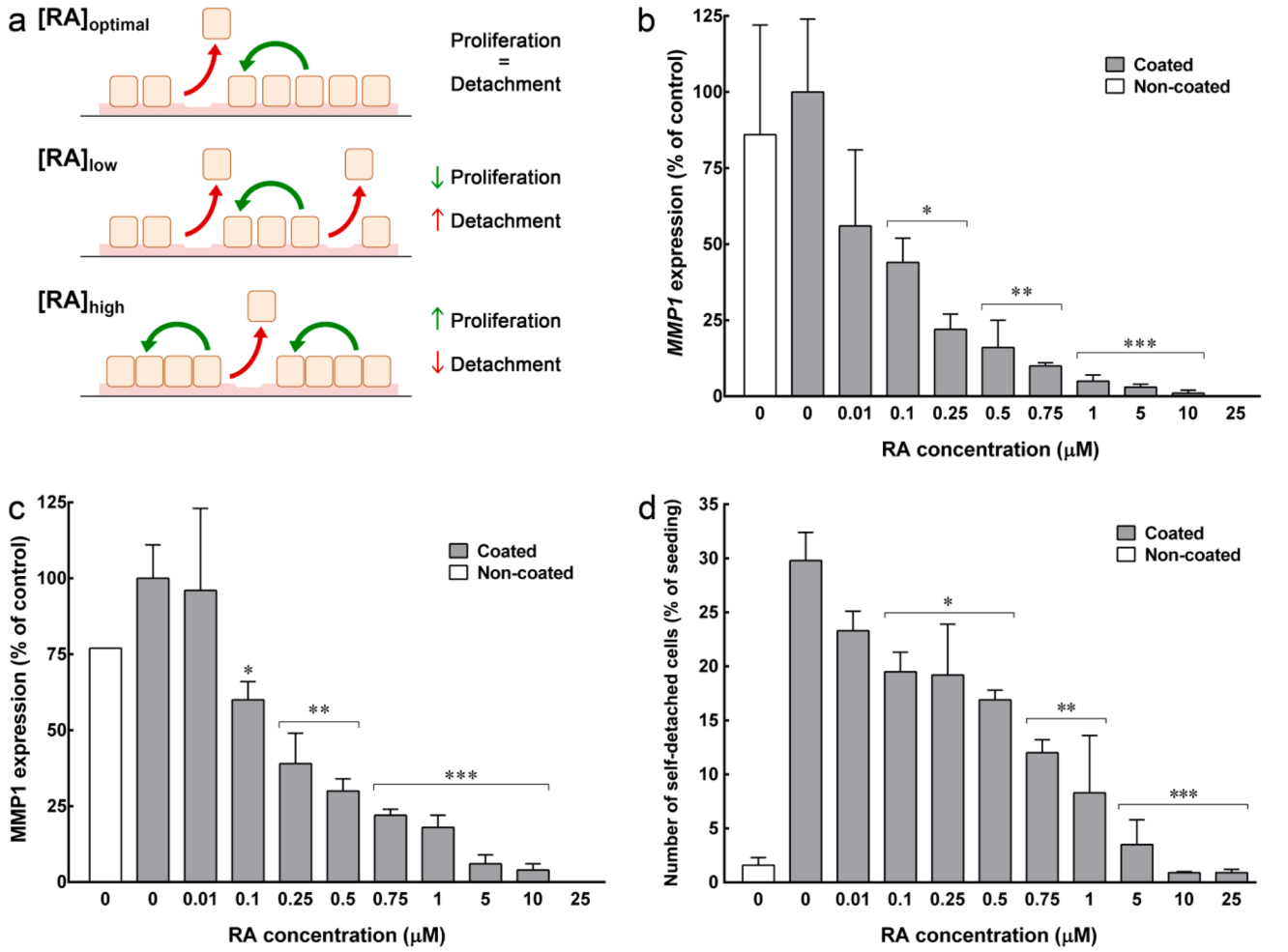

Figure 3. Optimizing RA concentration to control MMP1-regulated cell self-detachment. (a) Schematic illustrating the cell self-detachment model via modulation of RA concentration. CSCs were cultured with different concentrations of RA on either PA-coated surfaces (coated) or TCP (noncoated), and MMP1 expression was analyzed at gene (b) and protein (c) levels. Values were normalized as percentage expression of the control (PA-coated, 0 μM RA). CSCs cultured on PA-coated surfaces with 25 μM RA were undetectable because of the low quantity of mRNA extracted, possibly due to toxicity effects. (d) Self-detached CSCs were counted using the AlamarBlue assay. Values were normalized as percentage of seeded cells. Data (mean ± SD) was obtained from three independent experiments (n = 3) and compared to the PA-coated control using one-way ANOVA with Bonferroni's *post hoc* correction (*, **, and *** corresponded to $p < 0.05$, 0.01, and 0.001, respectively).

and 5% acetic acid for 15 min. The gels were washed thoroughly in ultrapure water to remove the alcohol, which were then incubated with anti-MMP1 antibody (ab23375; Abcam) diluted 1:1000 in blocking solution containing 5% bovine serum albumin (BSA). This was followed by the corresponding secondary antibody diluted in 1:1000 diluent for 1 h with gentle shaking. Antitubulin was used for protein loading normalization. The In-Gel Western detection was carried out using an LI-COR system (LI-COR Biotechnology), and the quantification was performed by densitometry analysis of imaged bands using ImageJ v1.46 software.

2.8. Immunofluorescence and Phase-Contrast Microscopy. CSCs cultured in the dynamic cell culture system for 30 days with 0 or 0.1 μM RA onto PA-coated or noncoated coverslips were fixed in 4% paraformaldehyde for 30 min, washed thrice with PBS for 5 min, blocked for 1 h in PBS supplemented with 2% BSA, and incubated with antikeratocan and anti-αSMA primary antibodies (sc-66941, Santa Cruz Biotechnology, Dallas, TX; VPS281, Vector Laboratories) in blocking solution (1:1000) for 2 h; washed thrice with PBS for 5 min and incubated with 1:1000 fluorescein-labeled goat antirabbit and Texas Red-labeled horse antimouse IgG antibodies (Vector Laboratories) and DAPI (Thermo Scientific), respectively, for 1 h. Cells were washed three times with PBS for 5 min and imaged using an A1R confocal laser microscope (Nikon) with constant illumination and capture parameters. Micrographs were analyzed using the NIS-Elements and ImageJ v1.46 software packages. Cells attached to the PA-coated coverslip or reattached in tissue culture polystyrene (TCP) plates were monitored throughout the 30 days in dynamic cell culture using either a DM IL Led inverted microscope or a Lumascope 500 time-lapse microscope (Etaluma Inc., Carlsbad, CA). Images were acquired at different time points.

2.9. Statistical Analysis. Error bars represent the standard deviation of the mean, analyzed *a priori* for homogeneity of variance. Replicates from each independent experiment were confirmed to follow a Gaussian distribution, and differences between groups were determined using one-way analysis of variance, followed by Bonferroni's multiple comparison *post hoc* test. Significance between groups was established for $p < 0.05$, 0.01, and 0.001, with a 95% confidence interval, and R^2 values of 0.9496, 0.9248, and 0.9586 for the viability, *MMP1* gene expression, and MMP1 protein expression assays, respectively.

3. RESULTS AND DISCUSSION

3.1. Structure, Stability, and Bioactivity of the Multifunctional PA. MMP/RGDS−ETTES is a binary system PA comprising two different PAs, the cell-adhesive, matrix metalloprotease (MMP)-sensitive MMP/RGDS, and the ETTES spacer, and was previously characterized in solution, i.e., analysis

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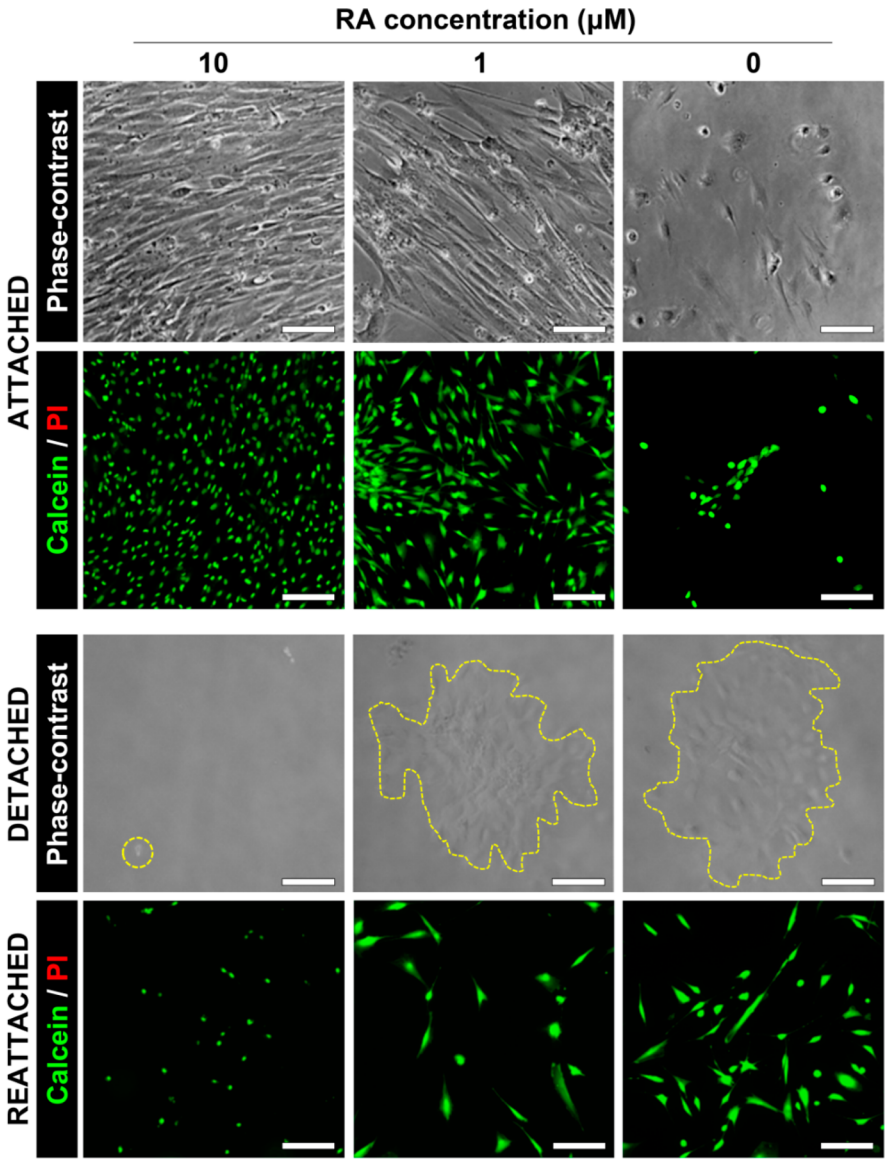

Figure 4. Representative micrographs of attached, self-detached, and reattached cells on static SFM cultures supplemented with 0, 1, and 10 μM RA. Phase-contrast images of cells grown on PA coatings (attached) and of self-detached cells at day 3 of culture (detached), making evident the inverse correlation between the number of detaching cells and RA concentration. Fluorescence micrographs corresponded to live (Calcein-AM; green)/dead (PI; red) staining at day 7 of culture, showing high viability of the cells remaining attached to PA coatings (attached), as well as of the self-detached cells on new TCP surfaces (reattached). Scale bars: 100 μm.

of its critical aggregation concentration, nanostructure, and cleavage specificity.[10] Here, we investigated the nanostructure of this binary system PA, along with its stability as a surface coating, using atomic force microscopy (AFM). The MMP/RGDS−ETTES PA was drop-spotted and homogeneously distributed as a 0.125 mM solution onto normal tissue culture (i.e., glass coverslips and polystyrene) or low-attachment surfaces, and then allowed to dry over the entire surface of the different substrates (Figure S1) and create thin film coatings comprising self-assembled PA nanotapes.[10] The self-assembled binary PA formed long, regular nanotape structures 89 ± 19 nm wide (Figure 1) that retained original shape and density both on normal- and low-attachment surfaces (Figure S2) even after extensive washing (Figure S3a). In contrast, coatings formed by the one-component, single-system PAs MMP/RGDS and ETTES were comprised of significantly (p = 0.002) wider (161 ± 35 nm) or narrower (55 ± 15 nm) nanotapes, respectively (Figure 1). This suggested that the multifunctional MMP/RGDS−ETTES PA nanotapes were composite structures resulting from the mixed self-assembly of individual MMP/RGDS and ETTES PA molecules, and not from two subpopulations of single-system PA nanotapes. This notion was supported by previous studies on PA self-assembly and nanostructure characterization.[10]

Coatings formed by the multifunctional MMP/RGDS−ETTES PA presented little or no interruptions, with nanotapes showing no evident organization across the coating (Figure 1). In addition, the arrangement of the nanotapes provided a suitable platform to grow cells on, directing them to interact preferentially with the PA coating and restricting their contact with the underlying surfaces. As such, the biocompatibility and bioactivity of PA-coated surfaces were tested by evaluating the proliferation and viability of primary corneal stromal cells (CSCs) seeded onto the multifunctional PA coatings.

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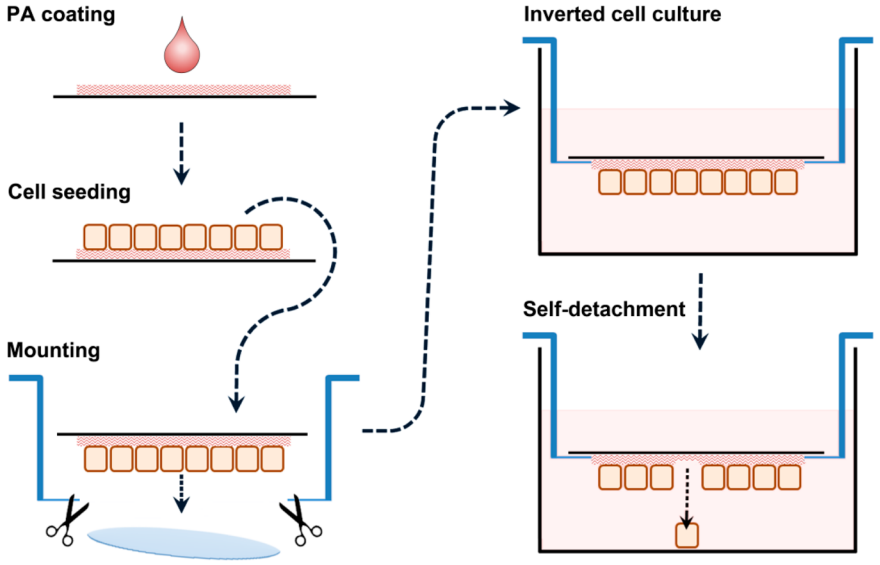

Figure 5. Schematic setup of the dynamic cell culture method allowing the immediate reattachment of self-detached cells. CSCs were seeded onto PA-coated glass coverslips and transferred upside-down into Transwell cell culture inserts that were previously cut *ad hoc*. In such system, CSCs were cultured in SFM containing RA for 30 days, during which self-detached cells were able to reattach to the bottom of the TCP plate.

Uncoated tissue culture polystyrene (TCP) and low-attachment plates (low-attachment) were used as positive and negative control surfaces, respectively. CSCs seeded on PA-coated surfaces were shown to attach and remained viable for the extent of the culture (Figure 2a). In addition, PA-coated surfaces enhanced cell proliferation compared to uncoated TCP, whereas uncoated low-attachment surfaces failed to sustain cell attachment altogether (Figure 2b). Specifically at day 7, PA-coated surfaces were shown to support significantly (p = 0.0046) more cells compared to TCP, corresponding to 324 ± 3% and 270 ± 19% of the initial number of seeded cells, respectively (Figure 2b). In contrast, cells on uncoated low-attachment surfaces were 3 ± 1% of the initial seed (Figure 2b). Together, these results showed that PA coatings provided a biocompatible, bioactive surface that promoted CSCs adhesion and proliferation while maintaining their viability. The PA's bioactivity was probably due to its RGDS peptide motif, previously shown to enhance cell proliferation via direct integrin interaction and activation.[12]

3.2. Using Retinoic Acid Supplementation to Fine-Tune Cell Proliferation/Detachment from PA-Coated Substrates. In order to maintain the generation of additional cells (proliferation) as part of a continuous bioprocess, a balance between cell proliferation and cell detachment from the cell culture substrate has to be achieved. Moreover, such a system should ensure maintenance of cell phenotype throughout this process (attachment, proliferation, and detachment). To that end, we first investigated whether a precise regulation of matrix metalloprotease 1 (MMP1) expression could be attained in cells cultured under different RA supplementation concentrations. Previously we have shown that RA dramatically suppresses MMP production across a range of human stromal cells.[11] Thus, herein, our working hypothesis was that a specific RA concentration could allow cells to express and maintain precise MMP levels that would subsequently promote controlled cleavage of the MMP1-sensitive PA coating, which in turn, with similar levels of precision, would cause the release of attached cells at a rate comparable to that of proliferation. In other words, we aimed to create a RA dose-dependent steady-state between cell proliferation and release from PA-coated surfaces (Figure 3a).

The levels of MMP1 expressed endogenously by CSCs were measured both at transcriptional and protein levels after growing for 7 days in static cell culture, using serum-free media (SFM) supplemented with increasing concentrations of RA (0.1−25 μM). Results showed that RA finely regulated MMP1 expression in a dose-dependent manner (Figure 3). In particular, *MMP1* transcription was significantly lower in CSCs cultured with 0.1−10 μM RA compared to that of cells without supplementation (0 μM RA) (Figure 3b). Moreover, *MMP1* transcription was inversely correlated to RA concentration, with a gradual decrease in expression following increasing RA concentrations (Figure 3b). RA supplementation had a correspondingly similar effect on MMP1 protein expression (Figure 3c), with a linear inverse correlation between RA concentration and MMP1 expression. These results clearly indicated that relatively small changes in RA concentration (i.e., within 2 orders of magnitude) can precisely modulate endogenous MMP1 expression from CSCs, in a dose-dependent manner.

The ability to finely regulate MMP1 expression from cultured CSCs via the level of RA supplementation allowed us to explore the potential of PA coatings as a platform for the controlled self-detachment of individual cells with maintained functionality, as a basis for continuous cell bioprocessing. For a further test of this concept, cells that had self-detached from the PA-coated surface (from the previous RA concentration optimization study) were collected from the supernatant (by removal of media and centrifugation), and their number was quantified. As expected, the results showed an inverse correlation between the number of detached cells and RA concentration (Figure 3d). Specifically, cultures supplemented with 0.1 μM or more RA showed a significantly lower number of cells detaching from the PA coatings compared to 0 μM RA coated control conditions (Figure 3d). Furthermore, cell self-detachment in this static culture system was observed starting at day 3, with an evident higher number of self-detached cells floating in cultures without RA supplementation (Figure 4; *attached* versus *detached*). This suggested that 3 days were

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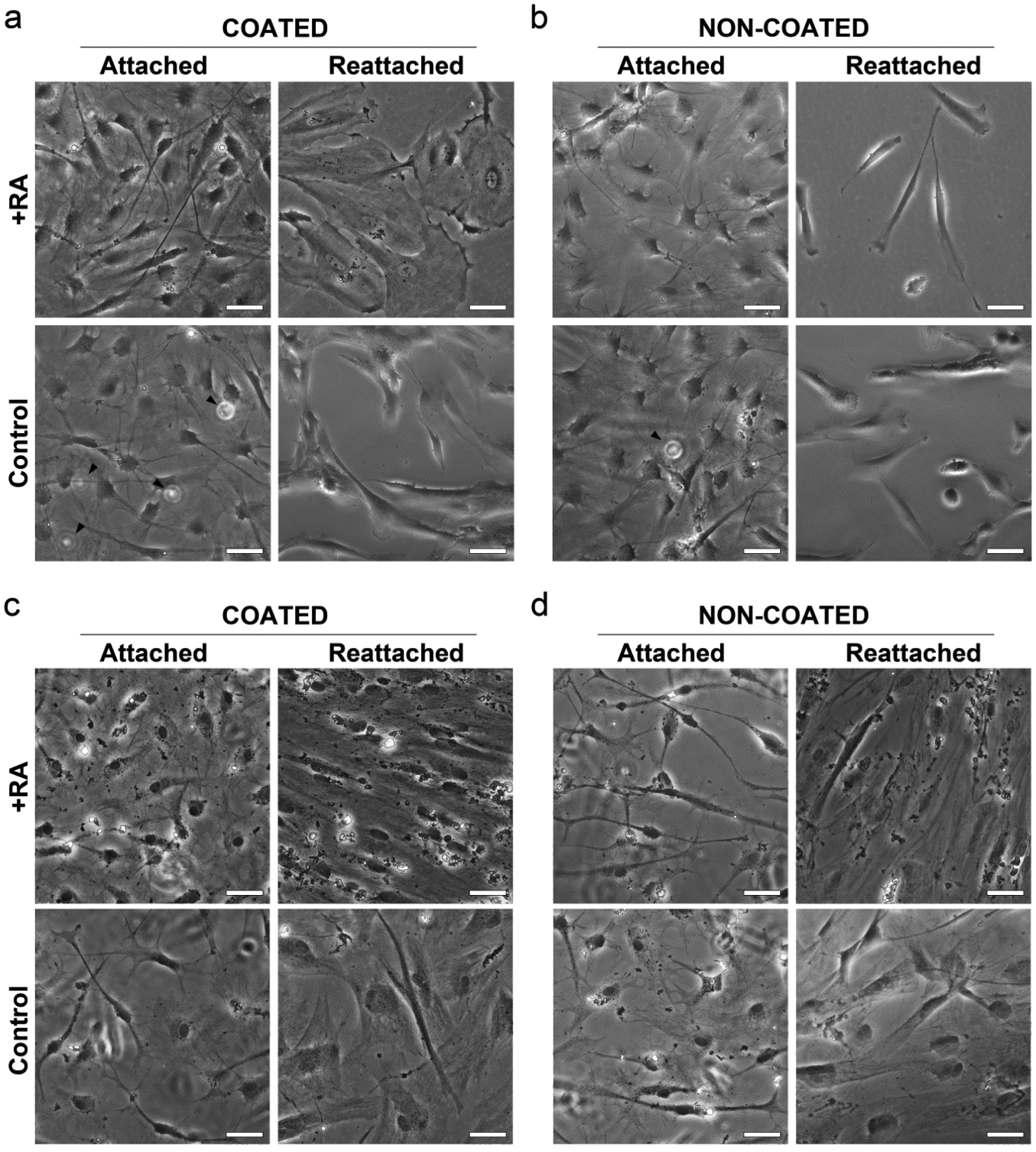

Figure 6. Representative phase-contrast micrographs of CSCs in dynamic culture. Cells grown on PA-coated or noncoated glass coverslips (attached) and self-detached cells reattached to TCP (reattached) were maintained with RA-supplemented (+RA) or nonsupplemented SFM (control) and imaged after 15 (a, b) and 30 (c, d) days in culture. Scale bars: 50 μm.

sufficient for CSCs to secrete enough MMP1 molecules to start cleaving the enzyme-responsive, cell-adhesive PA coating and elicit their self-detachment from the surface. The inverse correlation between the number of detaching cells and RA concentration was further supported by the live/dead cell staining assays (Figure 4). Importantly, these assays confirmed that CSCs collected from the supernatant after 7 days in culture were able to reattach to new TCP surfaces while maintaining high viability levels and suitable morphologies (Figure 4, *reattached*), suggesting a maintained phenotype following the MMP1-induced self-detachment.

The correlation between RA supplementation and PA cleavage was further investigated using a distinct static cell culture setup, with PA coatings incubated for 15 days together with CSCs as

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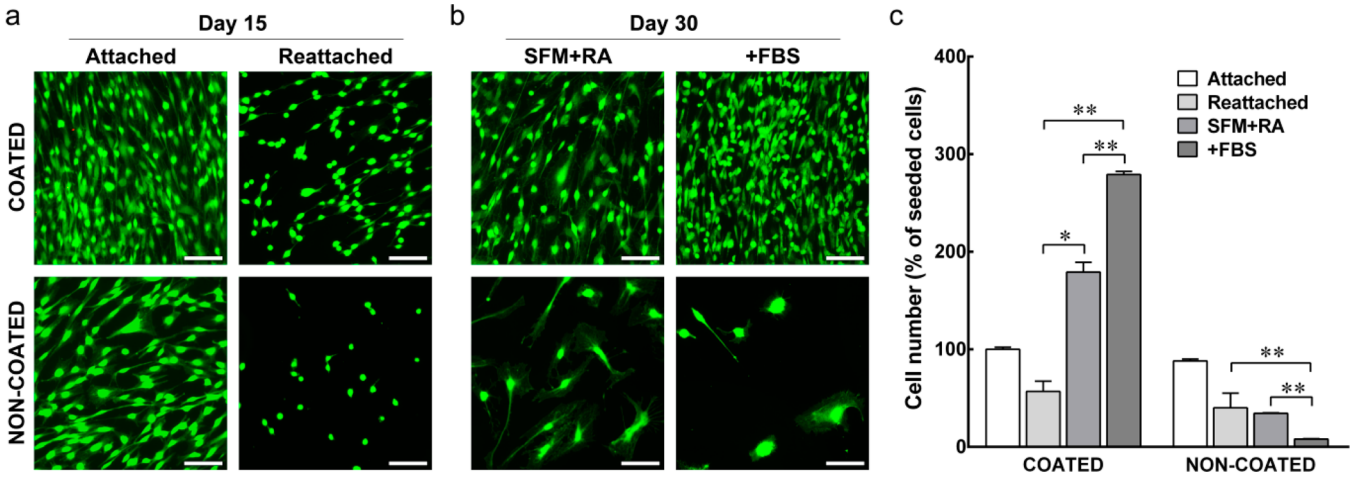

Figure 7. Proliferation and viability of CSCs in dynamic cell culture supplemented with 0.1 μM RA. (a) Representative live/dead staining micrographs of cells attached on PA-coated and noncoated surfaces (left panels) and cells detached from such surfaces and reattached to the bottom of wells (right panels) during a 15 day period in culture (Day 15). (b) Representative live/dead staining micrographs of reattached cells cultured for a further 15 days (Day 30) in either SFM+RA or +FBS medium. Live, calcein-positive (green) and dead, PI-positive (red stained) cells showed dynamic cell cultures maintained high cell viability. Scale bars: 100 μm. (c) Cell quantification using the AlamarBlue assay throughout the period in culture. Data (mean ± SD), represented as percentage of initial cell seeding number, was obtained from three independent experiments (n = 3), and analyzed statistically using one-way ANOVA with Bonferroni's *post hoc* test (* and ** corresponded to p < 0.05 and 0.01, respectively).

source of MMPs, and supplementing the medium with 0.01−10 μM RA to modulate MMP levels (i.e., increasing RA concentrations to elicit decreasing MMP expression). PA coatings were shown to be protease-sensitive, as indicated by the clearance of the coatings and the remodeling of their component nanotape structure (Figure S3b). Specifically, media conditioned with higher MMP levels (i.e., using lower RA concentrations) were shown to markedly reduce the amount of coating, as well as alter the PA nanostructure, with accumulation of 50−500 nm wide, globular aggregates (Figure S3b, insets). PA coatings incubated with conditioned medium with the lowest MMP concentration (i.e., using 10 μM RA) showed comparably less degradation, as well as the permanence of PA nanotapes, albeit greatly truncated (Figure S3b, right panel). Importantly, the structure of MMP/RGDS−ETTES molecules in coatings incubated with cell-derived MMPs was comparable to that of this PA in solution after recombinant MMP1 cleavage, previously characterized by transmission electron microscopy.[10] Together, these results demonstrated a strong, dose-dependent correlation between the level of RA supplementation, expression of CSC-derived MMPs, and the degree of PA cleavage. This correlation indicated that our proposed mechanism of using RA supplementation in SFM to finely control CSC self-detachment was feasible, and without apparent impact on cell viability or phenotype.

3.3. Proof-of-Concept for a Steady-State Cell Culture System. The static cell culture adopted in the previous experiments allowed us to achieve a considerable understanding on how the RA−PA system affects MMP1 expression and cell self-detachment. For further exploration of its potential within a continuous bioprocessing system, cells were cultured for longer times using a modified experimental setup. Specifically, CSCs were seeded onto PA-coated glass coverslips (as before), but which were now mounted in an inverted position upon the membrane of Transwell cell culture inserts, within standard 6-well TCP plates, and with membranes each having had an aperture (11 mm Ø) cut into it (Figure 5). Using this dynamic system, self-detached cells were transferred by gravity from the inverted glass coverslip to a new remote surface (bottom of the wells). These displaced cells were tested for their ability to reattach, proliferate, and maintain their phenotype. The specificity of cell self-detachment was tested by additionally culturing CSCs on noncoated surfaces. Concomitantly, we tested the ability to maintain the initial cell population (those on the glass coverslip) in a steady-state, i.e., the system's capacity to create a balance between the rate of cell proliferation and cell detachment within those cells on the glass coverslip.

On the basis of our previously optimized culture conditions, CSCs were cultured in SFM supplemented with 0.1 μM RA to explore the possibility of maintaining steady-state for a period of 30 days. Cultures maintained in nonsupplemented medium were used as controls. Inverted CSCs grown on a PA-coated glass coverslip displayed adherent cells both migrating and proliferating (Video 1, arrowheads) without any obvious signs of cell or PA coating detachment in the initial 12 h of post-seeding. This lag period was probably related to the time required for MMP synthesis, secretion, and accumulation in the medium. This notion was supported by the results from the static cell culture system and from previous studies where tissue self-release from PA coatings occurred only after 48−72 h of inducing MMP synthesis.[10]

In presence of RA, the inverted cultures analyzed by phase-contrast microscopy showed that cells grown on PA coatings maintained high confluence levels at day 15 (Figure 6a) and day 30 (Figure 6c), whereas cells on noncoated surfaces showed slightly lower densities (Figure 6b,d). Cells in this dynamic culture system maintained high viability levels independently of their growth surface (Figure 7a), and even after cell detachment, reattachment, and prolonged culture up to 30 days in SFM conditions (Figure 7b). In addition, cells detaching from PA coatings (in the presence of RA) precipitated onto the new (lower) surfaces in greater numbers and with greater retention of their adhesion potential, compared to those from noncoated surfaces, as indicated by their spread morphology, both at day 15 and at day 30 of culture (Figure 6). Specifically, the number of cells that detached from PA-coated surfaces and reattached to the bottom surface during the initial 15 days corresponded to 57 ± 11% of the original seeding

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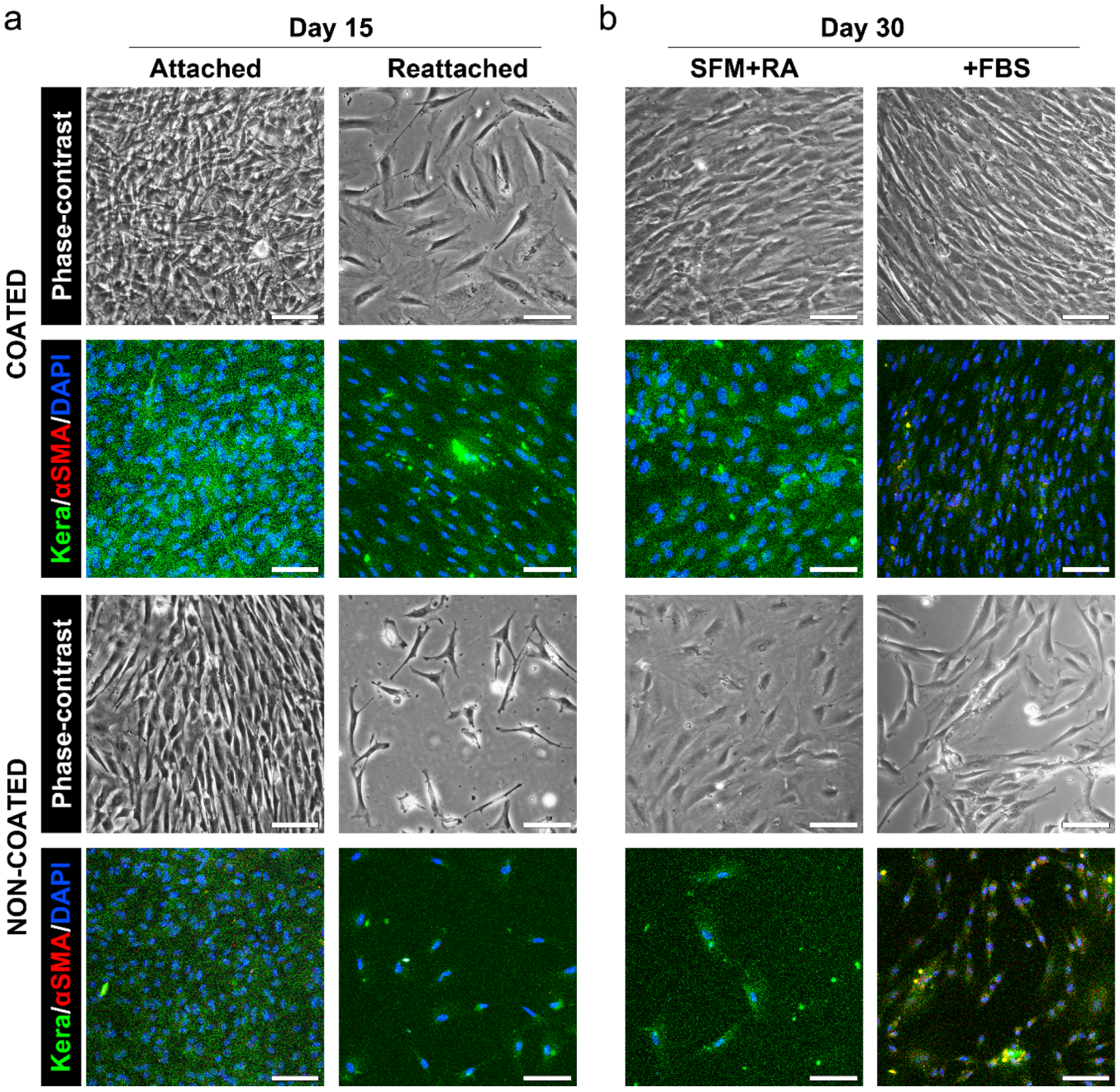

Figure 8. Phenotype of CSCs in dynamic cell culture supplemented with 0.1 μM RA. (a) Representative phase-contrast and Z-stack projection of confocal fluorescence micrographs of cells attached on PA-coated and noncoated surfaces (left panels) and cells detached from such surfaces and reattached to bottom of wells (right panels) during a 15 day period in culture (Day 15). (b) Representative phase-contrast and Z-stack projection of confocal fluorescence micrographs of reattached cells cultured for another 15 days (Day 30) in either SFM+RA or +FBS medium. CSC-specific marker keratocan (green) and fibrotic marker αSMA (red staining) were used to characterize cell phenotype. Cell nuclei were stained with DAPI (blue). Scale bars: 100 μm.

number (Figure 7c). Moreover, cells that detached from a PA-coated surface were able to proliferate once reattached, either in RA-supplemented SFM or FBS-containing medium (Figure 7b,c). The number of cells in SFM+RA and +FBS cultures at day 30 was significantly higher (p = 0.02 and 0.003, respectively) than that of reattached cells at day 15, and corresponded to 179 ± 10% and 279 ± 3% of the initial cell seeding, respectively. This represented a 3.1- and 4.9-fold increase in the number of reattached cells during a 15 days period in SFM+RA and +FBS conditions, respectively. The significantly higher (p = 0.005) cell proliferation in FBS-containing medium was expected, as serum has been shown to promote CSC activation and transition into a fibroblast-type phenotype.[13] In contrast, the number of reattached CSCs originated from noncoated surfaces during the initial 15 days corresponded to only 40 ± 15% of the original seeding, and these cells were unable to proliferate, independently of the medium used (Figure 7). Instead, the number of cells cultured up to day 30 in SFM+RA and +FBS conditions was further reduced (Figure 7c). These results indicated that the PA coatings were essential for the continuous detachment of viable and proliferative CSCs, up to 30 days in culture.

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Cells grown in the absence of RA (Figure 6; control) showed a higher number of round, detaching cells from PA-coated surfaces compared to noncoated surfaces (Figure 6, arrowheads). The small amount of detachment observed from noncoated surfaces was not unexpected, as previous work had noted poor cell−cell adhesion in CSC cultures under SFM conditions.[10] This loss of cell adhesion in the absence of RA was probably due to the higher turnover of extracellular matrix during culture, with higher expression of endogenous proteases leading to increased cleavage of deposited matrix, and detachment of the stratifying (apical) CSCs from their underlying cell monolayer.[11] In addition, cells cultured without RA or on noncoated surfaces showed substantial lower densities and a change in morphology from day 15 onward. These results indicated that to achieve a steady-state culture of CSCs for 30 days, both PA-coated surfaces and medium supplementation with 0.1 μM RA were required.

Tracking an individual cell's self-detachment from a PA-coated surface was not feasible using time-lapse microscopy because of the difficulty in focusing on the detaching cells (due to its inherent random nature across the surface). However, reattachment of self-detached cells in a remote location (i.e., the bottom of the TCP well) was observed (Video 2), both in small clumps (due to the aggregation of 2−3 cells) and individually. In addition, self-detached cells were also able to migrate and proliferate after spontaneous reattachment (Video 3). Note the relative differences in the migration of CSCs on TCP (Video 3) and PA-coated surfaces (Video 1) over the same 2 h period. This loss of cell migration on RGDS-functionalized surfaces has been previously reported.[14]

Finally, the effect of the dynamic cell culture system on CSC phenotype was further investigated. To this purpose, the expression of keratocan (a characteristic marker of CSCs[15]) and αSMA (a marker of myofibroblastic differentiation[13]) was evaluated by immunofluorescence microscopy (Figure 8; Figure S4). Results showed that CSCs detached from both PA-coated and noncoated surfaces maintained keratocan expression without expressing the fibrosis marker αSMA (Figure 8a). Furthermore, reattached cells maintained for up to 30 days in SFM+RA conditions showed further accumulation of keratocan, whereas cells cultured in +FBS medium assumed a fibroblast-type phenotype, as shown by their expression of αSMA (Figure 8b). Importantly, these experiments demonstrated that the dynamic system of CSCs production, based on this particular PA coating, did not compromise the phenotype of either attached (Figure S5) or self-detached cells during the 30 days culture period. Interestingly, cells derived from coated surfaces and subsequently cultured with FBS showed lower αSMA expression compared to those detached from noncoated surfaces in similar conditions (Figure 8b; Figure S4). Thus, these results suggested that the influence of PA coatings may extend beyond the period of direct cell−surface interaction, with phenotype persisting well after self-detachment and reattachment. A similar integrin-dependent mechanism, albeit related to mechanical memory, was previously described for human mesenchymal stem cells.[16]

3.4. Potential Applications of Continuous Bioprocessing. In light of these results, such a method could be applied to the future design of a closed, dynamic system of continuous bioprocessing. Here we demonstrated that human adherent cells grown on PA-coated surfaces (in the presence of 0.1 μM RA) were able to maintain a steady-state between generation of new cells through proliferation and their MMP-mediated detachment (with reattachment at a remote place) for at least 30 days with no impact upon their normal functional phenotype. We envision that this system can be further developed, using microfluidics to wash, sort, or just transport cells to different and remote compartments,[17] where they would subsequently be used for multiple applications (Figure 9). For instance, the

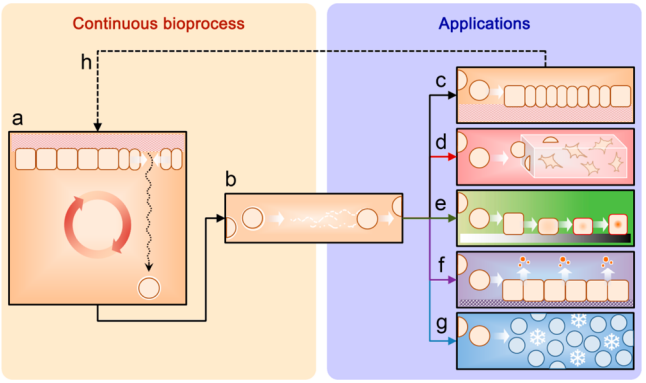

Figure 9. Schematic diagram of the potential applications of the newly developed system. CSCs seeded on PA-coated surfaces can be cultured with 0.1 μM RA in a dynamic continuous bioprocess system, thus maintaining a steady-state between cell proliferation and self-detachment (a), where self-detached cells can be collected and washed using microfluidics (b). Subsequently, self-detached cells may be collected and used for different applications, such as cell expansion and/or *de novo* tissue biofabrication (c), encapsulation (d), differentiation (e), secretion of growth/therapeutic factors (f), freezing and/or storage (g), or even to expand/replace cells in the continuous bioprocess system (h).

self-detached cells could be seeded on a new PA-coated surface to further expand their numbers[14] or produce bioprosthetic, self-lifting tissues;[10,18] cells could instead be incorporated within specific biomaterials for tissue engineering purposes,[19,20] used for cell-therapy,[21] or storage,[22] or sorted for cryogenic or hypothermic preservation.[23,24] In addition, the newly generated and remotely positioned cells could be differentiated toward a specific phenotype using distinct chemical[25] or physical factors.[26] Alternatively, they could be used as growth factor factories or feeder layers[27,28] (Figure 9). As a demonstration of its commercial potential one can easily conceive of scaling up this early proof-of-principle continuous bioprocessing approach. We have shown here that 7×10^3 cells can be continuously produced in a day via release from an area of 1 cm^2. Thus, translating to an area of approximately 155 cm^2 (similar to the area of standard cell culture flask), this system would result in the production of over 1 million cells every 24 h. Remarkably this small area could theoretically continuously produce approximately 100 million cells in 3 months. While this number of cells is comparable to existing bioprocessing approaches in terms of time, the associated footprint and materials requirement would be a very small fraction of that currently required, with accompanied cost savings.

4. CONCLUSION

We postulated that a dose-dependent, RA-mediated response to the endogenous production of MMPs from CSCs was achievable. This allowed us to regulate the number of cells self-detaching from a cell-binding, MMP-cleavable PA substrate coating. Overall, these results demonstrate the real possibility of having a culture system that, through the precise control of

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MMP1 expression, allows cells to steadily degrade their anchoring substrate and detach, while allowing others to continue to proliferate and replenish those lost cells continuously and for relatively long periods. Moreover, during these extended culture periods, both attached and self-detached/reattached cells maintained a CSCs-characteristic phenotype, and thus their functionality. Coupling this type of steady-state culture with sophisticated clearance, sorting, and/or concentration systems (i.e., by using integrated microfluidics) potentiates the future creation of high-throughput, automated cells factories with little or no need for eliminating the substrates cells grow on. As such, this can be considered the first successful proof-of-concept design for a closed, continuous bioprocessing system for therapeutic cell production.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.7b09809.

PA coating coverage, structure, and stability; immunofluorescence and time-lapse microscopy of dynamic cell culture system (PDF)

Video 1: Time-lapse micrograph sequence of CSCs on PA-coated coverslips, showing proliferation of attached cells in the dynamic culture system, 12 h after initial cell seeding (AVI)

Video 2: Time-lapse micrograph sequence of CSCs floating onto the bottom of a TCP plate well after self-detachment from the PA-coated surface (AVI)

Video 3: Time-lapse micrograph sequence of CSCs proliferating on the bottom surface of the dynamic culture system after self-detachment from the PA-coating (AVI)

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The authors declare no competing financial interest.

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