A durable and biocompatible ascorbic acid-based covalent coating method of polydimethylsiloxane for dynamic cell culture

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Abstract

Polydimethylsiloxane (PDMS) is widely used in dynamic biological microfluidic applications. As a highly hydrophobic material, native PDMS does not support cell attachment and culture, especially in dynamic conditions. Previous covalent coating methods utilize glutaraldehyde (GA) which, however, is cytotoxic. This paper introduces a novel and simple method for binding collagen Type I covalently on PDMS utilizing ascorbic acid (AA) as a crosslinker instead of GA. We compare the novel method against physisorption and GA crosslinker based methods. The coatings are characterized by immunostaining, contact angle measurement, atomic force microscopy and infrared spectroscopy, and evaluated in static and stretched human adipose stem cell (hASC) cultures up to 13 days. We found that AA can replace GA as a crosslinker in the covalent coating method and that the coating is durable after sonication and after six days of stretching. Furthermore, we show that hASCs attach and proliferate better on AA crosslinked samples compared to physisorbed or GA based methods. Thus, in this paper we provide a new PDMS coating method for studying cells, such as hASCs, in static and dynamic conditions. The proposed method is an important step in the development of PDMS based devices in cell and tissue engineering applications.

Keywords

1   Introduction

In microfluidic cell applications, silicone elastomers based on polydimethylsiloxane (PDMS) composites are often the materials of choice [1]. PDMS composites, such as Sylgard 184, are readily available for purchase and processing, and prototyping with the material is straightforward. They are also suitable for cell culture applications due to their biocompatibility and sterilizability. For these reasons, PDMS composites are favoured materials for engineers aiming for new biological microfluidic applications over polystyrene and glass, for example [2]. For simplicity, Sylgard 184 elastomer is abbreviated as PDMS in the following chapters.

PDMS is very hydrophobic when cured, thus it does not support cell attachment in this state [3]. Therefore, surface modifications have recently been the main focus in many cell related PDMS studies [1]. Many types of modifications, such as surface plasma treatment [4-14], protein physisorption [4-8,11,15-20], layer-by-layer methods [4,9,11,21], chemical immobilization [10-14,22-24], gels [23,25] and combinations of two or more methods have been studied to overcome the inert PDMS surface properties and to better support cell attachment. Table 1 gathers various surface modification methods that have been used in recent cell culturing studies. The information is organized along the used modification type with added critical information about the chemical and physical environment on the surface, and the types of cells cultured.

Even though the common and fairly simple surface modification methods, such as plasma treatments or physisorbed coatings, have been successfully used in PDMS cell culture applications [4,5,8,15-18,20,26,27], more durable PDMS modification methods are needed to provide longer cell culture periods, to study more delicate cell types and, especially, in dynamic culture conditions. In more complex applications, where the surfaces and the cells are under various stresses, or the timeframe of the culturing exceeds few days, the coating is often not durable enough and it begins to deteriorate leading to the detachment of coating and/or cells. For example in stem cell research, the differentiation process of stem cells usually takes weeks or even months, which highlights the

importance of the durability of the substrate coating. In addition, the long culture time on the coated substrate demands not only durability but also requires coating techniques harmless for living cells.

Surface treatments coupled with crosslinker molecules covalently attached to proteins and peptides have proven to be successful in providing durability to the coatings [10-14,22-24], but only rarely details about the used chemicals and their cytotoxicity are assessed or mentioned in the studies. The covalent linking process is typically started with plasma oxidation of the PDMS surface followed by aminosilanization [10-12]. This creates free groups of primary amines on the PDMS surface, which can react with a crosslinker with multiple amino reactive groups, such as carbonyl, azide, epoxide or *n*-hydroxysuccinimide (NHS) ester. GA is a commonly referenced crosslinker with this type of functionality [11,28,29]. When proteins or peptides that naturally contain at least one primary amine group are brought in contact with the surface containing the crosslinker, they will covalently bond with the crosslinker and attach to the surface. Currently, the chemistry of the coupling reactions is well understood and it is proven to create adherent PDMS surfaces for various cell types. A critical downside, however, is that the used chemicals are often highly cytotoxic [30-32], and the effect of a residue on the culture surface is unknown. Thus, there is an increasing need for developing durable coatings for demanding cell applications, such as long-term cultivation and stretching, without using cytotoxic chemicals.

This study introduces a novel covalent coating method which exploits ascorbic acid (AA) chemistry to promote protein binding on PDMS surfaces. The paper describes the preparation of the coatings with collagen Type I which was chosen as the model extracellular matrix (ECM) protein, and their implementation in PDMS pneumatic cell stretching devices (PCSD). New AA cross-linked collagen Type I coatings were compared to two conventional (physisorption and glutaraldehyde (GA) cross-linking based) collagen Type I coatings. As a proof-of-concept, we evaluated the capability of the coatings to enable and support the attachment and proliferation of human adipose stem cells (hASCs) in a 13-day culture and under cyclic stretching. The method has room for expansion for binding of other ECM proteins or peptides, and as Tiller *et al*. demonstrated [33], enzymes.

2 Materials and methods

2.1 Preparation of stretching devices

Tailor-made PCSDs from PDMS (Sylgard 184, Dow Corning, MI, USA) and glass were applied. The design and preparation of the devices, illustrated in Figure 1, have been described earlier by Kreutzer *et al*. [6]. The PDMS membrane served as a cell cultivation substrate in the devices. The dimensions are illustrated in Figure 1; the culture well is 12 mm in diameter (1.13 cm²) while the whole PCSD is 31 mm wide.

Curing of the PDMS was done uncovered in an oven (Binder GmbH, Tuttlingen, Germany) in 60 °C for 10 hours to reduce the possibility of a solvent residue. The individual pieces were treated with oxygen plasma (Pico-SR-PCCE, low pressure plasma system, Diener Elect., Ebhausen, Germany) to bond them permanently together. The plasma cleaner parameters were same in all the treatments (power 30 W; treatment time 18 s; chamber pressure 0.30; gas flow rate 1.4 sccm). The unstretched reference devices were prepared from the same PDMS batch and with the same dimensions for the cell culturing well as PCSDs. The vacuum chamber was removed from the reference devices to save manufacturing costs and time.

All devices were mounted on specifically developed polycarbonate rings in order to prevent the PDMS membrane touching the surface under the devices. The rings for the reference devices had an outer diameter of 25 mm and an inner diameter of 13 mm. The rings for PCSDs had an outer diameter of 40 mm and an inner diameter of 20 mm. All the rings were 0.8 mm thick.

The PDMS membrane was manufactured using the same PDMS curing protocol as for the devices. In order to attach the membranes to the devices, the membranes were first cleaned with isopropanol and deionized (DI-) water, after which they were treated with oxygen plasma and bonded to the devices.

2.2 Preparation of the coatings

The membranes of the devices were functionalized with Type I collagen from rat tail (Invitrogen, Life Technologies, Carlsbad, CA, USA) using five different methods: physisorption coating (PHY), covalent coating using glutaraldehyde as the crosslinker (GLA), and three ascorbic acid cross-linked covalent coatings (AA1, AA2 and AA3). Figure 2 illustrates the covalent coating process step-by-step and predicts the chemical reaction pathways.

For the PHY samples, the PDMS membrane was treated with oxygen plasma, and then incubated at room temperature for 60 min with collagen solution (50 μg/mL; diluted in 0.02 M acetic acid). The volume used to treat the wells contained 17 μg/cm² of collagen Type I. Following the incubation, the samples were washed thoroughly with DI-water, dried for 15 min in a ventilation cabinet, and stored at +4 °C.

For the GLA samples, oxygen plasma, (3-aminopropyl)triethoxysilane (APTES; Sigma-Aldrich, St.Louis, MO, USA), and glutaraldehyde (Sigma-Aldrich) were used to covalently bond collagen Type I onto the membrane [11]. After the plasma treatment, the membrane was treated with 10 % APTES solution in methanol for 2 min at room temperature to create a monolayer with primary amine groups on top. The samples were then washed once with methanol and rinsed twice with DI-water. Then the GLA samples were treated with 3 % glutaraldehyde solution in DI-water for 20 min and rinsed five times with DI-water. Finally, the devices were treated with collagen as in the PHY coating.

The AA1, AA2 and AA3 samples were prepared identically to the GLA samples, except the glutaraldehyde as the crosslinker was replaced by L-ascorbic acid (Sigma-Aldrich, cell culture grade, ≥98 %) and the incubation time was prolonged from 20 min to 60 min followed by only two washings with DI-water. For the AA1 coatings, the ascorbic acid powder was dissolved in Dulbecco's phosphate buffered saline (200 mg/mL; DPBS; Lonza, BioWhittaker™, Verviers, Belgium), whereas for the AA2 and AA3 coatings the ascorbic acid powder was dissolved in methanol (20 mg/mL). In the AA3 coating protocol, 5 μl of 30 % hydrogen peroxide, which is 2 % of the used volume, was added to the ascorbic acid coating solution in methanol to improve ascorbic acid reactivity [33].

In this study, all the coated samples were used for characterization and cell culture within 24 hours of the coating preparation. For the cell culture experiments, the devices were first wiped outside with 70 % ethanol, then placed in a laminar hood and finally sterilized under ultraviolet light for 20 min, after which they were rinsed once with DPBS and placed in sterile cell culture plates.

2.3   Stretching system and parameters

The vacuum stretching system was similar to the one used by Kreutzer *et al.* [6] and it was composed of a laptop computer, LabVIEW-based controller software, a measurement board (National Instruments, USB-6229 BNC, USA), a computer controlled pressure regulator (T-2000, Marsh Bellofram, USA) attached to a high pressure outlet, and an ejector pump (Festo OY, VAD-1/8, Finland) which creates the vacuum. The PCSD on Petri dishes were placed inside a cell culture incubator and attached to the ejector pump outside the incubator using a silicone rubber tubing system. The stretching was conducted under standard cell culture conditions in a humidified atmosphere (+37 °C, 5 % CO_2). Cyclic equiaxial stretching (sine wave, 0.5 Hz) was applied with an effective stretching period of 12 hours, following a 12 h relaxation period per day. The strain magnitude was increased from 2 % at the first stimulation period to 3.5 % at the second period and finally to 5 % for the rest of the stimulation periods.

2.4   Characterization of the coatings by fluorescent microscopy imaging

The collagen Type I coatings prepared by all the five methods were first characterized without cells by using immunofluorescent staining. Three parallel samples of each coating method were stained and imaged before (Day 0) and after a 6-day incubation period (Day 6) in both static and dynamic conditions to see the durability of the coating under mechanical stimulation. DPBS was used as medium in the wells. To further test the durability of the coatings, two parallel samples were exposed to sonication (45 kHz, 60W, Ultrasonic cleaner, VWR international, Radnor, PA, USA) in 50 °C in DI-water bath for 60 min, and then compared to untreated coatings. The staining protocol began with four quick washings using DPBS. After the washings, the unspecific binding of antibodies was blocked

using 1 % bovine serum albumin (BSA; Sigma-Aldrich) diluted in DPBS. The blocking solution was incubated in the samples for 60 min at room temperature. Then, the samples were incubated overnight at +4 °C with the anti-collagen Type I primary antibody (ab90395, Abcam, Cambridge, UK) diluted 1:200 in the blocking solution. Next day, the samples were washed four times for 3 min with DPBS. The Alexa Fluor 488 ® conjugated secondary antibody (Life technologies) was diluted 1:800 in the blocking solution and the devices were incubated with the secondary antibody solution for 60 min at +4 °C in dark. After the incubation, the samples were washed again four times for 3 min with DPBS and quickly rinsed once with DI-water before mounting them onto objective glasses and storing at +4 °C in dark. Finally, the devices were imaged with a fluorescent microscope (Zeiss Axio Scope.A1, Carl Zeiss, Oberkochen, Germany) using a 100× oil immersion objective.

2.5   Image-based quantification of coating properties

CellProfiler (Windows version 2.2.0) [34-36] was applied to images converted from CZI to 16-bit TIF format using the BioFormats package [37]. First, background was estimated and subtracted for each image separately using a median filter of 256 x 256 pixels via the CorrectIlluminationCalculate and CorrectIlluminationApply modules. Pixels representing coating were detected using the ApplyThreshold module via Otsu's three-class entropy-minimizing thresholding [38] with the middle class assigned to background. No smoothing or threshold scaling was applied. Lower and upper bounds of 0.01 and 0.2 were applied to the threshold to avoid false positives in images with very little coating and false negatives in images with dense coating, respectively. The resulting binary images were saved in TIF format. The percentage of pixels covered by coating was calculated to quantify the total amount of coating in each image and the binary images were further analysed in MATLAB R2016b (The MathWorks Inc, Natick, MA, USA) to quantify the amount of coating variation. Each image was divided into blocks of 256 x 256 pixels, the percentage of pixels covered by coating in each block was computed, and the standard deviation of the percentages within an image was calculated to quantify the consistency of the coating in the corresponding image.

2.6   Characterization of the coatings by contact angle measurement

Contact angle measurements were conducted to characterize the hydrophilicity of the collagen Type I coatings as well as all of the intermediate phases of the coatings. Intermediate samples included pristine PDMS, oxygen plasma, APTES, APTES with GA, and APTES with all the three variations of AA treated PDMS samples. Left and right static contact angles, and images of the droplets, of three parallel samples of each final and intermediate coatings were recorded using an optical tensiometer (Attension Theta Lite, Biolin Scientific AB, Stockholm, Sweden) with attached GASTIGHT® precision syringe (Hamilton Inc., Reno, NV, USA).

The statistical significance of the results were evaluated with SPSS (Version 22, IBM) using non-parametric Mann-Whitney U test with Bonferroni adjustment (15 comparisons). The results were considered statistically significant when $p < 0.05$.

2.7   Characterization of the coatings by atomic force microscopy

The coatings prepared by all the five methods and their changes to the surface morphology of PDMS were further studied by atomic force microscopy (AFM). Topographical images and root mean square (RMS) surface roughness values were obtained using XE-100 (Park Systems Corp, Suwon, Korea) atomic force microscope in non-contact mode. Silicon ACTA (Applied Nanostructures Inc., Mountain View, CA, USA) cantilevers with 15 μm pyramidal tip, spring constant of 37 N/m, and resonance frequency of around 300 kHz were used to take one 10 x 10 μm and three 1 x 1 μm images per sample. The resolution of the images was kept at 256x256 pixels and the scan rate at 0.33 Hz. Surface topography images and root mean squared (R_q) surface roughness values were extracted from the AFM scan data by AFM image analysis program XEI (Park Systems Corp).

2.8   Characterization of the coatings by infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) with attenuated total reflectance (ATR) mode was used to study the chemical nature of the final coatings and the intermediate phases after the crosslinking step with GA or AA. Two parallel samples of each coating were scanned 8 times with a Spectrum One

FTIR spectrometer with a diamond ATR accessory (Perkin Elmer Instruments, Waltham, MA, USA) in the range of 4000 to 650 cm^{-1} with a resolution of 2 cm^{-1} and their total transmittance spectra saved and normalized against the background spectrum. In addition to the normal coated samples, three control samples were also scanned. A pristine PDMS sample was used as a negative control for all samples, while a modified AA1 (CONAA1) coated sample was used as a positive control for the intermediate samples, and a modified PHY (CONPHY) coated sample was used as a positive control for the collagen Type I coated samples. 25 µl of 35 % hydrogen peroxide solution, enough to oxidize all AA molecules, was added to CONAA1 during the AA treatment in DPBS. In CONPHY, the acetic acid solvent was replaced with methanol that would evaporate during collagen Type I treatment and leave all of the collagen in the solution to the surface of the sample for easier detection by FTIR-ATR.

2.9   Isolation, characterization and culture of adipose stem cells

The study was conducted in accordance with the Ethics Committee of the Pirkanmaa Hospital District (Tampere, Finland, R15161) and with patients' written consents. The isolation of hASCs from adipose tissue was carried out using previously described mechanical and enzymatic procedures [39,40], and the hASCs were maintained and expanded in T-75 cm^2 polystyrene flasks in basic medium (BM) containing Dulbecco's modified Eagle medium/Ham's nutrient mixture F-12 (DMEM/F-12 1:1, Invitrogen, Life Technologies), 5 % human serum (PAA Laboratories, Pasching, Austria), 1 % L-glutamine (GlutaMAX, Life Technologies) and antibiotics (PEN-STREP; 100 U mL^{-1} penicillin, 100 U mL^{-1} streptomycin; Lonza).

The mesenchymal origin of the cells was confirmed after primary culture in T-75 flasks at passage 1 by flow cytometry (FACSAria, BD Biosciences, San Jose, CA, USA) as described earlier by Lindroos *et al*. [41]. The hASCs used in the study showed strong (> 88 %) expression of CD73, CD90 and CD105 surface proteins, whereas expression of CD14, CD19, CD34, CD45 and HLA-DR was lacking or low (<15 %) verifying the mesenchymal origin of the cells.

The thawed hASCs were seeded at passage 3 onto the pre-incubated stretching devices and reference samples. After the pre-incubation of 3-4 hours, the incubation medium (BM) was first

removed, 400 µl of fresh BM added, and 1130 hASCs (1000 cells/cm^2) were seeded onto the samples in 100 µl of BM. Three parallel samples were used in all experiments and the culture medium was changed twice a week. Different hASC donors were used for preliminary static experiments and for stretching experiments.

2.10 Attachment, viability and proliferation of hASCs on the coatings

The attachment and viability of hASCs were first studied with all five coating types (PHY, GLA, AA1, AA2 and AA3) in static conditions using light microscopy imaging. The same samples were followed and imaged by a light microscope (Zeiss Axio Vert.A1 microscope, Carl Zeiss) at time points of 3, 7 and 14 days.

Three selected coating methods (GLA, AA1 and AA2) were further studied with hASCs in dynamic conditions. The cyclic equiaxial stretching was started on Day 3, three days after plating, using the same parameters as for the coating characterization without cells. The unstretched reference samples were cultured in the same incubator at the same time. The stretched and static samples were imaged with the light microscope after 0 (Day 3+0), 3 (Day 3+3) and 10 (Day 3+10) days of stretching.

The cell morphology in GLA, AA1 and AA2 methods was studied in more detail by staining the cytoskeleton and the nuclei of hASCs 5 h after plating, at the beginning of stretching (Day 3), and on Day 3+10 cultured in static and dynamic conditions. The staining protocol began with fixing and permeabilisation in 4 % paraformaldehyde (Sigma-Aldrich) supplemented with 0.2 % Triton-X 100. After the blocking in 1 % BSA, the samples were incubated with Phalloidin-TRITC (#P1951, Sigma-Aldrich) diluted 1:5000 in the blocking solution together with DAPI (Molecular Probes; 1:1000), and then thoroughly washed. The mounted samples were imaged with a fluorescent microscope (Olympus IX51) using a 20× air objective.

The proliferation in GLA, AA1 and AA2 samples was studied quantitatively on Day 3+10 using a CyQUANT proliferation assay (Life Technologies) based on total DNA content in the samples. The method was performed as described earlier [42]. Briefly, the hASCs were lysed in 0.1 % Triton-X 100 buffer (Sigma-Aldrich). After a freeze-thaw cycle, three parallel samples from each lysate sample were

mixed with CyQUANT® working solution, and the fluorescence was measured at 480/520 nm using a microplate reader (Wallac Victor 1420 Multilabel Counter; Perkin Elmer).

3   Results

3.1   Immunofluorescent characterization of the coatings

The coatings of the five methods were first characterized using immunological staining of collagen Type I on Day 0 and after the 6-day incubation period in static and stretched conditions. The fluorescent images (Figure 3A) show that all coating methods bound collagen Type I onto the PDMS surface (Day 0). However, the amount and homogeneity of the attached proteins differed between the coating methods, as shown in quantifications (Figure 3B and C). By using the PHY coating method, collagen accumulated on the plasma treated PDMS surface in thick layers, whereas by using the other methods the collagen layer was thinner and more evenly distributed, especially compared to AA1. On Day 0, the amount (area %) of the coating was smallest in group AA1. However, the coating was also most evenly distributed in AA1 having smallest standard deviation across the sample images. In addition to PHY, also GLA and AA2 samples contained notable drifts.

The 6-day stretching as well as the 6-day incubation in static conditions had a large deteriorative effect on the coatings. The quantified coating area decreased from Day 0 in all the groups except for GLA. By Day 6 in both static and dynamic conditions, GLA-coated collagen had transformed and aggregated into bigger dots. In addition, stretching removed the bigger aggregate dots linearly from the GLA samples. In the PHY samples, almost all of the thicker features vanished, especially in dynamic conditions, and the amount of collagen was quantified as smallest on Day 6 between all the groups, whilst it was largest of all the samples on Day 0. To conclude, the AA1 coated samples kept their homogeneous form from Day 0 to Day 6 in both stretched and static conditions.

The durability of the covalent coatings was proved by sonication experiment (Figure 4). While the physisorbed coating detached almost entirely during sonication, covalently attached collagen Type I

was observed in GLA and AA1-3 samples also after sonication. However, the sonication decreased the amount of collagen in all groups, and also made it more homogeneous possibly due to the detachment of physisorbed collagen from the covalently coated samples. Secondary antibody blanks can be found in Supplementary Figure 1.

3.2   Physical characterization of the coatings

The water contact angle analysis (Figure 5) revealed that all of the coating methods significantly decreased the hydrophobicity of PDMS. The GLA coated samples were significantly more hydrophobic (larger contact angle) compared to all AA based coatings. During the intermediate phases of the coating methods, the plasma treatment first makes the surface totally hydrophilic (Contact angle < 5°), then after the APTES treatment the surface is more hydrophobic, and then GA and AA slightly increase the hydrophilicity of the surfaces. In the APTES + AA1 phase, the surface is significantly more hydrophilic compared to all other phases and methods, except for Plasma.

The AFM analyses (Figure 6) revealed that the roughness of plain PDMS is highest compared to all the coated samples. However, the surfaces of all the samples are smooth and differences minor. The lowest roughness was achieved using the AA1 coating method. The membranes of the devices are subjected to slight stretching during the detachment from the base, shown as cracks in the AFM images.

3.3   Chemical characterization of the coatings

Figure7A shows the FTIR-ATR spectra for collagen Type I coated samples and Figure 7B for intermediate samples without collagen. The spectral region was limited to 4000-1300 cm^{-1} to exclude the PDMS substrate dominated fingerprint region. While being generally very weak, several peaks of interest were found in this region. Table 2A and B shows the wavenumbers and the assigned chemical functionalities for these peaks.

The samples with collagen did not show many strong peaks apart from the CONPHY. However, quite many indications for groups found in collagen can be pointed out (Table 2A). Amide indications can

be seen at 3350, 1685-1659, and 1540-1508 cm^{-1}, while several peaks for other carbonyl compounds can be found between 1743-1696 cm^{-1}. Bands for CH_2, which cannot be found from PDMS, can also be seen at three locations in the control sample. Similarly, the samples without collagen showed several interesting peaks. Especially the control CONAA1, which was created for maximum reactivity, showed a strong indication of amide bonds forming between AA and APTES as was concluded by Tiller *et al.* [33]. In addition, the peaks at 1792 and 1744 cm^{-1} indicate that AA has not fully degraded, despite of the strong oxidizer. Overall, the FTIR-ATR results show a strong indication that covalent bond can form between the amine functionalized PDMS and AA.

3.4 Cell attachment and viability in static conditions

To prove the suitability of the new coatings for cell culture, all the five coating methods were first studied with hASCs in static conditions using light microscopy imaging on Days 3, 7 and 14. On Day 3, all the five coating types possessed attached hASCs indicating that all the coatings supported initial cell attachment (Figure 8). However, only four days later, on Day 7, almost all hASCs detached from the PHY coated samples seen as round floating cells. The other coatings on Day 7 supported cell attachment, but the number of cells had not distinguishable increased from Day 3. The attached hASCs took a star-shaped morphology with long extensions, which is typical for hASCs on soft substrates. At Day 14, the PHY coated membrane was empty indicating poor support for cell attachment and collagen binding. On the GLA coating, hASCs had not proliferated compared to both earlier time points, and the size of the few attached hASCs was large. Additionally, some debris was observed on the surface of the GLA coated membrane at Day 14. By Day 14, the AA1 and AA2 coated samples had both reached confluency and the hASCs grew in many layers. Cell clusters were also observed in AA2 samples. The hASCs in AA3 samples showed as weak proliferation as in GLA samples at Day 14. The amount of debris on the substrate also increased extensively and the morphology of the cells changed indicating a problem in cell viability. Judging by the light microscopy images of hASCs in static culture, the AA1 and AA2 coatings supported the attachment and long-term culture of hASCs best, and are

suitable coating methods for PDMS cell culture devices, whereas the PHY, GLA and AA3 methods did not support cell viability and proliferation.

3.5 Cell growth and proliferation on the GLA, AA1 and AA2 coatings

GLA, AA1 and AA2 coatings were further studied under stretched conditions using light and fluorescent microscopy imaging at the beginning of stretching on Day 3+0, after three days of stretching on Day 3+3, and after ten days of stretching on Day 3+10. As shown in Figures 9 and 10, no differences between the samples were observed on Day 3+0. At Day 3+3, stretching had clearly detached most of the cells in GLA coated samples while in the static samples the hASCs were still growing. AA1 and AA2 coated samples were not affected by stretching at this time point and their growth and proliferation were comparable to the static samples. At Day 3+10, even the static GLA samples showed problems with cell viability, further proving that the GLA coating is unsuitable for culturing hASCs sufficiently. At Day 3+10, AA1 samples showed the best proliferation in both static and stretched conditions, which was confirmed using the quantitative method shown in Figure 11. In addition, the stretched samples had a slightly smaller number of cells compared to the static samples in both groups AA1 and AA2. The AA2 samples showed some debris at Day 3+10, especially in the stretched conditions. At Day 3+10 in Figure 10, the cells in all groups can be seen growing in multiple layers already. Furthermore, Figure 10 shows that at Day 3+10 the cells in the stretched samples are more uniformly aligned than in the respective static samples, where the alignment is more random, regardless of the coating method.

4 Discussion

PDMS, or PDMS composites such as Sylgard-184, are widely used materials for microfluidic and cell culture applications because of their elasticity, transparency and biocompatibility. They have been studied as culture substrates for most cell types, but PDMS alone does not provide adequate support for cell adhesion [11]. This is even more evident in longer culture periods and especially in dynamic

conditions. This realization has led to various techniques to functionalize the PDMS surface to promote cell adhesion and proliferation while being durable enough to withstand stretching.

It has been previously demonstrated that covalent immobilization of collagen on the PDMS surface has been the most efficient technique to increase cellular affinity to the surface [11]. However, many crosslinking chemicals used for immobilization, such as GA, are cytotoxic, so using them can be detrimental to the cell culture [30-32]. In this study, we replaced the cytotoxic GA with AA, which is an essential vitamin for cell growth and maintenance. The chemical principles behind the AA crosslinking reactions and the subsequent fragmentation have been proposed earlier by Tiller *et al*. [33]. Here, we provide a new method for formation of durable coating with three versions and test their applicability in cell stretching; namely collagen Type I coating based on ascorbic acid as a crosslinker (referred as AA1, AA2 and AA3).

The three coating methods are otherwise identical, except for the solvent used for the distribution of AA, and the amount of AA dissolved. In the AA1 method, the AA powder was dissolved and distributed in DPBS; in AA2 method, the AA powder was dissolved in methanol; and in AA3, the AA powder was dissolved in methanol with added hydrogen peroxide. AA1 solution contained 200 mg/mL and AA2 and AA3 solutions contained 20 mg/mL AA, amounts which relate to AA's solubility. For comparison, we also tested physisorbed collagen Type I coating and glutaraldehyde crosslinker based coating and we can confirm previous results [11,43] that PDMS functionalized with the glutaraldehyde crosslinked protein collagen Type I can support static cell culture for three days. However, in our study the cell growth on physisorbed coating was so poor, especially after three days, that the method was excluded from the stretching tests. Moreover, we demonstrate that AA1 and AA2 coatings on PDMS successfully promote hASC adhesion and proliferation in static and stretched conditions. We also demonstrate that AA1, and to a lesser extent AA2 coating, improve adhesion and proliferation when compared to PHY and GLA coatings. The fluorescent study on the coatings also revealed differences in the amount and formation of the bound collagen. We demonstrated the presence of collagen on the covalently coated devices after stretching and also after sonication, and showed the detrimental

effect of physical loadings to the physisorbed collagen. The possible advantage of the large amount of collagen on the PHY samples visible in the fluorescent images, as well as hinted in the FTIR spectra, was completely nullified by mechanical load and the impact of the cells. Furthermore, the fluorescent images taken after 13 days of culture from phalloidin and DAPI stained samples revealed a well-developed cytoskeleton and hints of cellular alignment after stretching.

AA, albeit often in phosphate form, has been commonly used as a supplement in osteogenic differentiation culture media to facilitate ECM formation and to act as an antioxidant [44]. For example, the osteogenic culture medium used by *Tirkkonen et. al.* [45] included 250 µM of AA 2-phosphate. Compared to AA, the AA 2-phosphate is shown to be more stable in standard cell culture conditions [44]. Nevertheless, it is uncertain in the case of this study, if using AA phosphate in coatings would produce different results during cell culture and increase cell growth. The different chemistry of AA phosphate and most notably the loss of acidity could inhibit the critical initial imine formation reaction between APTES and AA, because the reaction is acid catalysed [46].

AA has been previously utilized by Tiller *et al.* [33] and Oliveira *et al.* [47] in covalent immobilization of enzymes by exploiting its reactivity with primary amines, but AA has not been used as a crosslinker in cell cultivation systems before. According to the findings of Tiller *et al.* and the previous evidence from food chemistry studies, AA readily reacts with primary amines [33,48,49]. They reported that AA reactivity could be further increased in alcohols or by adding hydrogen peroxide, due to oxidation. After linking itself with two primary amines, AA generally degraded via autoxidative fragmentation into an oxalic acid bis-amide species. Interestingly, the fragmentation leads to change in the crosslinking chemical bonds by turning the relatively unstable imine bonds into stable amide bonds. This was also indicated in our FTIR results. While this suggests that oxidation could also increase collagen binding, we did not see it positively affecting in cell culture.

Judging by the fluorescent images and image analyses on Day 0, the amount of collagen was lowest in AA1 compared to all other groups; still, the distribution of coating was most homogeneous in AA1. Incubation in static and dynamic conditions, and the sonication treatment decreased the amount and

evened out the distribution of collagen. This implies that some of the collagen was bound non-covalently in multiple layers that were removed during stretching, which was also proved by the sonication analysis. Cell proliferation was also weaker in AA2 and more in AA3 when compared to AA1. It was not expected in light of the results by Tiller *et. al*. [33], as they managed to increase the reactivity of AA with amines; however, their study did not include experiments with cells. Furthermore, during cell culture a large amount of debris accumulated in AA3 samples, possibly due to residue from dead cells staying attached to the substrate. It would mean that the coating is staying intact during cell culture, but also that there was an issue with cytotoxicity.

These results suggest that methanol and hydrogen peroxide increase the cytotoxicity during cell culture to the extent which overweighs their effect on better binding of collagen. Another explanation for the lower cell numbers could be the non-covalently bonded layers of collagen that detach during the experiments and take cells with them, but it would not explain the amount of debris that was seen. The effect of AA molecules and the possible leftover split products in the coating that are not bound to collagen is currently also not known, but it is unlikely that they are as cytotoxic as unbound glutaraldehyde, which is a known powerful fixative.

5   Conclusions

In this study, a novel covalent coating method based on AA as a crosslinker was investigated on stretched and static PDMS substrates. Three versions of the AA based covalent coatings were compared to physisorption based and previously used cytotoxic GA crosslinked coating methods. Immunofluorescent staining of the coated collagen Type I together with other surface characterization methods proved the covalent immobilization of collagen, and also showed the durability of the covalent coating after sonication and 6 days of stretching, which is important for dynamic cell culture studies. As a proof-of-concept we showed with hASCs that the novel AA1 coating method is suitable for long-term cell culture experiments, contrary to PHY and GLA methods. Furthermore, the AA1 method supports cell attachment and proliferation also in dynamic conditions for 10 days.

To conclude, the cytotoxic GA can be replaced by AA to bind collagen covalently on PDMS. The proposed method is simple, biocompatible and allows the binding of any molecule with a free amine group, similarly to GA, so it has room for expansion to other ECM proteins and peptides. Moreover, our coating method is an important step in the development of PDMS based devices, and may improve functionalization efforts of tissue engineering scaffolds and implants with durable but cell friendly coatings.

Acknowledgements

Data accessibility

All the images related to the study have been stored in a shared folder accessible by the research team. The CellProfiler pipeline file will be provided upon request.

Competing interests

We have no competing interests to declare.

Authors' contributions

JLe drafted the manuscript with SVi, had a major part in innovating, designing and implementing the ascorbic acid coating methods, fabricated the stretching devices and helped to build up and maintain the stretching system, and participated in study design, microscopy work and interpretation of data;

SVi planned, prepared and conducted most of the cell culture experiments and gathered data and images, had a major part in the fluorescent imaging of the coatings and interpretation of data and participated in study design and coordination; KKa performed the quantitative image analysis; PKa, JKr SMi and SVa participated in study conception, design and coordination, and contributed in manuscript editing. In addition to this JKr helped to coordinate and troubleshoot problems related to PDMS and the stretching devices.

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Ethics Statement

The study was conducted in accordance with the Ethics Committee of the Pirkanmaa Hospital District (Tampere, Finland, R15161) and with patients' written consents.

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Tables and figures with captions

Table 1: Details of recently used PDMS coatings.

| Modification type	Coating methods	Cell type	Ref.
Physisorption	Bovine collagen Type I on PDMS with nanogrooves	Human MSCs	[15]
Physisorption	Patterned polydopamine coating	Human fibro-sarcoma, mouse preosteo- and fibroblast	[16]
Physisorption	Fibronectin and poly-L-lysine coatings	Fibroblasts, chondrocytes	[17]
Physisorption	Collagen Type I on PDMS under stretch	Rat lung fibroblasts	[11]
Physisorption	Micro fibrillated cellulose coating	Rat hepatocytes	[18]
Physisorption	Graphene coating with PMMA support	Human MSCs	[19]
Physisorption	Poly-D-lysine coating on microgrooved PDMS	Human UCB-MSCs	[20]
Oxygen plasma/physisorption	Oxygen plasma, fibronectin, laminin or collagen Type I ECM coating or poly-D-lysine or L-α-phospha-tidylcholine electrically charged coating	Human intestinal epithelial cells	[4]
Oxygen plasma/physisorption	Oxygen plasma and BSA, collagen Type I or fibronectin coating	Human breast cancer cells	[5]
Oxygen plasma/physisorption	Gelatin on plasma treated PDMS under stretch	Human pluripotent stem cells	[6]
Argon plasma/physisorption	Pluronic F-68 on argon plasma treated PDMS	Mouse macrophages	[7]
Argon plasma/physisorption	Collagen Type I on plasma treated PDMS	Human endothelial progenitor cells	[8]
Argon plasma/polymer graft	PEGMA grafted PDMS	Mouse macrophages	[7]
Layer-by-layer	6 alternating layers of polyethylenimine and polystyrene sulfonate	Bovine vascular SMCs	[21]
Layer-by-layer	6 alternating layers of polyethylenimine and polystyrene sulfonate under stretch	Rat lung fibroblasts	[11]

Layer-by-layer	Oxygen plasma, 6 alternating layers of poly-ethylenimine and polystyrene sulfonate	Human intestinal epithelial cells	[4]
Layer-by-layer	Oxygen plasma, PAH, several PAA and PAM layers, PAA-azide layer with collagen Type I, PLL or PAH-g-PEG, UV irradiation patterned	C3A and L929 cell co-culture	[9]
Immobilization	Argon plasma, APTMS or MPTMS silanization, sulfosuccinimidyl cross-linked protein A, patterned	Rat endothelial cells	[10]
Immobilization	Gold layer with MMAAPA cross-linked bovine collagen Type I	Mouse pituitary tumour cells	[22]
Immobilization	Oxygen plasma, APTES silanization, glutaraldehyde cross-linked collagen Type I under stretch	Rat lung fibroblasts	[11]
Immobilization	Polyacrylamide hydrogel with Sulfo-SANPAH cross-linked collagen Type I	Human epidermal stem cells	[23]
Immobilization	Oxygen plasma, aminosilanization, sulfo-SANPAH cross-linked collagen Type I	Rat hepatocytes	[12]
Immobilization	Ammonia plasma, star PEG immobilized adhesion peptides	Human keratinocytes	[13]
Immobilization	Ammonia plasma, hydrogel immobilized fibronectin under stretch	Mouse skeletal myoblasts	[14]
Immobilization	Polyallylamine-azido cross-linked nerve growth factor on grooved PDMS		[24]
Gel scaffold	Collagen Type I gel scaffold in PDMS microfluidic device	Human endothelial cells	[25]

Table 2: FTIR-ATR spectral values of certain assigned peaks present in the spectra of the coatings with (A) or without (B) collagen Type I.

A	With Collagen Type I					
Assignment	CONPHY	PHY	GLA	AA1	AA2	AA3
H bonding (broad)/amide A NH stretch	~ 3350					
CH_2 stretch	2913					
CH_2 stretch	2849					
C=O stretch	1743	1740	1743		1743	
C=O stretch	1734	1734				
C=O stretch	1718	1718		1716	1718	
C=O stretch	1700			1696		
Amide I C=O stretch	1685	1676		1675	1675	1675
Amide I C=O stretch	1659					
Amide II NH bend				1578		

Assignment	CONAA1	GLA	AA1	AA2	AA3
Amide II NH bend		1540		1540	
Amide II NH bend		1512	1508	1508	1509
CH$_2$ bend	1473				
OH bend	1330				1329

B	Without Collagen Type I				
Assignment	CONAA1	GLA	AA1	AA2	AA3
H bonding (broad)	~ 3054		~ 3058		
CH$_2$ stretch	2840	2840	2840	2840	2840
C=O stretch	1792				
C=O stretch	1744				
C=O stretch			1718		
Amide C=O stretch	1653				
Amide C=O stretch	1628				
Amide II NH bend			1595	1596	1595
Amide II NH bend		1540	1540	1549	1549
Amide II NH bend	1513				
CH$_2$ bend	1468				
OH bend	1340			1346	1344
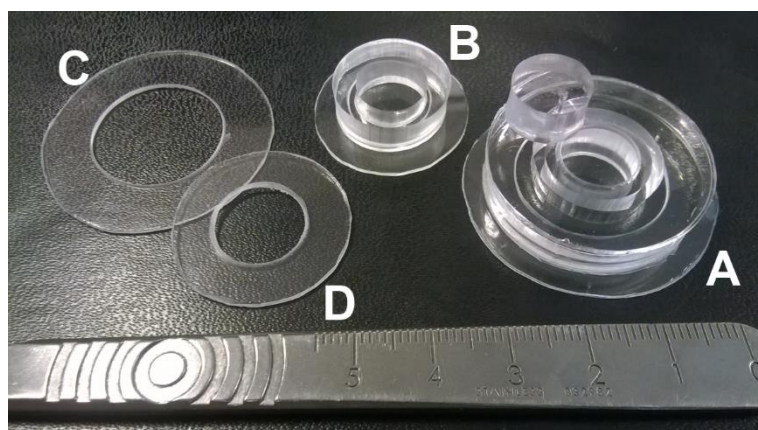
Figure 1: A PCSD with PDMS membrane (A), static reference device with the same cell culture area (B), and separate polycarbonate rings of the devices (C and D) for preventing PDMS membranes from touching the surface. The culture well is 12 mm in diameter (1.13 cm²) while the whole PCSD is 31 mm in width.
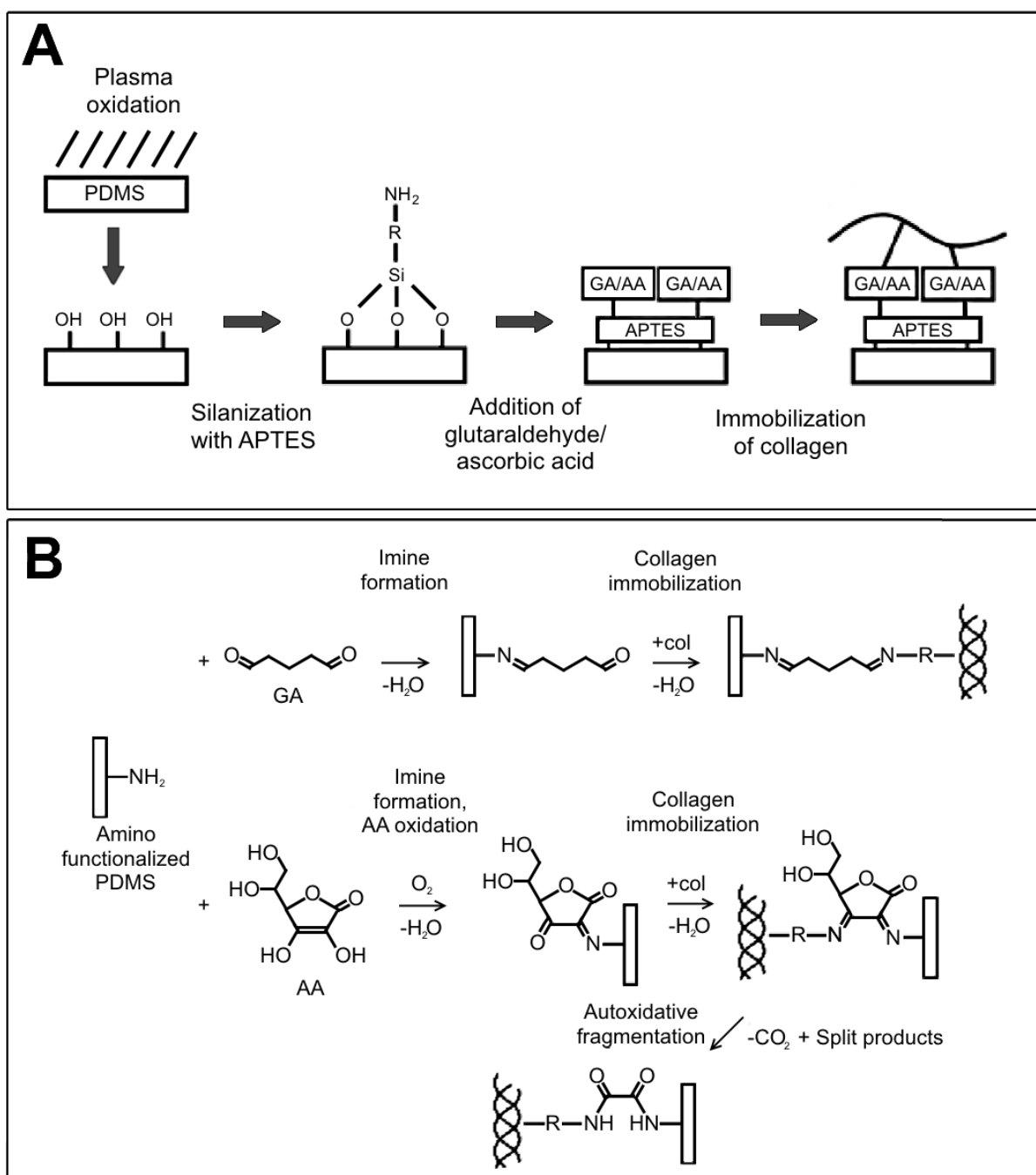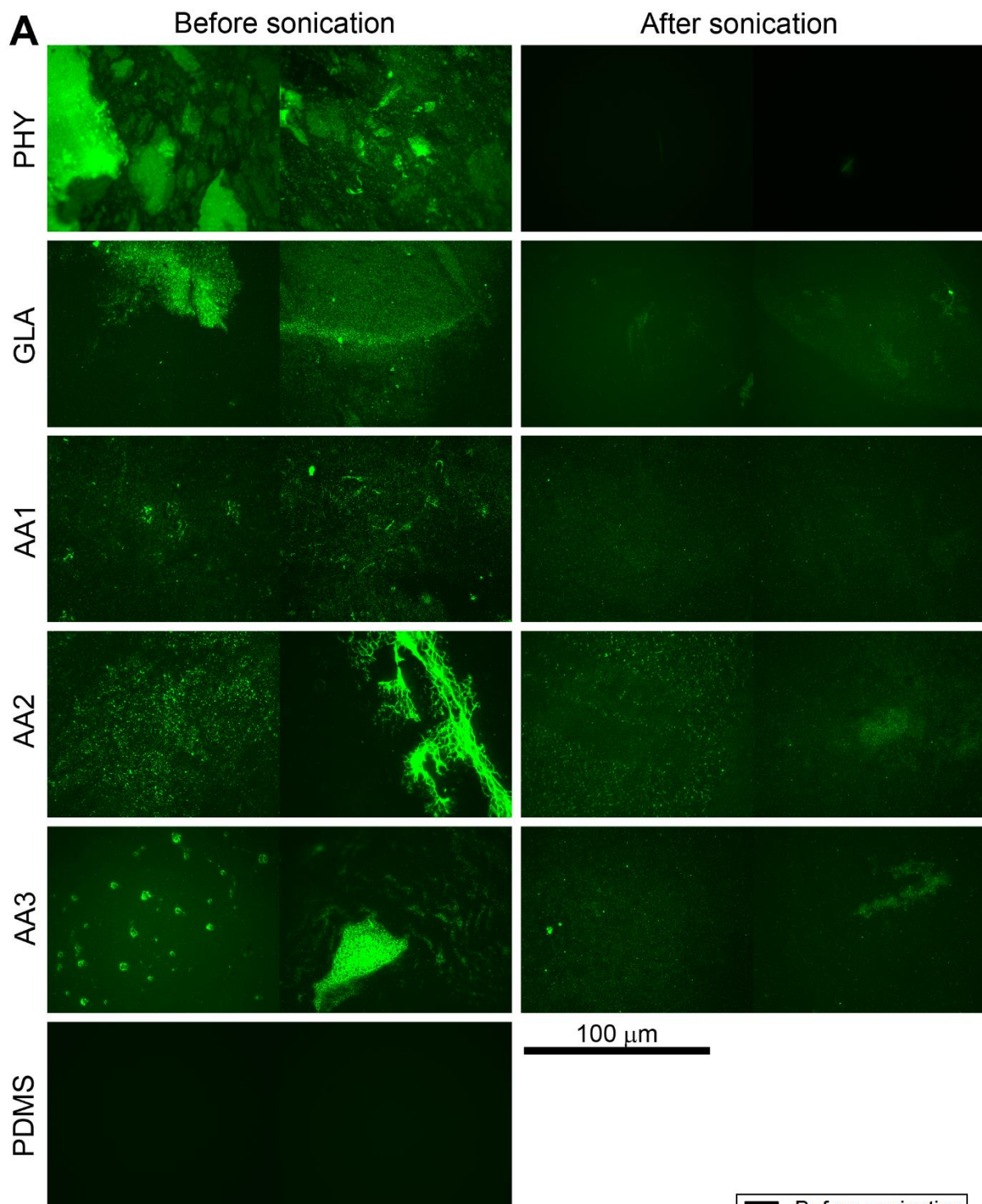
Figure 2: A schematic step-by-step illustration of the covalent coating process (A) and chemical depiction of the crosslinking reaction pathways between the substrate and glutaraldehyde (GA) or ascorbic acid (AA) (B). PDMS = polydimethylsiloxane, APTES = aminopropyltriethoxysilane, R = amino acid side chain of collagen that contains an amine functional group; lysine, hydroxylysine or arginine.
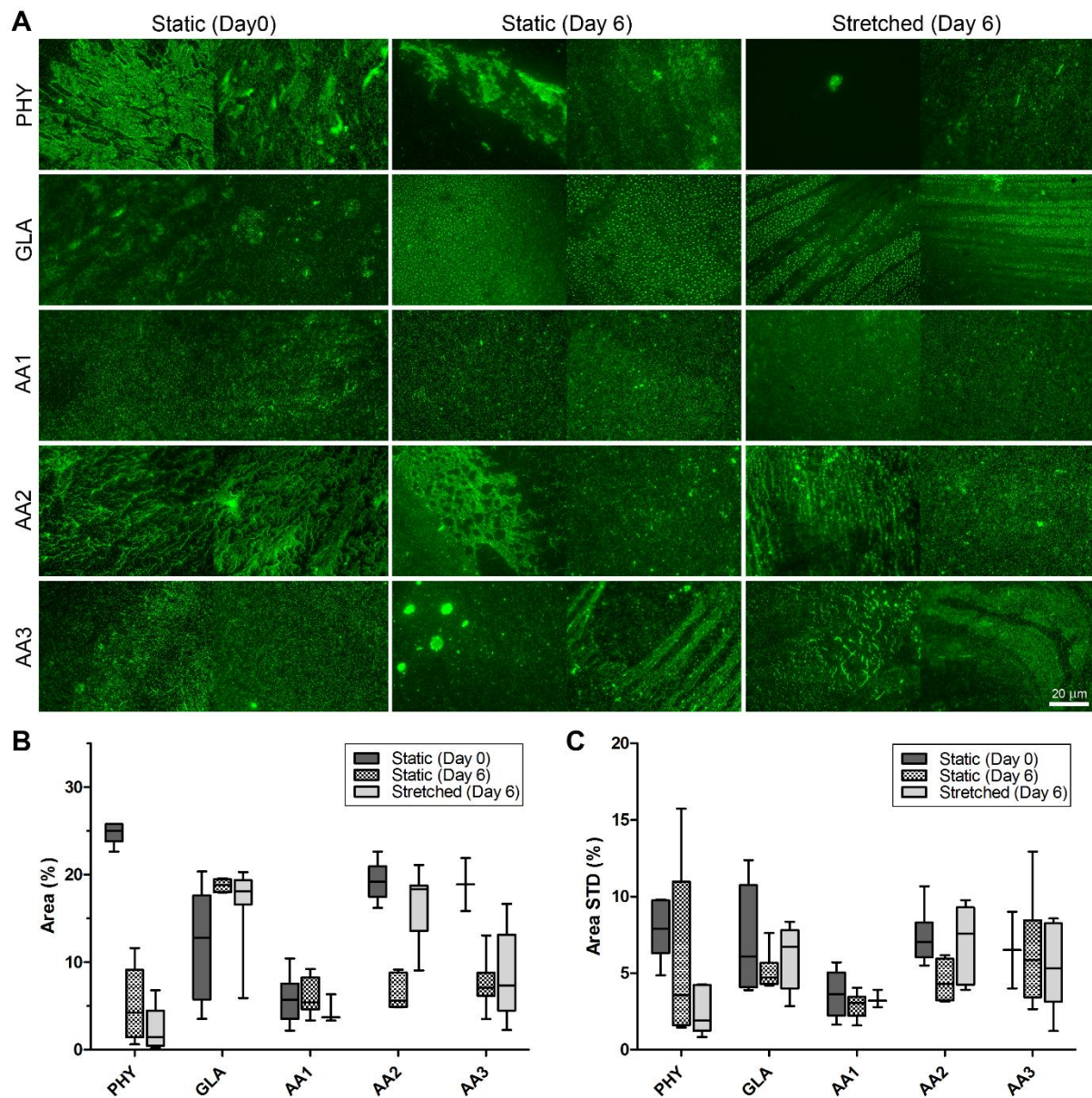
Figure 3. Immunofluorescent images of collagen Type I coatings (in green) without cells using PHY, GLA, AA1, AA2 and AA3 coating methods (A). The coatings were imaged before (Day 0) and after a 6-day incubation period in both static and stretched conditions. Scale bar = 20 μm. The amount (B) and consistency (C) of the coatings were quantified using image analyses.

A

Before sonication — After sonication

PHY

GLA

AA1

AA2

AA3

PDMS

100 μm

B

Area (%)

PHY   GLA   AA1   AA2   AA3   PDMS

Before sonication
After sonication

C

Area STD (%)

PHY   GLA   AA1   AA2   AA3   PDMS

Before sonication
After sonication
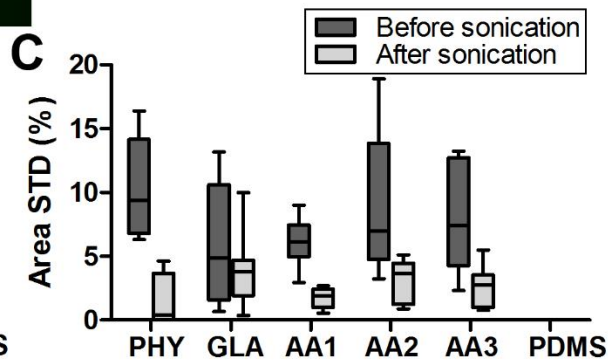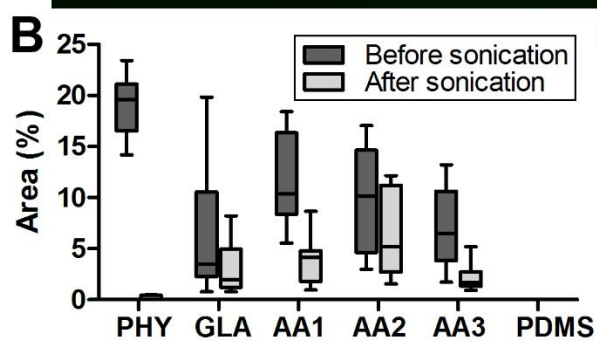
Figure 4. Durability of the coatings after sonication in 50°C for 60 min. Collagen Type I was stained in green (A), and the amount (B) and consistency (C) of the coatings were quantified using image analyses. Scale bar = 20 μm.
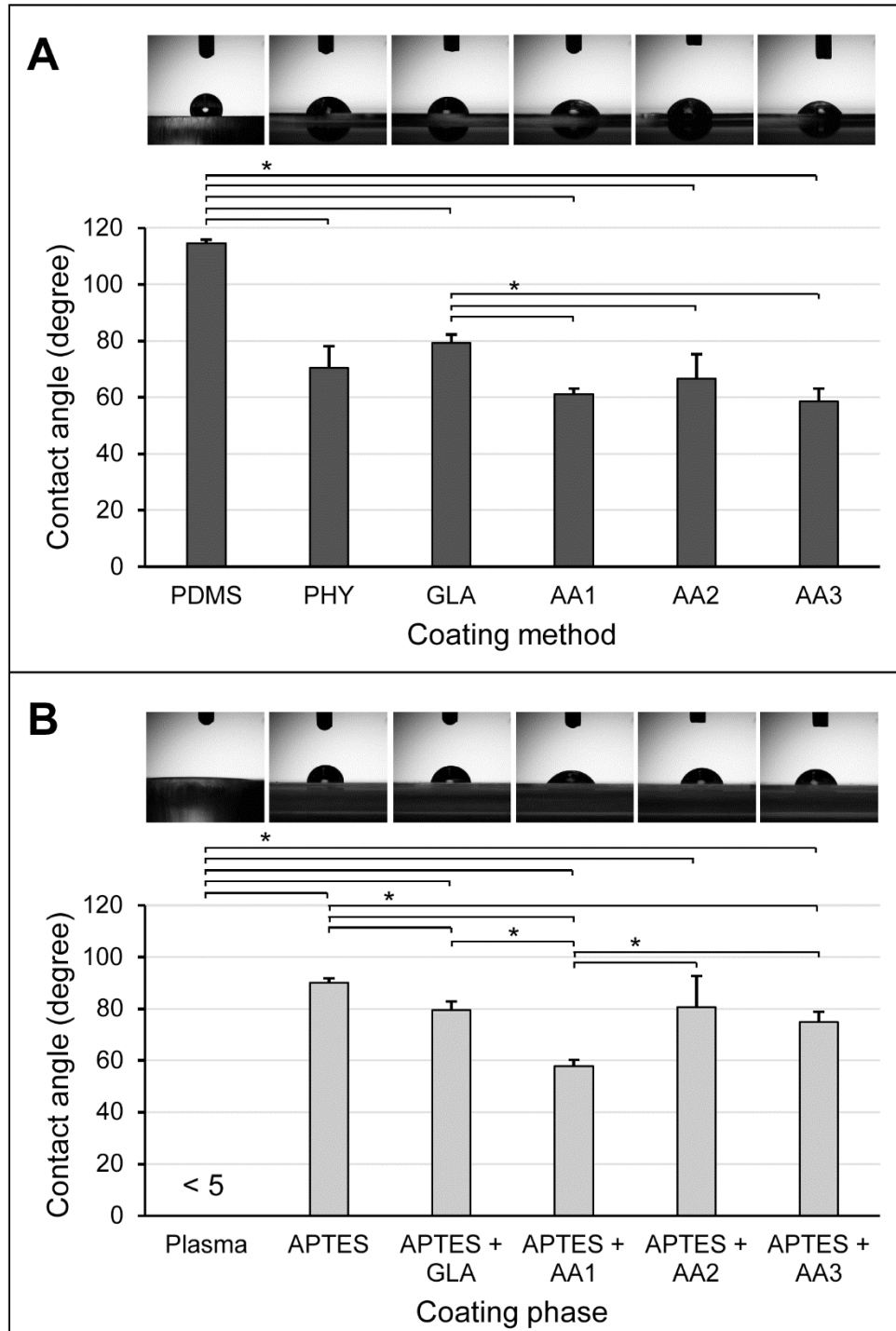
Figure 5. Water contact angle measurements of uncoated and coated PDMS using the five different coating methods (A), and after each of the intermediate coating phases (B). * p-value < 0.05.
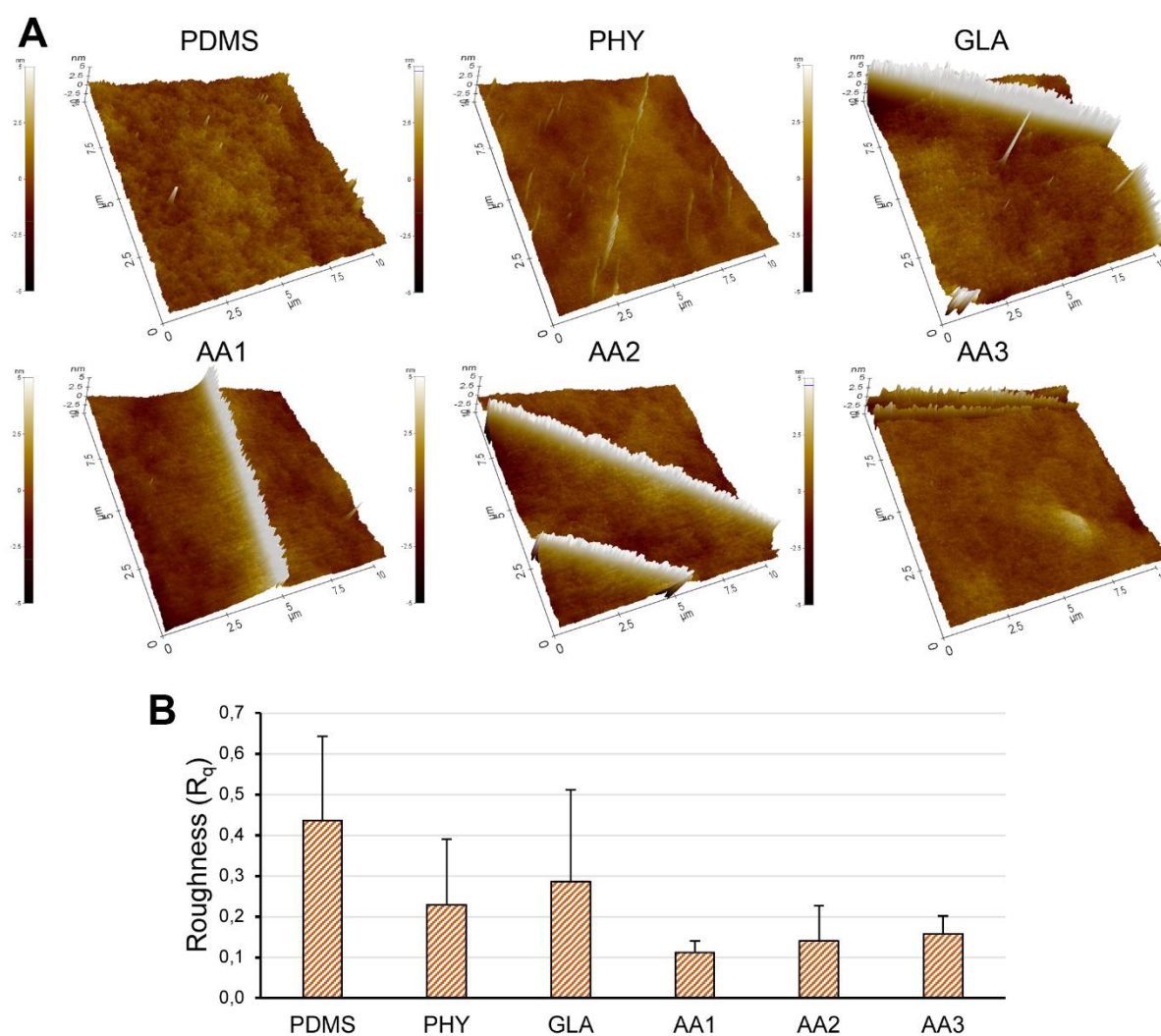
Figure 6. Atomic force microscopy images (A) of the plain PDMS surface and the collagen Type I coated surfaces using the five different coating methods: physisorption (PHY), glutaraldehyde based covalent coating (GLA) and three ascorbic acid based covalent coating methods (AA1-AA3)). Surface roughness (R_q) measured by AFM is presented as mean+SD (B).
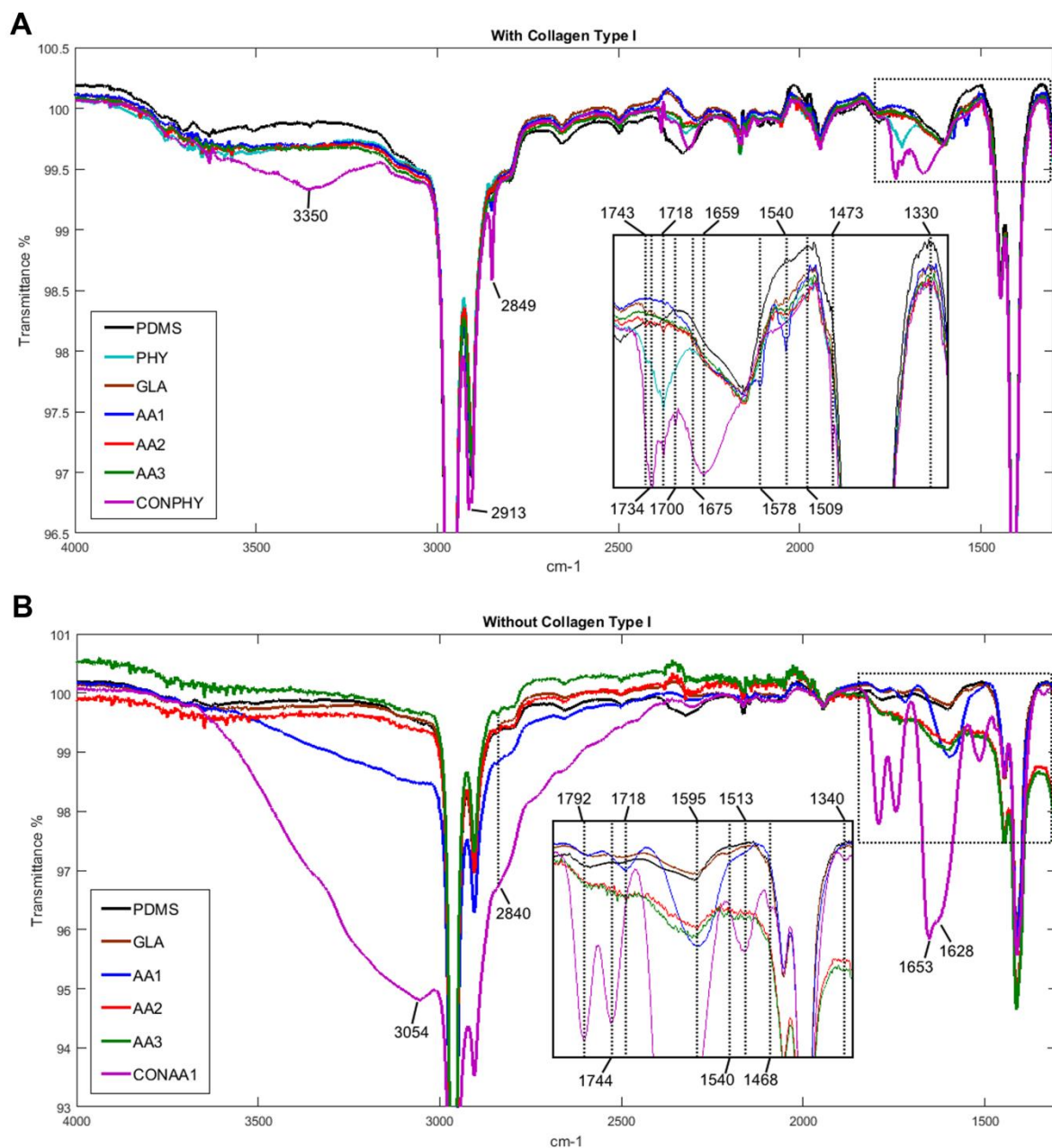
Figure 7. FTIR-ATR spectra of the coatings with (A) and without (B) collagen Type I in the range of 4000 to 1300 cm^{-1}. The areas marked with dash lined rectangles have been magnified, and the peaks of interest have been emphasized by numerical values.
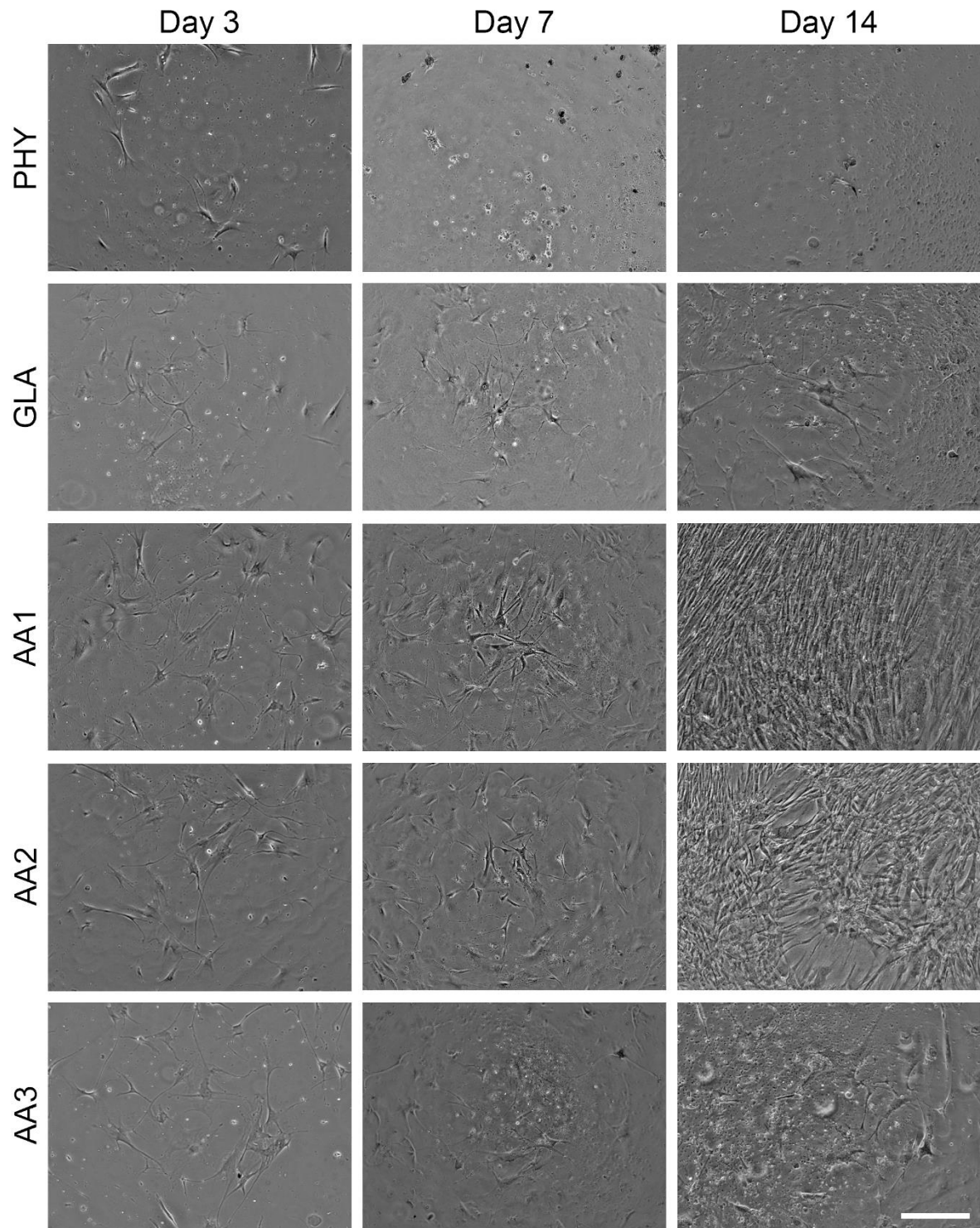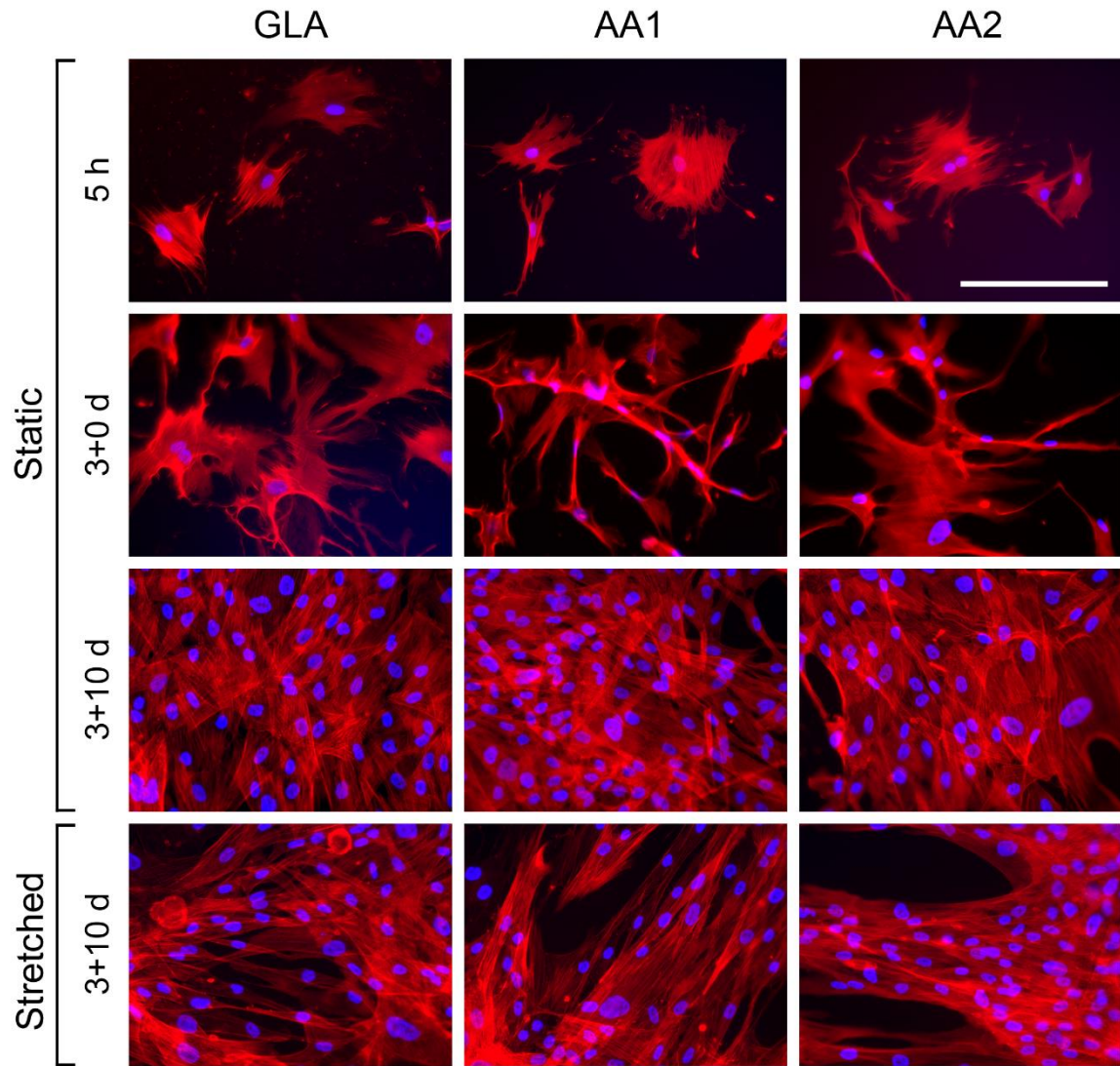
Figure 8. Light microscopy images of hASCs on PHY, GLA, AA1, AA2 and AA3 coatings at 3, 7 and 14 days in static conditions. Scale bar 200 μm.
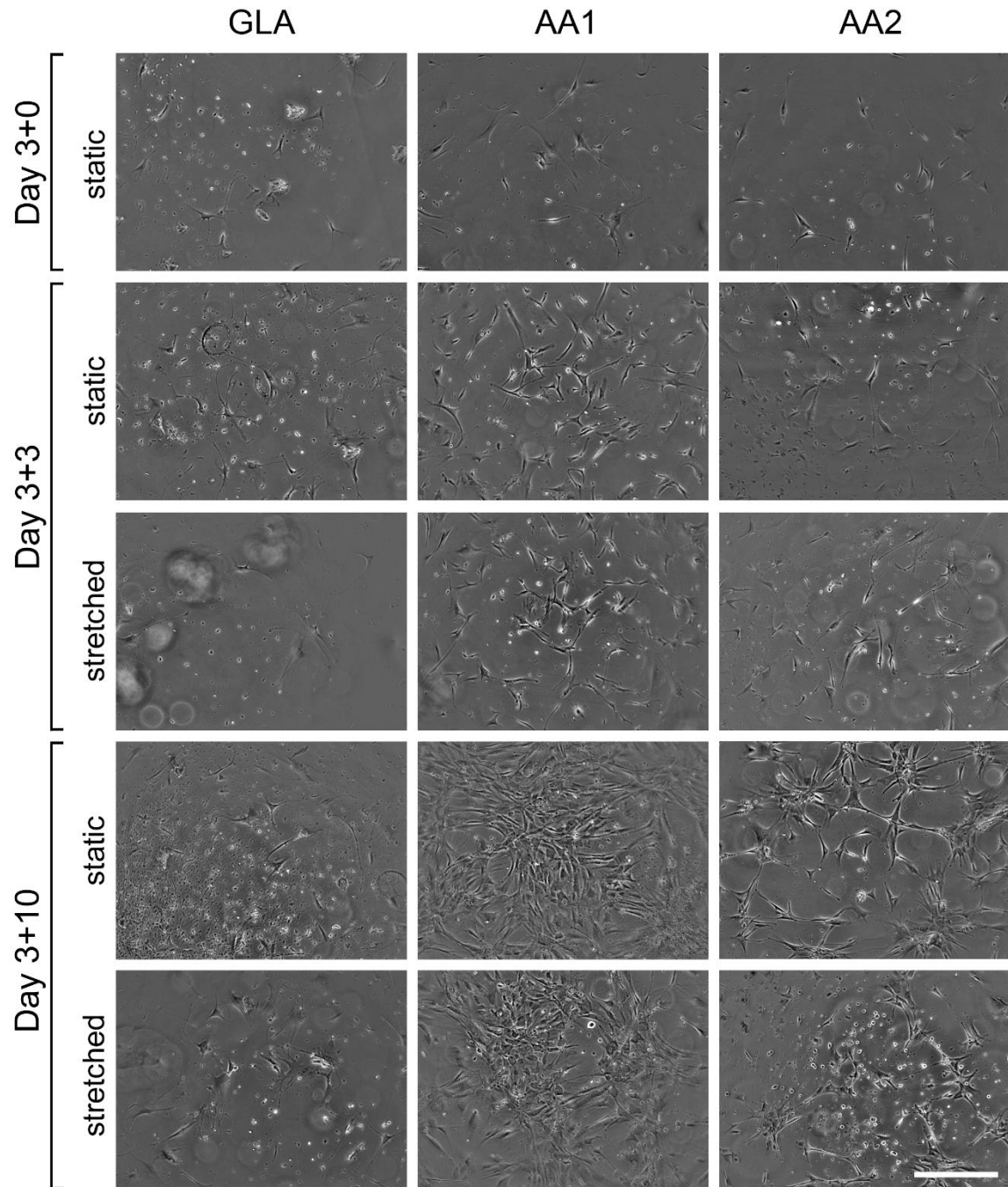
Figure 9. Light microscopy images of hASCs on GLA, AA1 and AA2 coatings in static and stretched conditions 0, 3 and 10 days after stimulation. The hASCs were plated three days before starting the stretching stimulation. Scale bar 500 μm.
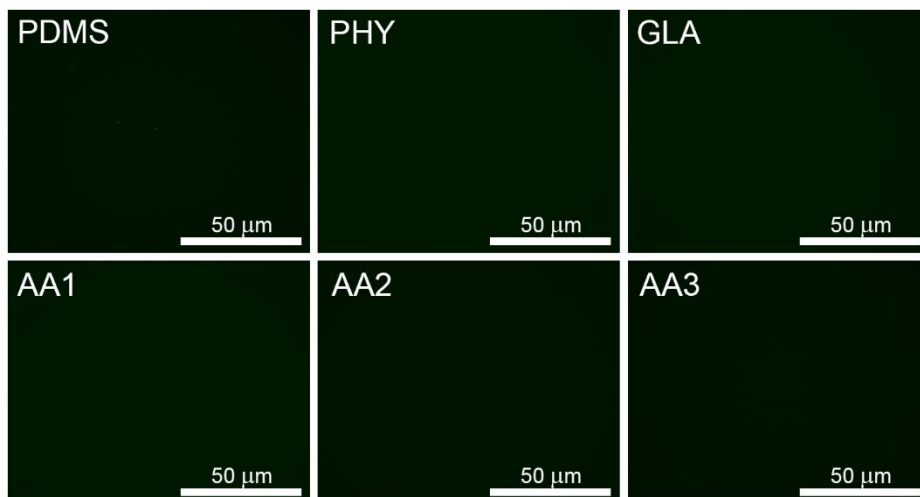
Figure 10. Fluorescent microscopy images of hASCs on GLA, AA1 and AA2 coatings 5 hours after seeding, 3 days after seeding at the beginning of stretching (3+0 d), and after 10 days of stimulation in static and dynamic conditions (3+10 d). The cytoskeleton was stained with phalloidin in red and nuclei with DAPI in blue. Scale bar 200 μm.
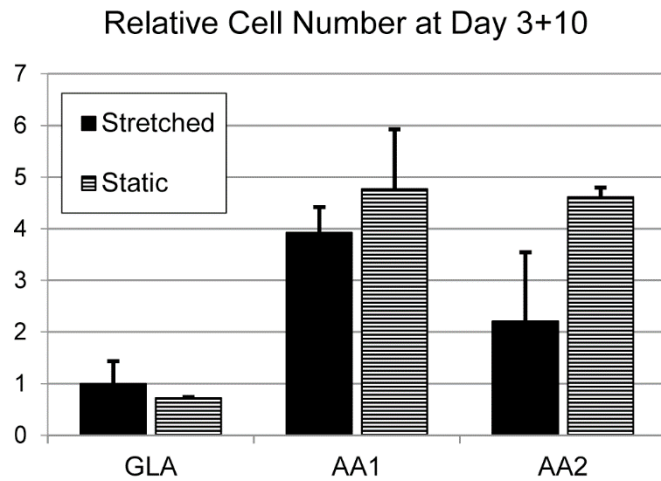
Figure 11. Relative cell number based on DNA amount of hASCs cultured in stretched and static conditions on GLA, AA1 and AA2 coated substrates on Day 3+10. Stretching was initiated after three days of static culture for the dynamic samples.

Supplementary material

Figure S1. Fluorescent microscopy images of secondary antibody blanks (Alexa 488, green; no primary antibody used) of the plain PDMS and the five coating methods showed that no unspecific binding of secondary antibody was observed.