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Assay for characterizing the recovery of vertebrate cells for adhesion measurements by single-cell force spectroscopy

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ABSTRACT

Single-cell force spectroscopy (SCFS) is becoming a widely used method to quantify the adhesion of a living cell to a substrate, another cell or tissue. The high sensitivity of SCFS permits determining the contributions of individual cell adhesion molecules (CAMs) to the adhesion force of an entire cell. However, to prepare adherent cells for SCFS, they must first be detached from tissue-culture flasks or plates. EDTA and trypsin are often applied for this purpose. Because cellular properties can be affected by this treatment, cells need to recover before being further characterized by SCFS. Here we introduce atomic force microscopy (AFM)-based SCFS to measure the mechanical and adhesive properties of HeLa cells and mouse embryonic kidney fibroblasts while they are recovering after detachment from tissue-culture. We find that mechanical and adhesive properties of both cell lines recover quickly (<10 min) after detachment using EDTA, while trypsin-detached fibroblasts require >60 min to fully recover. Our assay introduced to characterize the recovery of mammalian cells after detachment can in future be used to estimate the recovery behavior of other adherent cell types.

1. Introduction

Specific adhesive interactions between cells and extracellular matrix (ECM) or between cells play crucial roles in cellular communication, tissue organization, embryonic development and wound healing. Accordingly, a wide variety of diseases are associated with impaired cell adhesion [1–4]. Animal cells sense and adhere to their extracellular environment via cell adhesion molecules (CAMs), which are typically transmembrane proteins. Specific interactions between CAMs and their extracellular ligands induce intracellular signaling pathways, which regulate the adhesive and mechanical properties of cells besides other cellular processes. CAMs are classified into different families, including integrins, cadherins and selectins [5–8]. To strengthen the cellular attachment to an extracellular substrate, multi-protein complexes anchor CAMs to the cytoskeleton. Key cytoplasmic adaptor proteins include talin, kindlin, vinculin and catenins [9–12]. Due to the general importance of cell adhesion, the interaction of CAMs and their ligands are studied extensively using various, yet mostly qualitative methods [13,14]. However, as these qualitative methods can provide helpful insights, describing the adhesive interactions of cells benefits greatly from measuring quantitative parameters such as cell adhesion forces, kinetics and energies.

Single-cell force spectroscopy (SCFS) offers the possibility to measure adhesive forces and energies of single cells adhering to a biotic or abiotic substrate, another cell or tissue [15,16]. SCFS methods are based on force sensing devices such as optical or magnetic tweezers, micropipettes, or atomic force microscopy (AFM) [14,17,18]. In these SCFS-based methods the cell is brought into contact with an adhesive substrate or another cell for a given contact time and then separated. While approaching and retracting the cell, the interaction forces are recorded and provide a quantitative measure of the adhesive interactions between cell and substrate. Among all currently available SCFS methods, AFM-based SCFS cov-

Abbreviations: AFM, atomic force microscopy; BSA, bovine serum albumin; CAMs, cell adhesion molecules; ConA, concanavalin A; ECM, extracellular matrix; GFP, green fluorescent protein; FD, force–distance; FCS, fetal calf serum; MYH9, myosin heavy chain 9; PAR, protease-activated receptor; SCFS, single-cell force spectroscopy

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ers the largest dynamic force range from ≈10 pN to ≈100 nN [16,18,19]. This wide range permits quantifying the adhesive force of an entire cell down to the adhesive force established by single CAMs. AFM-based SCFS attaches a single cell to the apex of a tipless AFM cantilever (Fig. 1). To facilitate cell attachment, the cantilever is coated either with a substrate-mimicking ligand (e.g., cell surface receptors or ECM proteins including collagens, laminins, or fibronectin), concanavalin A (ConA) to bind carbohydrates on the cell surface, antibodies, or an unspecific adhesive (e.g., CellTak, poly-L-lysine) [15,20–32]. The cantilever-bound cell is then approached either to a protein-coated substrate, another cell, tissue explant or biomaterial. After a pre-determined contact time, during which the cell is allowed to initiate adhesion, the cantilever is retracted until cell and substrate are fully separated. During the approach and retraction cycle cantilever deflection (e.g., force) and cell-substrate distance are recorded in so-called force–distance (FD) curves (Fig. 1C). Analysis of the FD curves provides several quantitative insights into the cellular interaction with the substrate. The approach FD curve provides insight into the mechanical properties of the cell being pressed onto the substrate [18,26,33–35]. The retraction FD curve provides the maximum detachment force, also called adhesion force, of the cell. However, two types of smaller unbinding events contained in the retraction FD curves correspond to the unbinding of single or clustered CAMs [15,16,18,19,36]. These unbinding events are frequently named rupture and tether events, and differ in the molecular scenarios leading to their emergence. In rupture events, the CAMs remain anchored to the actin-cytoskeleton and upon exposure of mechanical stress detach from their extracellular ligand [22,31,32,37–39]. If the anchorage to the cytoskeleton breaks before the CAM unbinds from the extracellular ligand or if the CAM has not been attached to the cytoskeleton in the first place, the CAM is pulled away from the cell cortex on the tip of a membrane tether [19,36,40]. In this so-called tether event, the tether is mechanically extended until the receptor-ligand bond breaks. The force required to extend a tether from the cellular membrane does not depend on the strength of the CAM-ligand bond but rather on mechanical properties of the cellular membrane (e.g., bending rigidity, viscosity, and tension) [40], the velocity at which the tether is extracted from the membrane, and on cell membrane attachment to the cortical cytoskeleton. In rare cases, tether extension from the cellular membrane terminates when the tether fails or if the receptor is pulled out of the membrane [40,41]. In the later separating phase between cell and substrate, the cell body is not in contact with the substrate anymore and tethers exclusively mediate cell adhesion [31]. The analysis of tether unbinding events can provide information on the lifetime of single CAM bonds, the mechanical properties of the cell cortex, and cell membrane tension [31,37,40,42–45].

Although SCFS measurements and other methods applied to characterize cell adhesion provide quantitative and qualitative insights into cell adhesion, a drawback is that adherent cells must first be detached from culturing flasks in order to characterize their adhesion to a given substrate. Cells are commonly detached with trypsin and/or ethylenediaminetetraacetic acid (EDTA) [27,46,47]. Although some CAMs, such as α2β1 integrin [14], are trypsin resistant, other CAMs such as cadherins are sensitive to trypsin cleavage [48]. Furthermore, other proteins involved in the initiation of

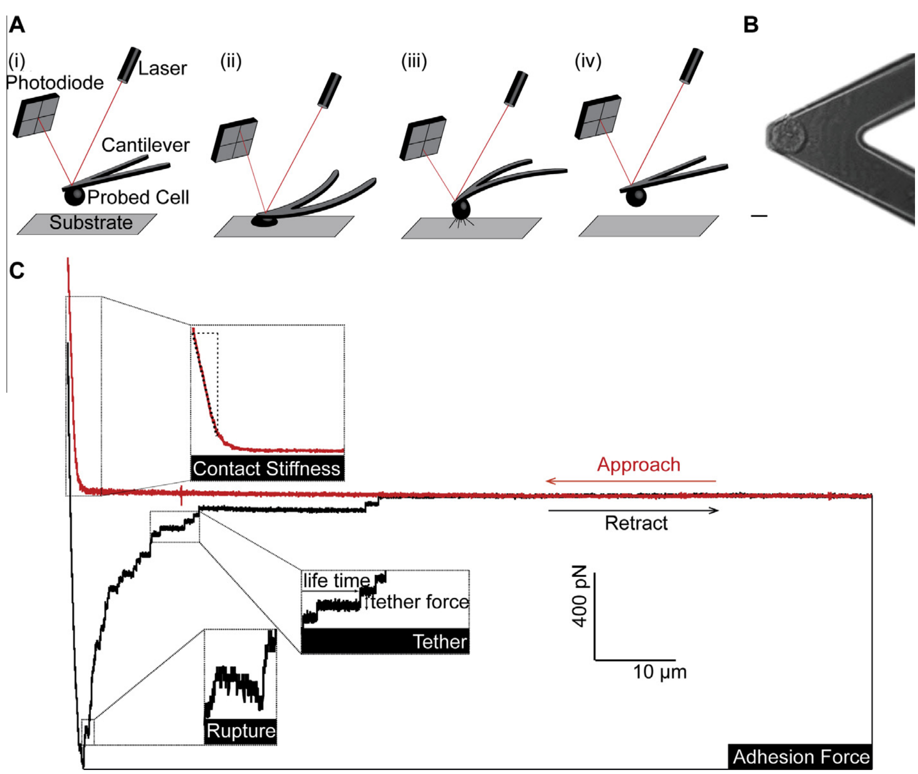

Fig. 1. Scheme of AFM-based SCFS. (A and B) To use a single cell as a probe it is bound to a concanavalin A (ConA)-coated tipless AFM cantilever (scale bar, 10 µm). (A) (i and ii) The cantilever is approached onto a protein-coated substrate until a preset contact force is reached. After a defined contact time (ii), the cantilever is retracted until the cell is fully separated from the substrate (iii and iv). During approach and retraction, the cantilever deflection and thus, the force acting on the cell is recorded in force–distance (FD) curves. (C) FD curves show different features: In the approach FD curve (red) the cantilever deflection measured upon pressing the cell onto the substrate correlates with the stiffness of the cell and is called contact stiffness [33]. The retraction FD curve (black) records the adhesion force of the cell, which represents the maximum downward force deflecting the cantilever and thus the maximum force needed to detach cell and substrate. After recording the maximum adhesion force, single receptor unbinding events are observed. Rupture events are recorded when the CAM-ligand bond of a cytoskeleton-linked CAM fails. Tether events are recorded when a membrane tether is extruded from the cell membrane with the CAM at its tip (tethers). In the latter case attachment of the CAM to the cytoskeleton is either too weak to resist the mechanical stress applied or non-existent [19,40,41].

cell adhesion may be indirectly activated by trypsin cleavage. For example, trypsin has been shown to cleave and activate protease-activated receptors (PARs), which regulate various cellular processes, including actomyosin cortex function and adhesion [49–51]. Moreover, trypsin cleaves proteoglycans, which can contribute to cell adhesion [52]. Because EDTA chelates divalent ions its presence can perturb calcium and magnesium dependent cellular processes [53,54]. Although some CAMs are not functionally dependent on divalent ions, many CAMs (e.g., integrins and cadherins) require the availability of divalent ions for stably interacting with their ligand and, thus, are inhibited upon EDTA treatment. However, it is not entirely clear if and how EDTA and trypsin treatment affects subsequent cell adhesion measurements, especially directly after the cells have been detached from culture flasks. To circumvent this uncertainty, in many SCFS studies the cells were explicitly left to recover for a certain time after detachment from the cell culture flask before characterizing their adhesion properties [15,22,25,26,32–34,55–57]. However, to our best knowledge a systematic approach to characterize the recovery time needed to conduct reproducible cell adhesion experiments has not been published. Here we introduce a simple assay to characterize the recovery time of selected eukaryotic cell lines to recover mechanical and adhesive properties after being detached from culturing flasks. For this assay we first detach vertebrate cells using either EDTA or trypsin, then allow them to recover from the detachment process for different time ranges and subsequently use SCFS to quantify their adhesive properties to collagen I, fibronectin fragments and bovine serum albumin (BSA). The experiments show that the recovery times of the cell lines depend on the detachment method and that trypsin treatment can highly upregulate cell adhesion to ECM proteins. After increased waiting times cells return to a 'normal' adhesion mode that is not influenced by the agents used for detaching cells from culture flasks. The approach described can be used to determine the 'recovery time' after detachment of virtually any eukaryotic cell type whose adhesive properties are to be characterized. The described protocol can thus be implemented in every SCFS-based study to exclude effects of the cell detachment process on the outcome of the experiments.

2. Materials and methods

2.1. Cell culture

HeLa (Kyoto) cells and mouse embryonic kidney fibroblasts were maintained in DMEM (Gibco-Life technologies, NY, USA), supplemented with 10% (v/v) fetal calf serum (FCS, Sigma, Steinheim, Germany), 100 units/mL penicillin (Gibco-Life technologies) and 100 μg/mL streptomycin (Gibco-Life technologies). HeLa cells were grown on untreated and fibroblasts on fibronectin (Calbiochem-Merck, Darmstadt, Germany) coated tissue culture flasks (Jet BioFil, Guangzhou, China).

2.2. Expression and purification of fibronectin fragments

Fibronectin fragment FN_{III}7–10 and RGD-deleted fibronectin fragment FN_{III}7–10ΔRGD were expressed from plasmid pET15b-FN_{III}7–10 in *Escherichia coli* BL21 (DE3) pLysS as described [58]. Briefly, cells were grown in Lennox L broth (Invitrogen, Carlsbad, USA) supplemented with 100 μg/mL of ampicillin (Sigma, Buchs, Switzerland) and 34 μg/mL chloramphenicol (Sigma) at 37 °C. Expression was induced with 500 mM isopropyl thiogalactose (IPTG, Sigma) at optical density $(OD)_{600} = 0.6$. Cells were harvested after 4 h, re-suspended in buffer (20 mM Tris–HCl, 150 mM NaCl, pH 8.0), and broken by sonication. Cell debris was removed by ultracentrifugation at 40000×g for 45 min. The soluble protein fraction was bound to nickel-nitrilotriacetic acid resin (Protino® Ni–NTA Agarose, MACHEREY–NAGEL, Düren, Germany) for 2 h at 4 °C. The resin was then loaded onto a column and washed with buffer (20 mM Tris–HCl, 150 mM NaCl, 10 mM imidazole, pH 8.0). FN_{III}7–10 was eluted with elution buffer (20 mM Tris–HCl, 150 mM NaCl, 500 mM imidazole, pH 8.0). Peak fractions were pooled and dialyzed against imidazole free buffer (20 mM Tris–HCl, 150 mM NaCl, pH 8.0). The protein concentration was adjusted to 1.0 mg/mL with dialyzing buffer and aliquots were stored at −20 °C.

2.3. Surface coating of cantilever and petri dishes

Cantilevers (NP-0, Bruker, USA) were prepared for cell attachment as described previously [27]. In short, cantilevers were plasma-cleaned prior to overnight incubation (at 4 °C) in ConA (2 mg/mL, Sigma) in PBS. The glass bottoms of Petri dishes (35 mm FluoroDish, World Precision Instruments, US) were overlaid with a PDMS mask to allow four different coatings of the glass surface [31]. Three of the four PDMS framed glass surfaces were incubated overnight in PBS at 4 °C either with collagen I (160 μg/mL, Inamed Biomaterials, Fremont, CA), fibronectin fragment FN_{III}7–10 (50 μg/mL), RGD deleted fibronectin fragment FN_{III}7–10ΔRGD (50 μg/mL) or BSA (Sigma). The fourth segment was left uncoated.

2.4. SCFS

For SCFS a CellHesion 200 (JPK Instruments, Berlin, Germany) mounted on an inverted microscope (Observer.Z, Zeiss, Jena, Germany) was used [59]. During SCFS cells were maintained at 37 °C using a temperature controlled incubator box (LIS, Basel, Switzerland). 200 μm long tip-less V-shaped silicon nitride cantilevers having nominal spring constants of 0.06 N/m (NP-0, Bruker) were used for adhesion measurements. The spring constant of every cantilever was determined prior the experiment using the thermal noise method [60] the accuracy of which lies at ≈10% [61].

Overnight serum-starved fibroblasts and HeLa cells grown in 24 well plates (Thermo Scientific, Roskilde, Denmark) to confluency of ≈80% were washed with PBS and detached with either 200 μL of 15 mM EDTA (BioUltra Grade, Sigma) or 0.05% (w/v) trypsin (Sigma), both in PBS, for four and two minutes, respectively. Detached cells were suspended in SCFS media (DMEM supplemented with 20 mM HEPES) containing 1% (v/v) FCS, pelleted and resuspended in serum free SCFS media. Throughout experiments the PDMS masks framing the four segments of glass surfaces remained on the Petri dishes. Each PDMS mask of a Petri dish was washed with SCFS media to exchange coating buffers and to remove weakly attached proteins of the individual glass segments. Cell suspensions were pipetted into the Petri dishes containing the substrate-coated glass supports and allowed to settle. To attach single cells, the apex of a calibrated, ConA functionalized cantilever was lowered with a velocity of 10 μm/s onto a cell until reaching a contact force of 3 nN. After 5 s contact, the cantilever was retracted from the Petri dish by 50 μm. Cells were incubated in SCFS media for different times to characterize cell adhesion after different recovery times. For adhesion experiments, cantilever bound cells were lowered onto a given substrate-coated glass segment with a velocity of 5 μm/s until reaching a contact force of 1 nN. The cantilever was maintained at this position (constant height) for 60 s and subsequently retracted with 5 μm/s for >90 μm until the cell detached from the substrate-coated glass segment. After detachment from the substrate segment the cell was allowed to recover for 60 s before probing adhesion to the next substrate-coated glass segment. Single cells were used to probe adhesion for all three recovery time ranges. As soon as cells showed morphological changes (e.g., spreading on cantilever) they were replaced. Cell adhesion at recovery times >60 min were quantified using additional cells. Cells were not characterized at recovery times >90 min after detachment from the culture flask. Adhesion forces were extracted from FD curves using the JPK data processing software (JPK Instruments). Cell stiffness, rupture forces, and tether forces were analyzed using in-house build routines, which were based in Igor 6 (Wavemetric, Oregon, USA). Rupture events were identified by the non-linear slope before of the force jump, while tether events were identified by the force plateaus (constant force) having a maximum tilt of 10° before of the force jump (Fig. 1C). Statistical test were done using Prism (GraphPad, La Jolla, USA).

2.5. Confocal microscopy

To image F-actin and non-muscle myosin IIA, we used a HeLa cell line expressing human MYH9-GFP and Lifeact-mCherry. Geneticin (0.5 mg/mL, Life Technologies) and puromycin (0.5 μg/mL, Life Technologies) were used for antibiotic selection. An inverted confocal microscope (Observer.Z1, LSM 700, Zeiss) with a 63x/1.3 LCI Plan–Neofluar water immersion objective (Zeiss) was used. Cells were maintained at 37 °C using a Petri dish heater (JPK Instruments). In all the representative images shown, contrast and brightness were adjusted to similar levels for visual comparison using Zeiss AxioVision software (Rel. 4.8).

3. Results

To characterize a potential influence of the detachment process of adherent cells from culture flasks on the cell's ability to re-establish adhesion, mouse kidney fibroblasts and HeLa cells were detached from flasks using either 15 mM EDTA or 0.05% (w/v) trypsin. After a certain recovery interval in media, cells were non-specifically attached to tipless AFM cantilevers functionalized with concanavalin A (ConA). SCFS was then used to characterize the adhesion of an attached cell to different substrates (Fig. 1). The parameters extracted from the approach and retract FD curves recorded in these experiments were contact stiffness of the cell pressed to the substrate, maximum adhesion force of the cell, and the force of the single rupture and tether events (Fig. 1C). In the following paragraphs we will report how the cell detachment procedure from culture flasks affects each of these parameters.

3.1. Characterizing the contact stiffness of cells after detachment from culture flasks

To conduct adhesion measurements by SCFS, single cells attached to the AFM cantilever are pressed onto a substrate for a given contact force and time. Normally contact forces on the range of a few nN are chosen, which distribute over the entire contact area of cell and substrate and result in a relatively small contact pressure applied to the cell. For example, when pressing mouse kidney fibroblasts onto the substrate at a contact force of 1 nN, the contact area estimated from optical microscopy is 70.4 ± 12.2 μm^2 (average ± S.D., $n = 8$). This results in a contact pressure of 14.6 ± 2.8 N/m^2 (e.g., Pa), which is much smaller than the typical intracellular pressure (≈10–10.000 Pa) generated by animal cells [62–64]. However, if the procedure applied to detach the cells from cell culture flasks influences the mechanical properties of the cell, pressing a softer or stiffer cell onto the substrate at a given contact force results in different cell-substrate contact areas. Variations of the contact area can have a direct impact on the number of CAMs that could bind their ligands and establish adhesion. Accordingly, if the mechanical properties of the cell would vary with the time after detachment from the culture flask this could have a considerable impact on the SCFS measurements.

Our SCFS experiments show that the contact stiffness of mouse kidney fibroblasts does not significantly change with increasing recovery time after detachment from the cell culture flask by trypsin (Fig. 2). After EDTA detachment from the culture flasks, the mean contact stiffness of fibroblasts shows small variations of less than 20% (940–1160 pN/μm) between different recovery times, and the contact stiffness of single cells distributed widely for each recovery time. We therefore consider this difference insignificant (P-values > 0.01). The independence of contact stiffness on recovery time is also observed for HeLa cells detached by EDTA or trypsin. These measurements suggest that at the contact force applied and within the sensitivity of the SCFS measurements, detachment

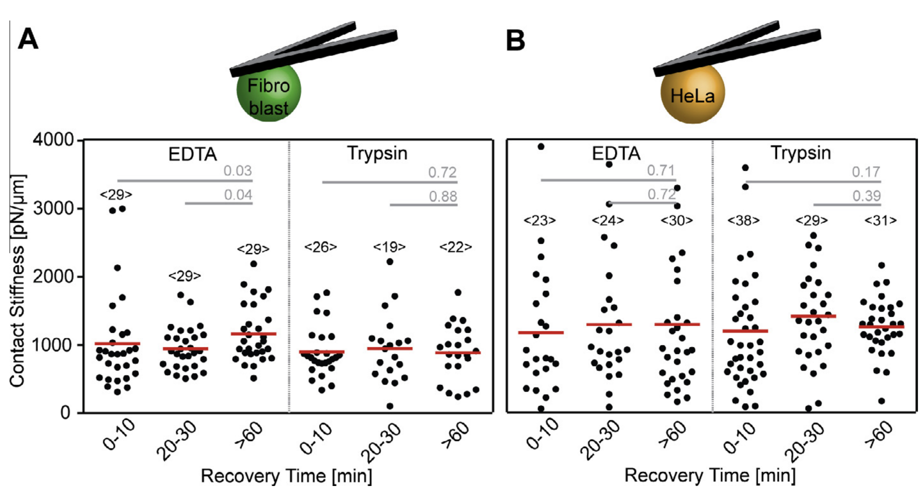

Fig. 2. Contact stiffness of (A) mouse kidney fibroblasts and (B) HeLa cells for different recovery times after detachment from cell culture flasks. The contact stiffness was determined as depicted in Fig. 1. SCFS experiments on different substrate coatings are combined for different recovery times. The recovery time denotes the time cells were allowed to recover after detachment from culture flasks using either EDTA or trypsin. Within this recovery time cell adhesion to the different substrates was characterized using SCFS. Each dot represents one SCFS measurement, approaching a single fibroblast or HeLa cell at 5 μm/s to the substrate until reaching a contact force of 1 nN. Red bars indicate average values. <n> Gives the number of measurements for each condition. Mann–Whitney P-values (in gray) indicating the significance of measurements compared to those made after a recovery time of >60 min.

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from the cell culture flasks by either EDTA or trypsin does not change the mechanical properties of the cell and, thus, does not change the contact area between cell and substrate.

3.2. Cortical actomyosin localization shows no significant changes during recovery after detachment from culture flasks

An AFM cantilever compressing a rounded cell by a few µm mainly measures the mechanical properties of the actomyosin cortex [65]. In the previous section we observed no changes of the contact stiffness of mouse embryonic kidney fibroblasts and of HeLa cells detached by trypsin or EDTA. Previous experiments suggest that the enrichment of cortical F-actin and myosin II correlates with higher cell cortex tensions in interphase cells [66,67]. Thus, our SCFS results showing that the mechanical properties of cells remain unchanged over the entire recovery time course suggest that the actomyosin cortex of the cells remains unchanged as well. To further investigate whether this is indeed the case we imaged the dynamics of actin and myosin in HeLa cells stably expressing Lifeact-mCherry and MYH9-GFP after detachment from culture flasks using EDTA or trypsin (Fig. 3). Regardless of the detachment method applied, the live cell confocal microscopy images revealed no significant elevation of F-actin or myosin IIA forming the actomyosin cortex thickness. The confocal microscopy images support the observation by SCFS that the cortical stiffness remained unchanged over the same time course.

3.3. Influence of recovery time on cell adhesion

Next, we investigated whether the adhesion force of mouse kidney fibroblasts or HeLa cells to different substrates depends on the detachment method from the culture flasks. For fibroblasts we used substrates featuring collagen I, a fibronectin type III fragment containing repeat 7–10 domains (FN_{III}7–10) and a fibronectin FN_{III}7–10 fragment lacking the integrin binding site (FN_{III}7–10ΔRGD). Whereas fibroblasts can specifically adhere to collagen I and to FN_{III}7–10 via integrins [58,68], they are unable to specifically adhere to FN_{III}7–10ΔRGD [58,69]. Thus, FN_{III}7–10ΔRGD was used as a control to characterize unspecific fibroblast adhesion. SCFS showed that the adhesion force of fibroblasts to the two specific substrates collagen I and FN_{III}7–10 does not depend on the recovery time of the cell if detached from culture flasks using EDTA (Fig. 4A). Although adhesion forces to the FN_{III}7–10ΔRGD control substrate decreases slightly after >60 min of recovery, the averages differ only by about 200 pN resulting in lower significance levels. However, fibroblasts detached from culture flasks in the presence of 0.05% (w/v) trypsin showed a different behavior. While adhesion of fibroblasts to collagen I did not depend on the recovery time after trypsin-induced detachment, adhesion to the fibronectin fragment FN_{III}7–10 showed a clear time dependence. To our surprise, also the adhesion to the non-specific substrate (FN_{III}7–10ΔRGD) depended on the recovery time. In both cases cell adhesion was at first strongly enhanced after detachment and only after recovery times >60 min showed values equal to those observed for fibroblasts detached from culture flasks using EDTA. This highlights that trypsin treatment to detach fibroblasts from cell culture flasks activates their adhesion to fibronectin. As fibroblast adhesion to the FN_{III}7–10ΔRGD control substrate can be seen as being unspecific the results suggest trypsin cleavage to slightly increase unspecific adhesion as well.

Using HeLa cells we characterized adhesion to collagen I, FN_{III}7–10, and BSA. Similarly to the fibroblasts HeLa cells adhered to collagen I and FN_{III}7–10 specifically via integrins [26,70,71]. However the integrin expression levels of both cell lines may be different and, thus, also the adhesion of HeLa cells differed from that observed for fibroblasts. In contrast to fibroblasts, HeLa cells showed a relatively high unspecific adhesion to FN_{III}7–10ΔRGD coated substrates (data not shown). Thus, we used BSA as substrate, which is frequently used to suppress unspecific cell adhesion to the supporting glass surface [72,73]. The adhesion force of HeLa cells to the three different substrates was largely independent on the detachment method (EDTA or trypsin) from culture flasks prior to SCFS measurements (Fig. 4). These results highlight that the adhesive properties of different cell lines are differently affected by the procedure used to detach the cells from culture flasks.

3.4. Rupture events do not depend on recovery time

After having characterized the maximum cell adhesion force of fibroblasts and HeLa cells to different ECM substrates, we analyzed the rupture events recorded during cell-substrate detachment

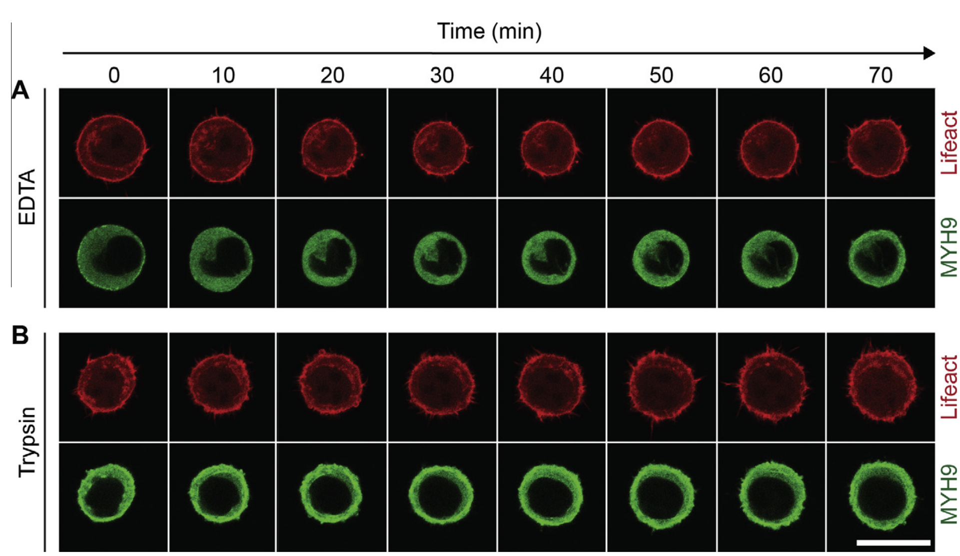

Fig. 3. Tracking the actomyosin cortex after detachment with (A) EDTA or (B) trypsin. Confocal images of HeLa cells expressing mCherry labeled F-actin (Lifeact-mCherry, red) and GFP labeled myosin II (MYH9-GFP, green). Images were acquired every 10 min through the center of the cell. Cells were detached from cell culture flasks with 0.05% trypsin or 15 mM EDTA, and seeded in SCFS medium devoid of either trypsin or EDTA. Scale bar 20 µm, applies to all images.

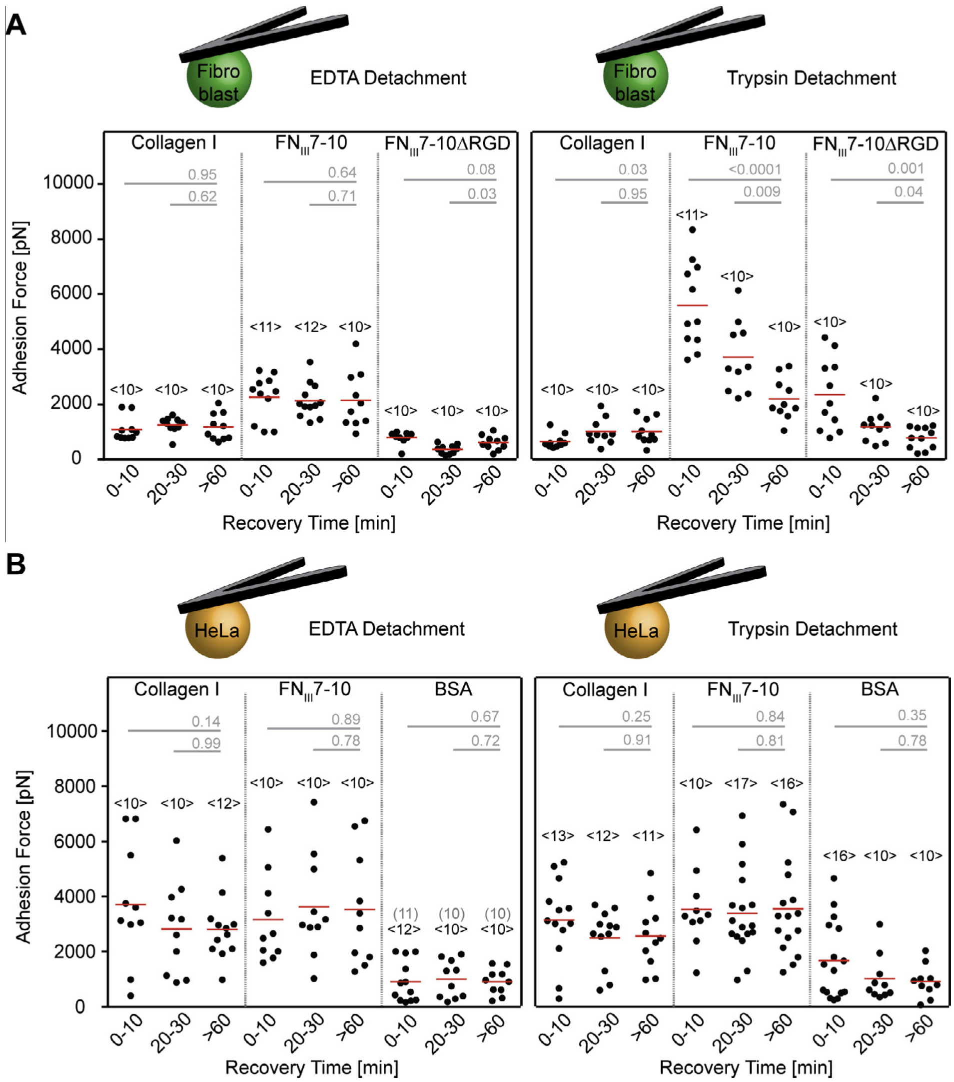

Fig. 4. Adhesion force of (A) mouse kidney fibroblasts and (B) HeLa cells recorded after different recovery times from trypsin or EDTA treatment. Fibroblast adhesion forces were recorded to collagen I, fibronectin type III fragment containing repeats 7–10 (FN_{III}7–10), or the same fibronectin fragment lacking the RGD sequence (FN_{III}7–10ΔRGD). Adhesion forces of HeLa cells were recorded to collagen I, FN_{III}7–10, or BSA. Cantilever-bound cells pressed on the substrate with a force of 1 nN were allowed to initiate adhesion for 60 s prior to retraction. Each dot represents a single cell characterized. Red bars indicate average values. <*n*> Gives the number of cells characterized for each condition. Mann–Whitney *P*-values indicating the significance of the measurements compared to those made after a recovery time of >60 min given in gray.

(Fig. 5). These rupture events correspond to the breaking of individual or clusters of CAM-ligand bonds exposed to mechanical stress. Rupture events recorded for fibroblasts and HeLa cells detached from culture flasks using EDTA or trypsin prior SCFS did not show significant dependency on recovery time (Fig. 5). This result may be seen in contradiction to the increased adhesion strength of fibroblasts to the fibronectin constructs, which depended strongly on the recovery time of the fibroblasts after trypsin treatment (Fig. 4). However, because the strength of the single rupture events (median rupture force ≈50 pN with data points spreading from 15 to 400 pN) were not affected by trypsin (Fig. 5) our result suggests that the increased fibroblast adhesion to FN_{III}7–10 originated from increased avidity (e.g., availability of CAMs binding to fibronectin) rather than increased affinity (e.g., binding strength of CAMs to fibronectin).

3.5. Tether forces do not depend on recovery time

Next, we characterized the forces required to extract single tethers from fibroblasts and HeLa cells while being detached from the three different substrates (Fig. 6). Although the median tether forces statistically sometime depended on the recovery time after detachment from the culture flasks, the differences were very minor (<10 pN) compared to the spread of the data points (Fig. 6). Thus, we do not consider the tether force differences as relevant for the detachment process using either EDTA or trypsin. Because the force required to extract tethers from cell membranes depends on the properties of the cell membrane and not on the CAM bond adhering the tether to the substrate, this result indicates that the properties of the cell membrane do not depend on the procedure used to detach the cells from the culture flasks.

4. Discussion

We investigated the time-dependent recovery of the adhesive properties of eukaryotic cell lines, which, prior to measuring these properties by AFM-based SCFS, have been detached from culture flasks using either EDTA or trypsin. Therefore, we quantified mechanical stiffness and adhesion forces of mouse embryonic kidney fibroblasts and HeLa cells at different recovery times after detachment from culture flasks. The mechanical stiffness of a cell determines the contact area of the cell pressed onto the substrate

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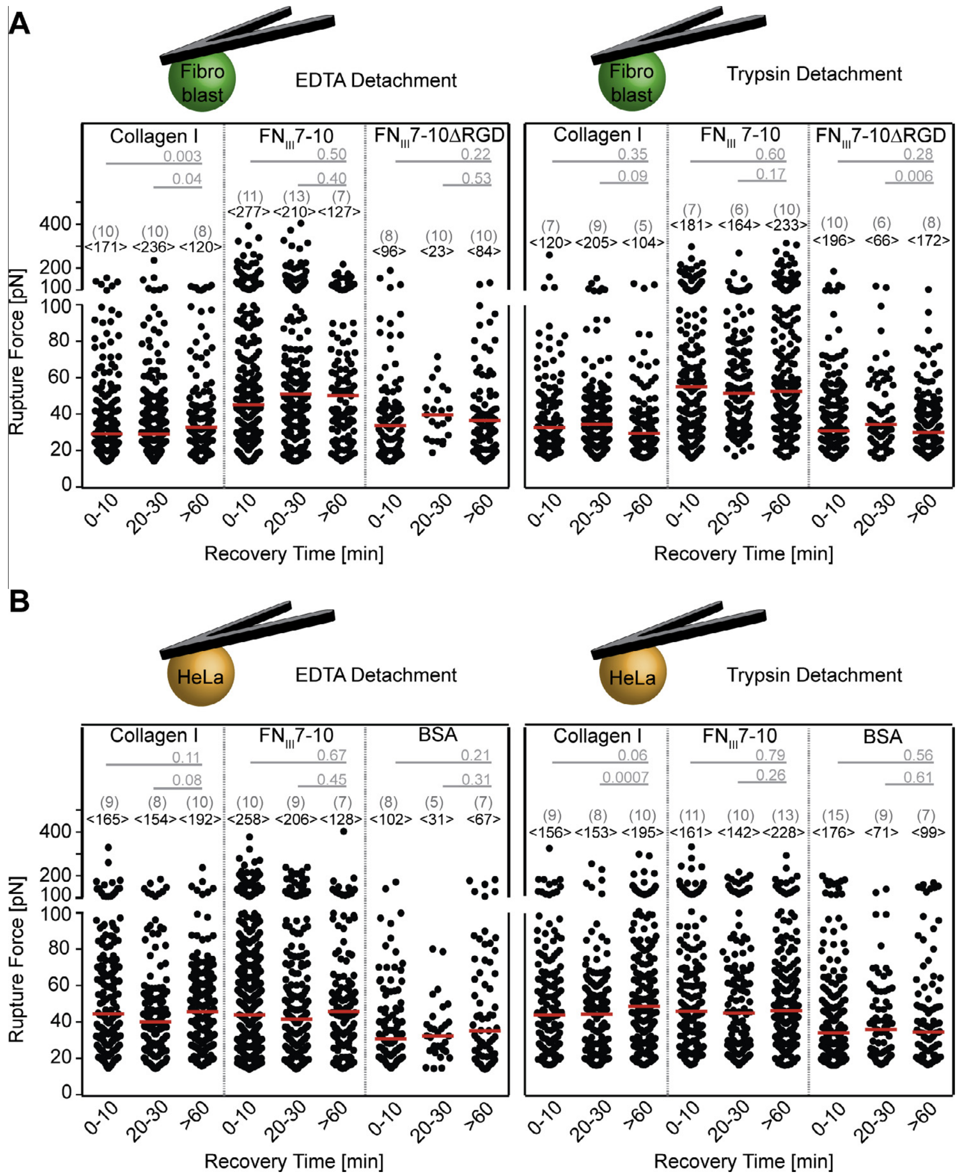

Fig. 5. Forces of single rupture events recorded for (A) mouse kidney fibroblasts and (B) HeLa cells after different recovery in times from EDTA or trypsin treatment. Rupture forces were recorded upon detaching single fibroblasts adhering to Petri dishes coated with collagen I, FN_{III}7–10, or FN_{III}7–10ΔRGD and upon detaching HeLa cells adhering to Petri dishes coated with collagen I, FN_{III}7–10, or BSA. Cells were pressed onto the substrates with a 1 nN contact force and were allowed to establish adhesion for 60 s. Subsequently, the cantilever was retracted at 5 µm/s for at least 90 µm. The recovery time denotes the time cells were allowed to recover after detachment from culture flasks using either EDTA or trypsin. After the recovery time passed, adhesion of the cells to the different substrates was characterized using SCFS. Each dot represents one rupture event with the red bars indicating median values. (*n*) Gives the number force–distance curves and <*n*> the number of rupture events analyzed for each condition. Mann–Whitney *P*-values indicating the significance of the measurements compared to those made after a recovery time of >60 min are given in gray. Distributions of rupture forces are shown in Supplementary Fig. S1.

and thus has a direct influence on the adhesion formed. Interestingly, the contact stiffness determined for fibroblasts and HeLa cells did not reveal any significant dependency on the detachment method applied or on the recovery time investigated. One reason may be that the low contact force of ≈1 nN applied by the cantilever on single cells while pressing them to the substrate only weakly deforms the cells and, thus, hardly stresses their actomyosin cortex. However, we applied only very little contact force to the cells in our SCFS measurements and applying much higher forces of 50–100 nN through the cantilever severely deforms pre-rounded interphase cells [74,75]. At such high forces the AFM cantilever probes different mechanical properties of the cell, which may depend on pretreatment using trypsin and/or EDTA. Such dependency would change the contact area between cell and substrate and, thus, the adhesion probed by SCFS.

There was also no significant influence on adhesive properties when detaching either cell types from culture flasks using EDTA. EDTA chelation of divalent ions inhibits CAMs that require divalent ions for establishing adhesive interactions [76]. Since the detached cells are transferred to EDTA-free buffer solutions this result suggests that CAMs recover quickly from EDTA treatment and can readily re-establish adhesion [33]. However, we can only make conclusions concerning mouse embryonic kidney fibroblasts and HeLa cells, and for CAMs facilitating adhesion to collagen I and fibronectin, and recovery from EDTA exposure may be characterized for every cell line and CAM by SCFS.

Trypsin severely affected the adhesive properties of fibroblasts. Shortly after trypsin-induced detachment of fibroblasts from cell culture flasks the adhesion force of these cells to the fibronectin constructs increased considerably. Fibroblasts needed >60 min to

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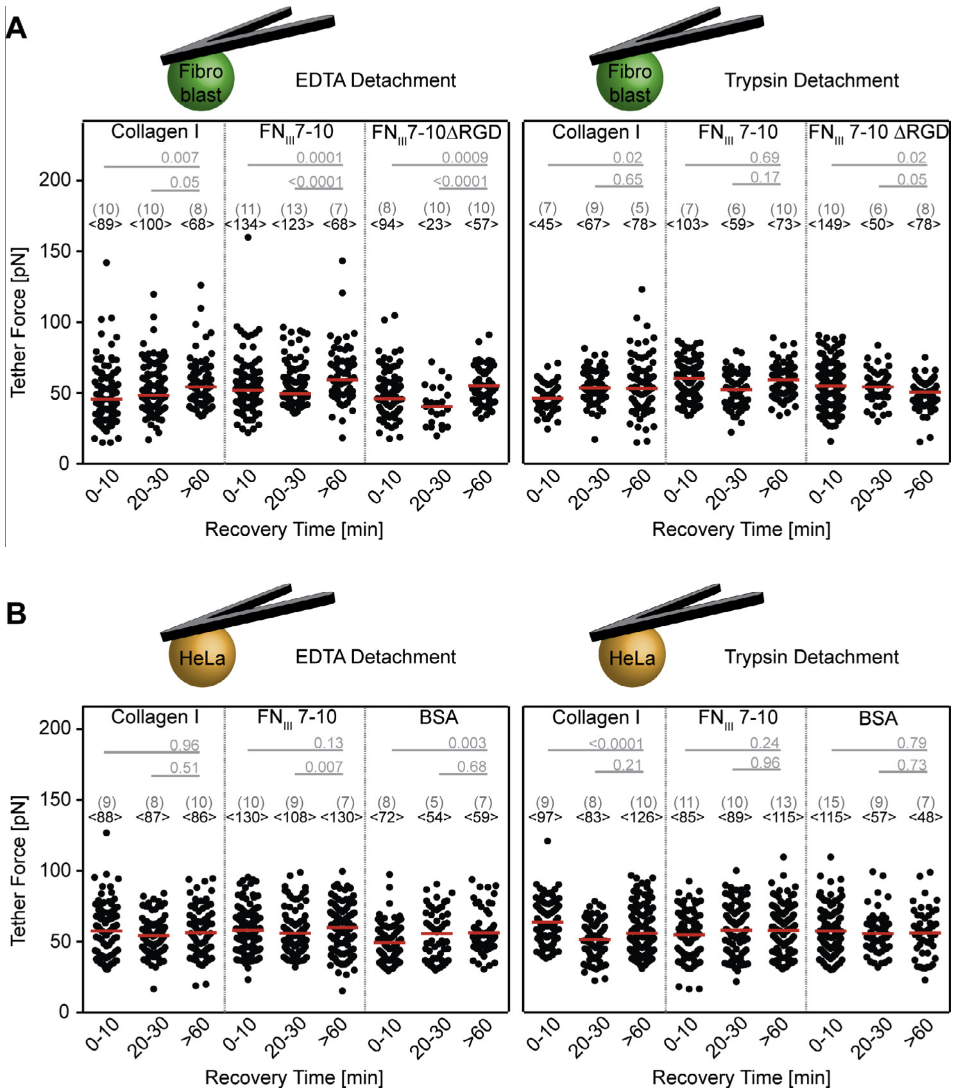

Fig. 6. Forces required to mechanically extract single tethers from (A) fibroblasts and (B) HeLa cells after different recovery in times from EDTA or trypsin treatment. Tether forces were recorded upon detaching single fibroblasts adhering to Petri dishes coated with collagen I, FN_{III}7–10, or FN_{III}7–10ΔRGD or upon detaching HeLa cells adhering to Petri dishes coated with collagen I, FN_{III}7–10, or BSA. Cells were pushed onto the substrates with a contact force of 1 nN and were allowed to establish adhesion for 60 s. Subsequently, the cantilever was retracted at 5 μm/s for at least 90 μm. The recovery time denotes the time cells were allowed to recover after detachment from culture flasks using either EDTA or trypsin. After this recovery time passed the adhesion of the cells to the different substrates was characterized using SCFS. Each dot represents one tether event with the red bars indicating median values. (*n*) Gives the number force-distance curves and <*n*> the number of tether events analyzed for each condition. Mann–Whitney *P*-values indicating the significance of the measurements compared to those made after >60 min recovery time are given in gray. Distributions of tether forces are shown in Supplementary Fig. S2.

recover adhesive properties from trypsin treatment. In contrast the adhesion force of fibroblasts to collagen I did not increase by trypsin pre-treatment. Although the adhesion of fibroblasts was just above background level, we also did not observe a decrease in adhesion force. This latter finding is in agreement with previous investigations showing that pre-treating CHO-A2 cells with trypsin does not cleave collagen I binding α2β1 integrins and does not affect cell adhesion to collagen I matrices [22]. Thus, pre-treating fibroblasts using trypsin specifically upregulated CAMs binding to fibronectin. Indeed, trypsin cleaves and activates human PAR2, which stimulates α5β1 integrin but not αVβ3 integrin dependent cell adhesion [77]. α5β1 integrins bind to the RGD site located in the FN_{III}7–10 fragment of fibronectin [69] and besides αVβ3 integrins are the main CAMs for fibronectin in mouse kidney fibroblasts [68]. These results highlight that only certain CAMs may be affected by the procedure used to detach cells from culture flasks whereas other CAMs remain unaffected. Our results furthermore show that the cell detachment procedures applied do not alter the affinity of fibronectin binding CAMs (e.g., binding strength remains unchanged), but rather increases the cell adhesion forces by increasing the avidity of these receptors (e.g., number of binding events).

To our surprise fibroblast adhesion to the FN_{III}7–10ΔRGD substrate increased after trypsin cleavage. Fibroblasts needed >60 min to lower their enhanced unspecific adhesion to FN_{III}7–10ΔRGD to their normal adhesion value. Because mouse kidney fibroblasts have no CAMs to specifically adhere to FN_{III}7–10ΔRGD [68], we speculate that this increased adhesion is due to an increased number of CAMs, which interact unspecific with the substrate. However, the strength of such unspecific cellular interactions may depend on the substrate.

In contrast to fibroblasts the adhesion of HeLa cells was apparently not affected by trypsin treatment within the recovery times tested and force sensitivity of our SCFS-based assay. This shows that cell lines can react differently to the detachment methods used and that the recovery of each cell line must be carefully stud-

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ied before characterizing its mechanical and adhesive properties by SCFS. Importantly, these results further demonstrate that the quantification of cell adhesion by SCFS and probably by other cell adhesion assays requires careful investigation whether the CAMs addressed in cell adhesion studies are affected by the detachment procedure and whether the cells characterized have sufficient time to recover from this detachment.

To date in most SCFS studies the cells were explicitly left to recover for a certain time from their detachment from the cell culture flask before being characterized by SCFS [15,22,25,26,32–34,55–57]. Thus, SCFS users have already allocated a certain time span to enable detached cells to recover. However, so far a quantitative approach to characterize this recovery has not been presented. Our approach can be applied to characterize the recovery time of any adherent cell after detachment from cell culture flasks. Our approach can also be used to optimize the detachment procedure for specific cell types. For example, our measurements show that mouse kidney fibroblasts and HeLa cells, detached from culture flasks by EDTA, do not need recovery times of more than 10 min, whereas cells detached using trypsin need to recover for up to 60 min. Thus, EDTA may be more suitable to detach the cell lines investigated here from culture flasks and to investigate their mechanical and adhesive properties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.06.012.

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