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Controlling the Hydrophilicity and Cohesion During Deposition of Highly Oriented Type I Collagen Films: an approach for biomedical applications

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Controlling the hydrophilicity and cohesion during deposition of highly oriented type I collagen films: an approach for biomedical applications

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Abstract: Using the Langmuir-Blodgett (LB) technology we have pioneered a straightforward and low-cost approach to fabricate highly oriented collagen in thin film format (thickness: ~20 nm, surface areas: 2.5 x ~6.0 cm). An important factor for the use of these films is their cohesion under various conditions. Film formation was studied by coating hydrophilic or hydrophobic glass substrates. The fresh or aged (2 ½ years, -18°C.) collagen solutions used for this purpose either contained collagen network-stabilizing agents (n-propanol or phosphate ions) or were prepared without these stabilizers. Film formation on the air/water interface was analyzed by pressure-area isotherms. Maximum surface pressures were ~ 4-7 mN/m and ~ 10-18 mN/m for isotherms using n-propanol or phosphate buffer saline (PBS), respectively, versus ~ 0.4-0.8 mN/m without using a stabilizer; with higher surface pressures for the combination fresh solution/n-propanol or aged solution/PBS. Deposited films were studied by optical and electron microscopy and fast Fourier transform analysis. Coatings (to both substrate types) exhibit a defined orientation of collagen aggregates within a matrix of oriented collagen when freshly made or aged collagen solutions were used and n-propanol was present during film formation. The higher degree of hydrophilicity of the aged solution does not adversely affect the cohesion and collagen orientation during film formation. Using physiological phosphate ions shows that deposition of defect-free and oriented collagen (on both substrate types) is only possible using fresh collagen solutions. Unlike n-propanol-containing solutions, films were most stable using hydrophilic glass substrates. Film formation failed in the absence of network stabilizers. Controlling the cohesion via (a) the water accessibility of collagen structures, (b) specific network stabilizers and (c) substrate properties enables tunable film characteristics for future biomedical approaches.

Keywords: Langmuir-Blodgett films; highly oriented collagen; cohesion; tunable hydrophobicity in collagen; hydrophilic/phobic glass substrates; protein stabilizer; network former; phosphate

1. Introduction

Collagen is the major structural protein in animals. In mammals, it comprises up to 30% of total protein weight and constitutes the foundation of the extracellular matrix [1,2]. The protein exists in several forms (so-called types), whereas the type I is the most abundant form found in e.g. bone, cornea, dermis and tendon [1,2]. Type I collagen molecules are composed of three left-handed helical polypeptide chains forming a right-handed triple-helical structure that is stabilized by hydrogen bonds. The resulting molecule (so-called tropocollagen) is ~300 nm long and 1.5 nm in diameter [1,3,4].

Tropocollagen molecules aggregate in a staggered fashion and form (micro) fibrils [1]. In further steps, bundles of these fibrils form collagen fibers. This process, called fibrillogenesis, is thermally driven and favored by a large positive entropy contribution due to the displacement of structured water around collagen molecules [5]. In other words: hydrophobic interactions between non-polar regions of adjacent molecules are governing collagen fibril formation which leads, in turn, to a negative temperature coefficient of the collagen solubility and to an endothermic nature of the in vitro fibrillogenesis [5,6]. However, fibrillogenesis is also driven by hydrogen bonding between polar residues [7] and the overall processes can be further controlled by variation of, e,g., the pH, or the nature and concentration of salts or other compounds [8,9]. Depending on the tissue and its function, collagen can show a lamellar order (in mature bone) a random orientation (in woven bone or dermis) [10-12], it is highly oriented (parallel to the long axis of the tendon) [13], or shows an orthogonal arrangement (in cornea) [14]. These differences in protein orientation and structure provide the appropriate mechanical properties for the specific tissue [15]. Based on these findings, collagen has been used extensively in medical applications [16], and is successfully incorporated into artificial constructs [16]. Similarly, collagen has been used for a variety of applications, including wound dressings, lip augmentation, drug delivery, and many other purposes [16].

Despite these successful applications in medicine, mimicking natural fiber arrangement and orientation is still a major challenge [16-18]. In principle, collagen solutions, gels or constructs need to be able to mimic the supramolecular assemblies they will be replacing in the tissues. However, in recent years, various approaches have been developed that meet these conditions to produce, e.g., films or scaffolds for biomedical applications. Alignment of collagen in films is accomplished by magnetic or electrical fields and methods such as stress/strain and Langmuir-Blodgett/Schaefer [19-24]. For the fabrication of thick films and membranes methods like micro fluidics, stress/strain, extrusion/compacting are used [22,25,26], while the production of scaffolds can be based on electro spinning, freeze drying, electrochemical treatment, micro fluidics and 'crowding" of collagen [27-34].

In a recent study the Langmuir-Blodgett (LB) technology [35] was introduced to fabricate highly oriented thin film coatings of type I collagen on glass substrates [36]. It was found that during film deposition the collagen molecules/fibrils behave similarly to a particular class of (hydrophobic) synthetic polymers known as 'hairy rod' polymers [37-39]. Most notably, both types of molecules show shape persistence [36-38]. During LB transfer collagen fibrils and fibrillar aggregates align preferentially along to the pull-out direction [36]. But they are also part of an "orientation arch", which forms immediately after the initiation of the LB transfer, with its orientation perpendicular to the pull-out direction and parallel to the air-water interface [36]. By systematically varying the shapes (e.g. rectangles, squares, circles, parallelograms, and variously shaped triangles) and dimensions of glass substrates during LB transfer it was also found that the collagen orientation and orientation distribution is directly depending upon the geometry of the substrate and the pull-out direction of that substrate with respect to the collagen/water subphase [40].

In the present study, we will test if collagen (from different stabilized and/or aged solutions) can be coated in a highly oriented fashion on both hydrophobic and hydrophilic glass substrates, and if the respective collagen residues (hydrophobic or hydrophilic) interact with sufficient cohesion to form ultra thin and intact films. To this end, either freshly prepared or aged (2 ½

years, -18°C) collagen solutions are used, which contain or omit the collagen network stabilizers n-propanol or phosphate ions. For all procedures, the film formation, the alignment and the cohesion of collagen is investigated qualitatively. Based on previous work (e.g. collagen/PBS gels, collagen/hydrophobic-hydrophilic substrates [8,20,22,24,36,40-45]), it is expected that ultra-thin film coatings (from fresh or aged (more hydrophilic) solutions) on both hydrophobic and hydrophilic substrates are feasible. It is, however, not clear whether, e.g., films exhibit still a stable and highly oriented collagen alignment when transferred to hydrophilic samples, or if collagen is transferable at all in studies without a stabilizer. In addition, the replacement of propanol by phosphate ions might result in a loss of film cohesion and collagen alignment by reducing intermolecular hydrophobic-hydrophobic interactions, but in a gain of film stability/alignment by phosphate's electrostatic interactions. In cases where films of aligned collagen are formed, it will be important to understand the underlying interactions, also in view of future studies on biomedical processing and handling of e.g. freestanding films (for wound dressing) or coatings for implants.

2. Materials and methods

2.1 Collagen solutions

Type I collagen (~300 kDa) was prepared from rat tail tendons as previously described [46]. The collagen material obtained has been shown to form a triple-helical structure (tropocollagen) when dissolved in an acetic acid solution [47]. For LB experiments, 2 µM collagen solutions were prepared following a protocol by Usha et al. [48]. Briefly, 0.6 mg collagen was dissolved in mixtures of 0.9 mL acetic acid (5 mM) and 0.1 mL n-propanol for 12 h at 4 °C (in motion). n-Propanol was used as it stabilizes the protein structure, supports the LB film formation [9] and shows a fast evaporation on the LB trough [36]. In addition, 2 µM collagen solutions were

prepared without n-propanol, dissolving 0.6 mg collagen for 12 h (at 4 °C, in motion) in 1.0 mL acetic acid (5 mM). For a further set of LB experiments aged collagen solutions (having the same composition as the two above) were used. These solutions had been stored for 927 days (30 months, 15 days) in a freezer (-18 °C). The individual solutions are referred to as F_{prop} and $F_{no\text{-}prop}$ (for fresh collagen prepared with or without n-propanol, respectively) or A_{prop} and $A_{no\text{-}prop}$ (for aged collagen solutions with or without n-propanol, respectively).

2.2 Circular dichroism (CD) spectrometry

All collagen solutions prepared for LB experiments were diluted (0.3 mg collagen/mL) using 5 mM acidic acid and then incubated for 2 h at 20 °C. For each solution, samples were transferred to a 1 mm path length quartz cuvette and the ellipticity measured from 185 to 260 nm using a J810 spectropolarimeter (Jasco; Easton, MD, USA) at 20 °C; the temperatures were maintained by a Peltier system (Jasco, PTC 4235). Spectra were also obtained at 60 °C, well above the denaturation temperature, to determine the effect of thermal denaturation. For analysis of CD data, relevant literature was consulted [9,49].

2.3 Preparation of hydrophilic and hydrophobic glass substrates for LB transfer

For hydrophilic glass surfaces, glass slides (Microscope slides, Bio Nuclear Diagnostics Inc.) were thoroughly cleaned by sonication in acetone and Milli-Q water ($\rho \geq 18$ MΩ·cm, Millipore) for 5 min. After the last step, the substrates were rinsed five times with Milli-Q water. The slides were then immersed in Piranha solution (4:1 [97% H_2SO_4] : [30% H_2O_2]; both Sigma Aldrich) overnight to create −OH groups on the substrate surface, then rinsed with copious amounts of Milli-Q water, and dried with N_2. Contact angle measurements (Goniometer NRL Model 100-00, Ramé-Hart, Montréal) indicated a strong hydrophilicity (creation of −OH groups) on the slide surfaces ($\theta < 2°$).

Hydrophobization of glass surfaces was achieved by thorough cleaning and preconditioning of glass slides followed by a self-assembly process of n-octadecyltrichlorosilane (OTS) on glass surfaces. First, for creation of −OH groups on glass surfaces, which are essential for silanizing of substrates, glass slides were cleaned and Piranha-treated as described above. After rinsing and drying the samples with N_2, contact angle measurements indicated strong hydrophilicity of the samples (contact angle: $\theta < 2°$). To generate hydrophobic glass surfaces, a 10 mM OTS solution (97%, Sigma-Aldrich) in anhydrous toluene was prepared. Since OTS is moisture sensitive the solution was always prepared under a nitrogen/dry atmosphere (using a glovebox) and used immediately. In the glovebox, the preconditioned glass substrates were then immersed in OTS for 6 h to perform OTS self-assembly, followed by a rinsing step with toluene and ethanol, and drying with N_2. The hydrophobicity of sample surfaces was confirmed by contact angles measured ($\theta > 100°$).

2.4 Langmuir−Blodgett (LB) technique

For the LB transfer of collagen onto hydrophilic or hydrophobic glass substrates, samples were immersed nearly completely in the Milli-Q water (or phosphate buffer solution (PBS); see below) subphase. Using a Hamilton syringe, 2 μM collagen solutions were applied dropwise to the entire surface of the aqueous subphase in the LB trough (KSV Instruments LTD Model KSV3000-2 LB). Following a 20-min waiting period (to allow for the volatile solvent to evaporate if present), either an area-pressure isotherm (surface pressure Π vs. trough area) was obtained at a barrier compression speed of 5 mm/min (at 25°C) or the barriers were allowed to compress the collagen up to ~3/4 of the highest achievable pressure [36] at a speed of 5 mm/min (at 25°C). For LB film transfer, the substrate was then moved out of the water or PBS subphase through the collagen LB film with the KSV film lift at a speed of 5 mm/min. For collagen deposition onto a further sample, the trough was thoroughly cleaned, refilled with Milli-Q water

or PBS, and the new glass substrate mounted nearly completely immersed in the aqueous subphase, before collagen was reapplied and the fabrication process repeated.

For some experiments, PBS was used as the subphase instead of Milli-Q water to transfer collagen onto substrates. PBS (tablets, Sigma-Aldrich) was prepared by adding 8 tablets in 1.6 L Milli-Q water; the final solution contained 10 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl (pH 7.4, at 25°C).

2.5 Optical microscopy

For analysis of collagen solution (droplets) or deposited LB films, bright and dark field microscopy (Zeiss Axioskop 2 MAT, Germany) was carried out.

2.6 Scanning electron microscopy (SEM)

A SEM (LEO 1540XB, Carl Zeiss, Germany) equipped with a Gemini field emission column was employed to determine the surface structure of the transferred collagen LB films. The samples were investigated without additional metal coating, using an acceleration voltage of 1 kV and a working distance between 3.5 and 4.0 mm.

2.7 Fast Fourier transform (FFT) analysis

FFT (ImageJ, version 1.48v; National Institutes of Health, USA) was used to get insight into the orientation of collagen matrix and whether the matrix consists of collagen molecules and/or fibrils.

3. Results and discussions

The present study deals with the question whether the ability of collagen molecules and fibrils to interact with each other under different conditions, is sufficient for coating films of highly oriented collagen on physicochemically different glass substrates. In other words, we will investigate the feasibility to fabricate ultrathin collagen films by varying the hydrophobicity/hydrophilicity of the collagen used, the substrate present for coating and effects of different collagen network stabilizers on collagen cohesion, film formation (e.g. sufficient collagen-collagen interaction and adhesion) and the alignment of the films. Tunable film properties would broaden the range of future approaches to medical and bioengineering studies.

3.1 Confirming tropocollagen molecules in aged and fresh LB solutions

To determine if the collagen solutions are functional for manufacturing highly oriented collagen films, we confirmed the presence of triple-helical structured type I collagen molecules (tropocollagen) in fresh and aged (2 ½ years, -18°C) solutions by CD spectroscopy. To this end, CD spectra of the respective solutions (with or without n-propanol as network stabilizer) were determined at 20°C and 60°C Figure 1 shows that all spectra from fresh and aged solutions taken at 20°C are consistent with triple-helical type I collagen without fibrillar components (Fig. 1(a)) [49]. Therefore, all four collagen solutions are suitable for a test on their efficiency for film formation, although freshly prepared solutions might be more appropriate as they exhibit a slightly stronger signal. On the other hand, collagen solutions treated at 60°C resulted in the loss of the collagen's tertiary structure: collagen was denatured (Fig. 1(b)) [49]. Bentz et al. [50] have reported that denaturation of unbound collagen sets in slightly above 37°C. This means that the triple-helical structure is stable up to a temperature ~ 37°C and films should be coatable.

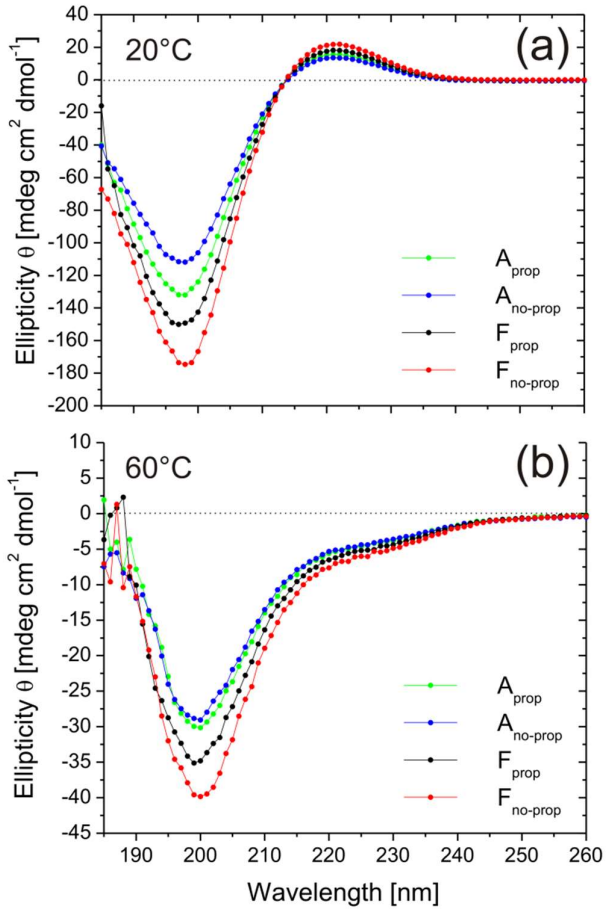

Fig. 1. CD spectra of fresh [black, red] and aged [green, blue] collagen type I dissolved in acetic acid (fresh: $F_{no\text{-}prop}$; aged: $A_{no\text{-}prop}$) or in acetic acid/n-propanol (fresh: F_{prop}; aged: A_{prop}), respectively and measured at (a) 20°C and (b) at 60°C. Please note: the vertical scales in (a) and (b) are different.

3.2 Ultrathin collagen coatings deposited from aged or fresh solutions (network stabilizer n-propanol) on hydrophobic or hydrophilic glass substrates

3.2.1 Compressing behaviour of differently prepared collagen on the air/water interface

To determine if the four differently prepared collagen solutions (aged/fresh, with/without n-propanol) can be spread, we applied 400 µL (2µM) of each solution drop by drop onto the air-water interface of the LB trough. However, following spreading of solutions without n-propanol, the drops smoothly sank into the subphase, implying that the collagen molecules might not have spread on the air-water interface but descended into the subphase. In contrast, spreading solutions containing n-propanol resulted in a change of the air-water interface and a barely visible film was observed. Compressing films from fresh collagen solutions led to visible "sticky foils" clearly floating on top of the air-water interface, compressing aged collagen instead resulted in films that were barely visible and gave the impression of not hovering on the air-water interface, but rather be located at the interface. It appears that these films have less cohesion and a higher water content (internalized into the structured collagen) than films from fresh collagen. The area-pressure isotherms of these films with a lateral pressure of ~7 mN/m for fresh vs. ~4 mN/m for aged collagen illustrate that behavior (Fig. 2(a)). The isotherms show a 'featureless' increase (e.g. no gas/liquid or liquid/solid transition) in pressure with decreasing surface area. Without n-propanol, collagen molecules do not interact sufficiently with each other. The formation of water structures in the vicinities of hydrophobic residues prevents stronger hydrophobic-hydrophobic-interactions; a sufficient film formation for both fresh and aged collagen does not take place (surface pressures are below 1 mN/m; Fig. 2(b)).

From these data it becomes evident that not only fresh but also aged collagen can be compressed if n-propanol is present. In both cases cohesion between the triple-helical structured collagen molecules is strong enough and leads to film formation. However, this process is less pronounced if aged collagen is used. These findings confirm a behavior also observed in previous studies [36,40] but obviously different from findings reported about stearic acids or similar systems [35,51]. It has been suggested that classical monolayer formation does not take place using collagen; instead layering of molecules within the LB film on the trough will result in thicker, multilayered films [36]. This would also explain the fact that collagen LB films are visible to the naked eye when compressed [36].

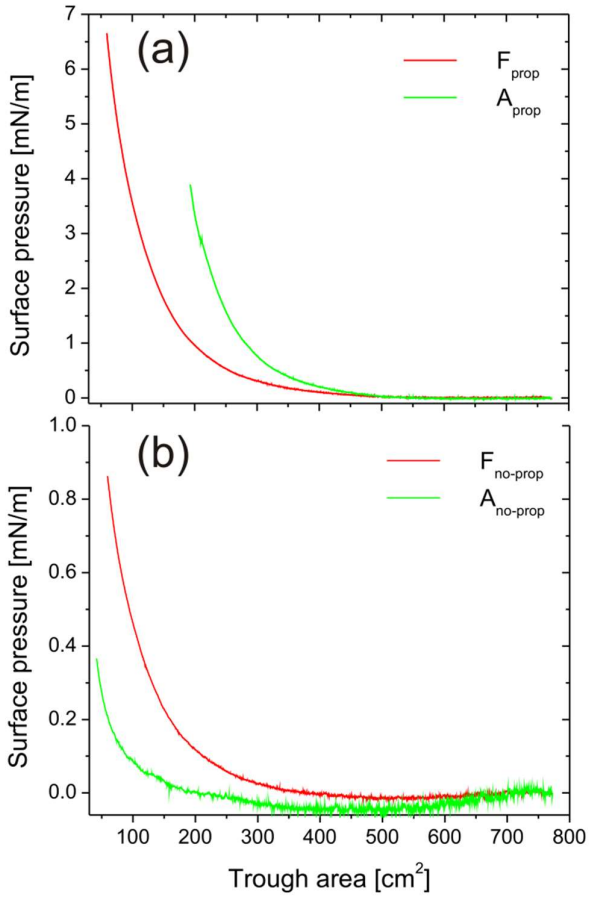

Fig. 2. Surface pressure - area isotherms of (a) fresh [F_{prop}, red] and aged [A_{prop}, green] collagen solutions containing n-propanol, and (b) fresh [$F_{no\text{-}prop}$, red] and aged [$A_{no\text{-}prop}$, green] collagen solutions prepared without n-propanol. Subphase: deionized water (Milli-Q). Please note: the vertical scales in (a) and (b) are different.

As mentioned above, after applying collagen solutions without n-propanol to the air-water interface, the molecules may not have spread on the air-water interface, but have (partially) sunk into the subphase. A hint for such a behavior is the strong background noise generated when individual molecules are shifted one above the other and into each other (e.g. aged solution, no n-propanol, Fig. 2(b)), perhaps resulting in some layered structures of low cohesion. Furthermore, both the lateral pressures of aged collagen solutions with and without n-propanol are lower than their respective counterparts of fresh solutions. This behavior suggests that (long-time) stored collagen solutions undergo a continual incorporation of water

molecules into the vicinities of hydrophobic residues, even with solutions containing n-propanol as a stabilizing agent.

3.2.2 Film deposition from fresh or aged collagen solutions (containing n-propanol) on hydrophobic or hydrophilic glass substrates

The former paragraph indicates that a stabilizer is needed to create cohesion between the triple-helical structured collagen and to form a stable film. Thus, collagen transfer on hydrophilic and hydrophobic glass slides was carried out exclusively using collagen solutions (aged or fresh; [hydrophobic/fresh as control]) containing the stabilizer n-propanol. For LB transfer, substrates were mounted with their long axes parallel to the pull-out direction and immersed with the lower part of the sample into the water subphase. After collagen was spread on the air-water interface, samples were pulled out and collagen transferred on the glass substrate. The coating resulted in a collagen film with fibrillar collagen aggregates forming an "orientation arch" (upper ~1/3 of the sample area) and an alignment of the fibrillar aggregated collagen (within a collagen film) parallel to the pull-out direction (lower ~2/3 of the sample area; for details see Tenboll et al. [36]). In Fig. 3 optical micrographs of deposited collagen structures are shown. The four panels exhibit collagen film formation using aged (Fig. 3(a)) or fresh (Fig. 3(b)) collagen coated on hydrophobic substrates, whereas Fig. 3(c) and (d) show the results using aged and fresh collagen, after deposition on hydrophilic samples, respectively. The films exhibit highly oriented collagen structures consisting of fibrillar aggregates (large elongated features in Fig. 3) embedded in a collagen matrix. None of the films indicate recognizable fissures or other flaws.

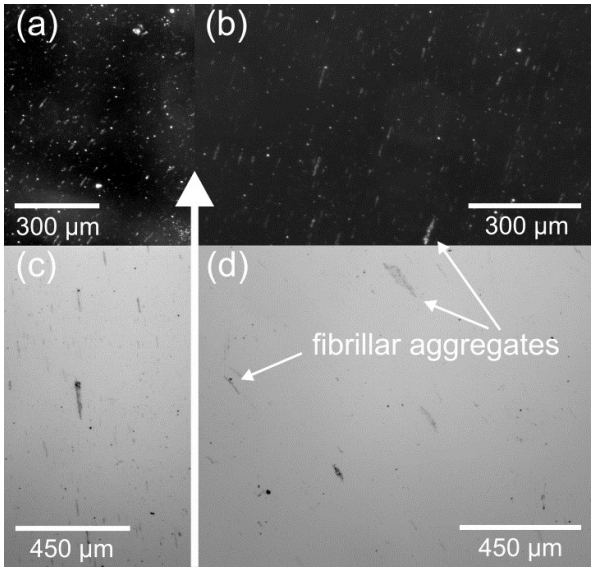

Fig. 3. Optical micrographs of collagen LB films deposited from propanol-containing solutions on hydrophobic or hydrophilic glass substrates. Structures of collagen on hydrophobic [(a). aged collagen; (b) fresh collagen] and hydrophilic [(c) aged collagen; (d) fresh collagen] substrate surfaces. (a) and (b) Dark field images of collagen films taken close to the area of the sample where the air-water interface was located, prior to lifting ("arch"). (c) Bright field image (in transmission) of a collagen film taken from an area which was immersed underneath the air-water interface prior to lifting. (d) Micrograph (bright field image in transmission) of a collagen film imaged close to the 'orientation arch'. Large arrow: direction in which substrates were lifted through the LB film (pull-out direction).

However, there is some uncertainty how the collagen matrix is organized, in which the fibrillar aggregates are embedded. Using SEM (Fig. 4) we found that each film transferred to a substrate developed collagen matrices with orientations identical to those of fibrillar aggregates. Fig. 4(a) shows the structure of a collagen film (from aged solution with n-propanol) transferred on a hydrophilic substrate. The structure of the film consists of the matrix and the larger fibrillar aggregates (micrometers in length). Performing fast Fourier transform (FFT) analysis (not shown) in areas exhibiting no fibrillar aggregates (e.g. bottom left of Fig. 4(a)), we were able

to additionally clarify the orientation of collagen within the matrix. We found that both the fibrillar aggregates and the matrix (fibrils) are aligned to each other and parallel (except the "arch") to the pull-out direction. Moreover, the higher magnification in Fig. 4(b) shows details of the collagen matrix (deposited from aged solution with n-propanol; hydrophilic substrate). Perpendicular to a zigzag structured fibrillar aggregate, aligned fibrils of the collagen matrix are identified.

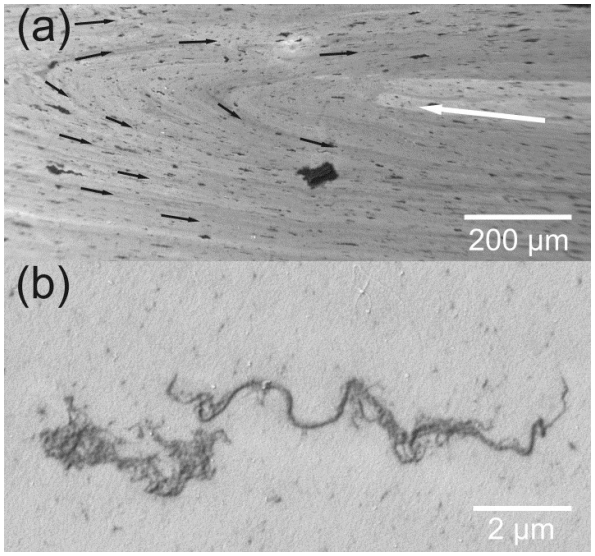

Fig. 4. Scanning electron micrograph of (a) a collagen LB film transferred on a hydrophilic glass substrate and (b) transferred on a hydrophobic substrate (both: aged collagen solution containing n-propanol). The section shown in (a) is located approximately in the middle of the sample (arch is getting very small). White arrow: pull-out direction. Black arrows: flow direction and orientation of collagen fibrils/fibrillar aggregates. (b) The higher magnification in (b) clearly shows the zigzag structure of fibrillar aggregates and, perpendicular to that, the aligned fibrils of the collagen matrix.

The zigzag shape might be a result of flow processes during film transfer. As the aggregate did not align with the pull-out direction (it stayed nearly perpendicular to it) flowing solution deformed the hitherto elongated shape, while drying might have fixed the structure.

Eventually, these findings demonstrate that not only fresh but also aged collagen solutions (containing n-propanol) can be used to coat collagen in an aligned fashion. Wegner and Mittler and coworkers have shown that slow LB transfer of collagen results in a convergent flow of the shape persistent molecules/fibrils towards the substrate and leads to an alignment parallel to the pull-out direction on the substrates [36-39]. At the same time, the hydrophobic-hydrophobic interactions between the fibers/aggregates induce sufficient cohesion for film formation [36]. In other words; long-range ordered highly oriented alignment of collagen occurs explicitly during film-coating; it is not generated on the air/water interface during compression as, e.g., Langmuir-Schaefer experiments show [24]. The fibrillar aggregates form when the solution is drying and they stretch up to a length of ~150 μm (collagen did not aggregate to large fibrils in solution between 4 - 37°C) [36,47]. n-propanol was found to enhance this process by altering the hydrophobic effect at the hydrated protein and promoting the formation of more compact structures [9]. The film thicknesses created under the given conditions are in the range between 16 – 20 nm [36], irrespective of whether fresh or aged collagen solutions were applied to hydrophilic or hydrophobic substrates. Our SEM studies confirm, in part, earlier work showing that both the fibrillar aggregates and collagen fibers are following the same orientation after transfer on substrates [36]. Moreover, these coatings can be carried out using hydrophobic but also the less expensive hydrophilic substrate surfaces. However, fresh collagen seemed to show a higher affinity to the hydrophobic substrates, whereas aged collagen interacted stronger with hydrophilic substrates. The reasons for this behavior are most likely to be found in the characteristics of the collage solutions and their behavior when transferred to substrates. It is assumed that compressing aged collagen on the air-water interface creates films with a higher content of water molecules (internalized in the structured film) than using fresh collagen. Hence, films made from aged collagen show a slightly lower inner cohesion and they are less affine to hydrophobic sample surfaces than using freshly prepared solutions. A similar behavior was reported by Ying et al. and Coelho et al. [42,43] who investigated the adsorption of collagen on hydrophobic and hydrophilic surfaces. They found that hydrophobic surfaces were preferred

over hydrophilic ones, but in competition with other (more hydrophobic) proteins collagen adsorbed to hydrophilic surfaces more readily. Thus, collagen demonstrates a certain hydrophilic-hydrophobic balance, which is affected e.g. by water molecules. In solution, water can be driven out (by e.g. n-propanol) or incorporated (by aging) into the vicinities of hydrophobic residues. Wood et al. [52] have shown that viscosities of collagen solutions decreased with increasing storage time, and attributed this behavior to a significant increase of electrostatic interactions between the soluble fibrillar aggregates.

3.3. Ultrathin collagen coatings deposited from aged or fresh solutions (network stabilizer: phosphate ions) on hydrophobic or hydrophilicglass substrates

3.3.1 Compressing behaviour of differently prepared collagen on the air/water interface and comparison to n-propanol-treated collagen

A further series of experiments was carried out to clarify whether n-propanol can be replaced by the physiological phosphate. To this end, fresh ($F_{no\text{-}prop}$) or aged ($A_{no\text{-}prop}$) collagen solution (400 µL; 2µM, without n-propanol) was applied drop by drop onto the phosphate ion containing air-aqueous interface (phosphate buffer solution, PBS) of the LB trough. Following spreading, the compression of the collagen molecules on the PBS subphase resulted in films (visible with the naked eye) indicating an intermolecular cohesion qualitatively much higher than films prepared without a stabilizer (those films were not visible, section 3.2.1), but not as developed as the "sticky foils" found after compression of fresh solutions in the presence of n-propanol (section 3.2.1). On the other hand, the area-pressure isotherms of these phosphate-stabilized films (Fig. 5) show values (~10-18 mN/m) even higher than those found for F_{prop} (7 mN/m; Fig. 2(a)), which indicates the highest value for n-propanol-stabilized samples.

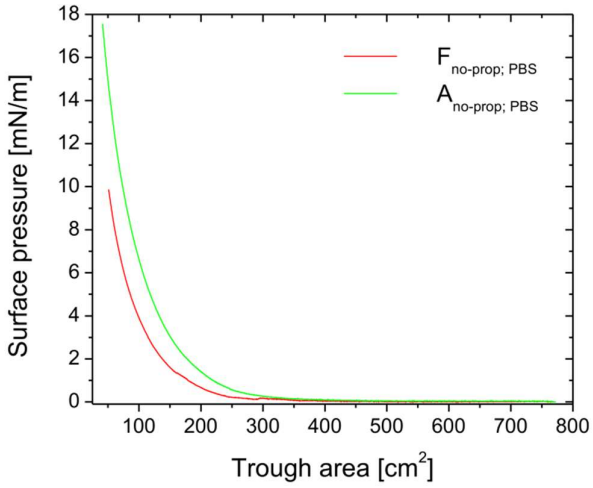

Fig. 5. Surface pressure - area isotherms of fresh [$F_{no\text{-}prop,PBS;}$ red] and aged [$A_{no\text{-}prop,PBS}$; green] collagen solutions prepared without n-propanol. Subphase: phosphate buffer saline (PBS).

These findings suggest that the absence of n-propanol provides an easier access for water molecules to drift into the vicinities of the collagen's hydrophobic groups and form hydrophilic structures. In addition, the strong isotherms of aged solutions point out that the phosphate ions interact with collagen molecules/fibers more readily if the overall hydrophilicity is increased (see Fig. 5). It is assumed that the phosphate ions electrostatically bind collagen fibrils (maybe molecules as well), therefore, stabilizing film formation and ensuring film transfer on substrates. Likewise, Li and Mertz et al. [8,53] have demonstrated that divalent phosphate ions bind to fibrillar collagen, a process they explain by the phosphate ion's promoted electrostatic interactions with collagen in solution. Under these circumstances, it is not surprising that the aged/non-stabilized ($A_{no\text{-}prop}$) solution with the more hydrophilic character is barely observable on the air-water interface (section 3.2.1), but that film formation in the aged/phosphate-containing subphase ($A_{no\text{-}prop,\ PBS}$) is more distinct (higher lateral pressure by phosphate-bound (stabilized) collagen) than using the fresh/phosphate containing subphase ($F_{no\text{-}prop,\ PBS}$; less hydrophilic).

3.3.2 Film deposition from fresh or aged collagen solutions (containing phosphate ions) on hydrophobic or hydrophilic glass substrates

The area-pressure isotherms of the phosphate-stabilized films (section 3.2.1) imply that deposition of collagen on glass substrates from aged solution is better or at least as good as from fresh solution. However, LB transfer from fresh solutions ($F_{no\text{-}prop\ PBS}$) resulted in less material failure, like fissures or complete/segmental tear off during transfer (see Fig. 6). This applies to coatings on both hydrophilic (Fig. 6(a) and (b)) and hydrophobic (Fig. 6(c)-(f)) substrate surfaces. Aged solutions ($A_{no\text{-}prop,\ PBS}$) always led to failing or a non-proper transfer (insufficient cohesion and low adhesion of collagen on the substrate).

After transfer from fresh solutions ($F_{no\text{-}prop\ PBS}$), the coated films exhibit fibrillar aggregates aligned with fibrils of the collagen matrix. This finding is very similar to the outcome found when n-propanol was used. These experiments show that physiological phosphate can be used instead of n-propanol or other additives to stabilize and transfer collagen on substrate surfaces.

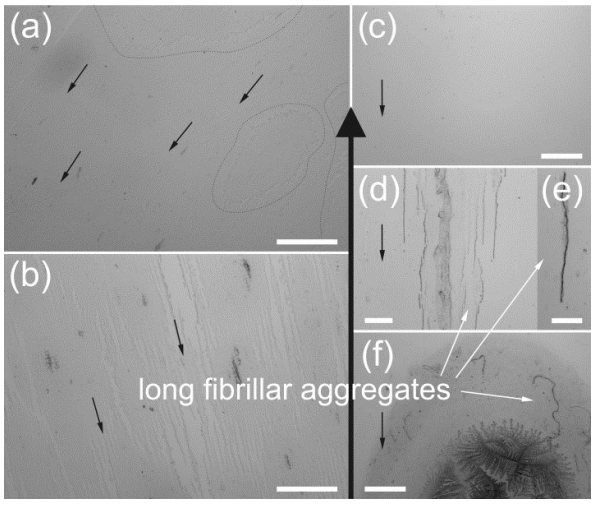

Fig. 6. Optical micrographs of deposited collagen (from fresh collagen solutions) structures on (a), (b) hydrophilic and (c)-(f) hydrophobic glass substrate surfaces. The transferred collagen is always accompanied by phosphate precipitates. All images: bright field transmission mode.

Large black arrow: substrate pull-out direction. (a),(b) Images taken close to a sample area where the air-water interface was located prior to substrate lifting ("arch"). Small black arrows: direction of collagen flow and subsequent alignment during deposition. (a) Some deposits (most likely collagen/phosphate accumulation; dotted lines) are recognizable. (b) Loose and open collagen structures; darker spots are most probably phosphate salt precipitates. (c)-(f) Images taken along the pull-out direction, but below the 'arch' (see Figure3). (c) Upper section, (d), (e) middle part and (f) bottom part of collagen films coated on hydrophobic substrates samples. Note the (d) moderate and (f) stronger aggregation (presumably collagen/phosphate salts); also very long fibrillar aggregates with varying diameters are formed (white arrows). Scale bars: (a), (b), (c), (f): 300 μm; (d): 50 μm; (e): 200 μm.

Another feature of the phosphate stabilized films is the extensive precipitation of phosphate salts. Fig. 6 shows a series of images, with panel (a) and (b) taken from sample sections near the so-called "arch" (hydrophilic substrates surfaces) and panel (c)-(f) taken along the pull-out direction, but below the 'arch' (hydrophobic substrates surfaces). In these images phosphate salt residues can be found, which accompany the fibrillar collagen. Precipitation is, however, stronger in areas near the bottom part of a sample (Fig. 6(f)), those areas which are leaving the aqueous subphase last during film deposition. Because of phosphate precipitations, film thicknesses will vary strongly and measurements would not lead to reliable data. Therefore, besides the fact that the concentration of phosphate salt needs to be controlled to avoid salt deposits on films, it is very likely that the way of delivering the phosphate ions needs to be adjusted as well. This could be achieved, for example, by the addition of phosphate ions to the collagen stock solution, similar to the procedure carried out with n-propanol. However, these studies are beyond the scope of this study.

After all, it is still unclear which effects resulted in the higher level of cohesion when using the fresh collagen subphase ($F_{\text{no-prop PBS}}$). It is possible that a certain degree of hydrophobicity between the collagen fibrils is needed to generate a sufficient stability of the films on the

subphase. In fact, Mertz et al. have found that the presence of phosphate ions results in two different kinds of collagen-collagen interactions [53]. Among fibrils, bound phosphate appear to form salt bridges between positively charged amino acid residues (within regions of high excess positive charge), whereas the increase in fibrillogenesis (increase of fiber formation) results from preferential exclusion of divalent phosphate from interstitial water within fibrils [53]. The latter points also out that in fibrillogenesis a high degree of hydrophobic-hydrophobic interactions occurs [5,6] inducing a self assembly process to form collagen fibers [20,54]. For isolated collagen molecules no phosphate binding was found, which suggests that collagen-collagen interactions during film formation are preferentially proceeding on the level of fibril-fibril networking and fibrillogenesis. Finally, interfibrillar hydrophobic-hydrophobic interactions in addition to the formed collagen-fiber-phosphate network (created via the hydrophilic fiber/phosphate bridges) appear to be essential to form stable and long-lasting collagen films in the presence of phosphate ions.

4. Conclusion

The present study has demonstrated that LB transfer of highly oriented collagen films is feasible on both hydrophobic and hydrophilic glass substrates, using either freshly prepared or aged (2 ½ years, -18°C) collagen solutions. The forming films contain fibrillar collagen embedded within a matrix of aligned collagen fibrils. These films are most stable when a network stabilizer (e.g. n-propanol) is present during film formation, which reinforces the collagen fiber structure on the water subphase and during LB transfer. Using n-propanol, the film stabilizing effect, the obstruction of water accumulation/structure formation in the immediate vicinity of hydrophobic residues (in and around fibrils), is strongest using fresh solutions (control experiments) and hydrophobic substrates. Films made from aged solutions show a slightly lower inner cohesion and less affinity to hydrophobic sample surfaces. However, these films still demonstrate

unrestricted usability for deposition of highly oriented collagen. Moreover, the findings show that the hydrophilic character of the collagen structure is controllable and that coatings can therefore be fabricated for specific applications. The study of film formation with fresh or aged solution, but without the stabilizer, showed that collagen fibrils do not interact sufficiently with each other on the air-water interface; they are not able to form a cohesive film for deposition, neither on hydrophilic nor for hydrophobic substrates.

When n-propanol was replaced by physiological relevant phosphate ions, only fresh collagen solution produced films with a sufficient cohesion and transferable film quality. Coatings made from aged solution often showed several fissures or an incomplete film transfer (low cohesion and adhesion), and was, therefore, classified as unsuitable. The most stable films were produced by transferring freshly prepared collagen solution on hydrophilic substrates. This is in contrast to films made from n-propanol stabilized fresh collagen; these films are most stable when transferred on hydrophobic substrates. It is suggested that (a) intermolecular hydrophobic-hydrophobic interactions of collagen (self assembly process: fibrillogenesis) and (b) the phosphate ion's strong electrostatic interactions with collagen fibrils (creating a collagen-phosphate network) result in this higher level of cohesion. Furthermore, stabilization of such films on hydrophilic substrates is, of course, also based on the film's higher hydrophilicity and its interactions with the hydrophilic surface of the glass substrate.

The control of the hydrophilic-hydrophobic balance via the water accessibility of collagen at a given concentration and the use of a specific collagen network stabilizer (both affecting the alignment and the cohesion of films) are the parameters available to engineer application-specific collagen films for coatings on surfaces exhibiting different properties. For the future, various areas of applications, such as wound dressing, coatings for bone repair and implants or use in regenerative dentistry and corneal transplantation are conceivable. Making freestanding films and testing them for some of these applications is the goal of future studies.

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