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PAPER

Collagen I and III and their decorin modified surfaces studied by atomic force microscopy and the elucidation of their affinity toward positive apolipoprotein B-100 residue by quartz crystal microbalance

Joanna Witos,[a] Julien Saint-Guirons,[ab] Kristoffer Meinander,[c] Lucia D'Ulivo[a] and Marja-Liisa Riekkola*[a]

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Collagen, the major component of extracellular matrix (ECM) and the most abundant protein in the human body, is implicated in the development of atherosclerosis. Collagen types I and III were immobilized on fused-silica capillary to investigate their shape, size and structure by atomic force microscopy (AFM). For comparison, collagen was also immobilized on a mica surface. Our studies demonstrated that not only does the adsorption pattern on the substrate vary with the type of collagen, but also the substrate material plays an important role in the fibril formation process. Decorin, which promotes the binding of low-density lipoprotein (LDL) particles with collagen, was investigated for its effect on the fibrillogenesis. On both substrate materials, addition of decorin clearly reduced the fibril diameter of collagen surfaces. Moreover, a quartz crystal microbalance (QCM)-based biosensor approach was applied to clarify and evaluate the affinity of different collagen coatings immobilized on a silicon dioxide sensor chip toward apolipoprotein B-100, the major protein of LDL. The results confirmed the importance of collagen type and their fibrillogenesis on the binding of the positive residues of apolipoprotein B-100 on negatively charged collagen surfaces.

Introduction

Collagens are the most abundant proteins in the human body and major structural components involved in the formation of extracellular matrix (ECM).[1,2] Each collagen molecule consists of the repetitive sequence Gly–X–Y, where X is usually proline and Y hydroxyproline, and the triple helix structure where three polypeptide α-chains are twisted around each other.[1,3] Among the twenty nine types, collagen I, II and III are the fibril-forming types, which are composed of an uninterrupted right-handed triple helix approximately 300 nm in length and 1.5 nm in diameter. Fibrils exhibit the cross-striated, D-periodic banding pattern and are formed from collagen molecules by self-assembly.[4] As is well recognized, collagens vary in composition: type I consists of two α_1-chains and one α_2-chain, while type III is composed of three identical α_1-chains and a greater amount of hydroxyproline, which result in lower hydrophobicity than type I.[1] Collagens as a principal constituent of ECM, which are usually associated with decorin,[5] take part in the development of several diseases, most notably atherosclerosis.

[a]*Laboratory of Analytical Chemistry, Department of Chemistry, University of Helsinki, P.O. Box 55, FIN-00014 Helsinki, Finland. E-mail: marja-liisa.riekkola@helsinki.fi; Fax: +358-9-191-50253; Tel: +358-9-191-50268*
[b]*Attana AB, Björnnäsvägen 21, SE-114 19 Stockholm, Sweden*
[c]*Accelerator Laboratory, Department of Physical Sciences, University of Helsinki, P.O. Box 43, FIN-00014 Helsinki, Finland*

Low-density lipoprotein (LDL) particles, the main cholesterol carriers in the circulation, with the physiological role of providing cells with cholesterol, is the major cause of atherosclerosis development by accumulating in the *intima*.[6,7] LDL particles are composed of a hydrophobic core containing cholesteryl esters, triglycerides, fatty acids and fat-soluble vitamins, and the surrounding hydrophilic layer consisting of apolipoprotein B-100 (apoB-100), a monolayer of phospholipids and unesterified cholesterol.[8] ApoB-100, the largest and the most hydrophobic known monomeric protein, controls and emphasizes the specific interactions of LDL particles with ECM structural components, proteoglycans and collagen. Borén *et al.*[9] noticed that the principal proteoglycan binding site of apoB-100, peptide residue 3359–3377, composed of six positively charged amino acids, is able to interact with negatively charged chondroitin-6-sulfate of proteoglycans.

In our previous studies we showed electrochromatography to be an excellent platform for the study of collagen surfaces under a variety of experimental conditions.[10] Collagens I and III, and collagen I–decorin and III–decorin coatings immobilized on the inner surface of fused-silica capillaries enabled studies on the interactions between collagen and selected peptide fragments of apoB-100. Decorin stabilized the coatings, and enhanced the binding between collagen and the peptides. Immobilization of collagen I on the inner wall of the fused-silica capillary was also exploited for glycation studies that allowed clarification of the interactions of native LDL and oxidized LDL with unmodified

or glycated collagen.[11] Although capillary electrochromatography serves as an excellent microreactor, it cannot provide detailed information about the collagen surfaces on the fused-silica capillary wall. To obtain such information, we applied atomic force microscopy (AFM), which is an excellent tool for elucidation of the structure and molecular properties of human materials. A highly sensitive and tiny probe, which is essentially pulled across a surface coated with human material, provides topographical images on nanometre scale. Over the last few years, AFM has been successfully applied to determine collagen structures, study the interactions of collagen with other ECM components and clarify the modality of collagen self-assembly on polymeric and other surfaces.[12–16] To our knowledge, no investigations of collagen have been carried out on hydrophobic surfaces, such as fused-silica that is a high purity synthetic amorphous silicon dioxide.

Our goal in the present study was to elucidate the structures of collagen types I and III by immobilizing collagen on fused-silica capillary surfaces. For comparison, collagen was also immobilized on the more polar mica surface. Moreover, we examined, on both types of surface, the ability of collagen to bind decorin during fibrillogenesis. In addition, a novel instrumental microanalytical technique, quartz crystal microbalance (QCM), that has been successfully employed for on-line detection of bioaffinity interactions,[17,18] and for surface adsorption and characterization studies,[19] was applied to elucidate and determine the interaction between collagen type I and III, and decorin modified collagen I, collagen III immobilized on the silicon dioxide surface and peptide residue 3359–3377 of apoB-100.

Results and discussion

Collagen, the most abundant protein in the human body, is involved in the formation of ECM. Modification of its structure can lead to several diseases, such as atherosclerosis, increasing the morbidity and mortality rates in industrialized countries.[20–23] In this study, AFM was used to examine the shape, size and structure of collagen types I and III immobilized on fused-silica capillary and mica surfaces. The ability of collagen to bind other ECM components and the effect of binding on fibrillogenesis were investigated. In addition, QCM was successfully applied to study and determine the affinity of the same collagen surfaces studied by AFM toward the lysine- and arginine-rich domain 3359–3377 of apoB-100. The immobilization procedures used for the fused-silica, mica and silicon dioxide substrates were almost identical.

Fibrillogenesis of collagen types I and III

The fibrillogenesis of collagens I and III was investigated on fused-silica capillary and mica surfaces. Collagens were immobilized on the inner surfaces of fused-silica capillaries by the relatively simple procedure developed earlier in our laboratory.[10] In our previous studies, collagen I and III were dialyzed at 4 °C at pH 7.4 to get homogeneous monomer solution for the immobilization, and in this way to assure the stability and repeatability of the complex collagen coatings. Moreover, our results showed that the phosphate buffer appeared to stabilize the collagen I coating, while HEPES buffer ensured the stability of the more hydrophilic collagen III film, and it was essential to use the same buffer solution for the dialysis as for the immobilization of the collagens on the substrates. In addition, because NaCl destabilized the collagen I film, it was employed only with collagen III.[10] As shown in Fig. 1, type I collagen adsorbed more strongly on the fused-silica than on the mica surface: on fused-silica capillary fibrils were long, thick and randomly distributed (Fig. 1a), while on mica short and thin fibrils formed a tightly packed netlike structure (Fig. 1e). In the case of collagen III, the situation was opposite: the adsorption on the mica surface was stronger, and fibrils were longer, thicker and more-evenly distributed than on fused-silica, where a fibrous branch network was seen (Fig. 1c and g). The differences in the adsorption patterns are attributable to the differences in collagen I and III structures. Collagen III is more hydrophilic than collagen I due to the larger number of hydroxyproline groups,[1] which results in weaker adsorption on hydrophobic surfaces such as fused-silica capillary.

For comparison of the thickness of the collagen I and III coatings, the full width at half maximum (FWHM) was calculated for the central peak in each AFM image (Table 1). The thickness follows the adsorption capability of the substrate; *i.e.*, the collagen I film immobilized on mica is much thinner (5.50 ± 0.97 nm) than the collagen I (7.72 ± 0.75 nm) and collagen III (7.05 ± 0.97 nm) coatings achieved on the fused-silica capillary surface. Again more hydrophobic collagen I adsorbed more easily on the hydrophobic fused-silica surface than collagen III. Moreover, the more uniform and even the coating, the thinner is the collagen film.

Influence of decorin on the adsorption pattern of collagens

Binding of low-density lipoproteins to the ECM of the inner layer of the arterial wall, the *intima*, has been implicated in the development of atherosclerotic plaque. The modifications of collagen can also affect the affinity of native and oxidized LDL for collagen, and promote the entrapment of LDL particles in the intima, accelerating the progression of atherosclerosis. Such binding is promoted by the presence of decorin.[23,24] To elucidate the role of decorin in the formation of collagen fibrils and the effect on the adsorption of collagen on different surfaces, dialysed collagen solutions containing NaCl were premixed with decorin before immobilization on fused-silica and on mica surfaces. Fig. 1 shows that in almost all cases the addition of decorin reduced the fibril diameter. Furthermore, collagen films were more even and comprised tightly packed, well-organized fibrous networks, and films were thicker when decorin was involved in the fibril formation. Interestingly, when decorin was mixed with collagen III before the immobilization on the fused-silica capillary surface, the collagen was modified and aggregated (Fig. 1d), and collagen fibrils were huge and irregular. The degree of collagen III–decorin surface coverage was low, even though FWHM was about 14 nm. While the reasons for the aggregation are unclear, one possibility could be the considerable differences in hydrophobicity between collagen III–decorin and the substrate material. Nevertheless it is clear that decorin, by promoting well-packed collagen fibrous networks with a high degree of order, stabilizes the collagen structures.

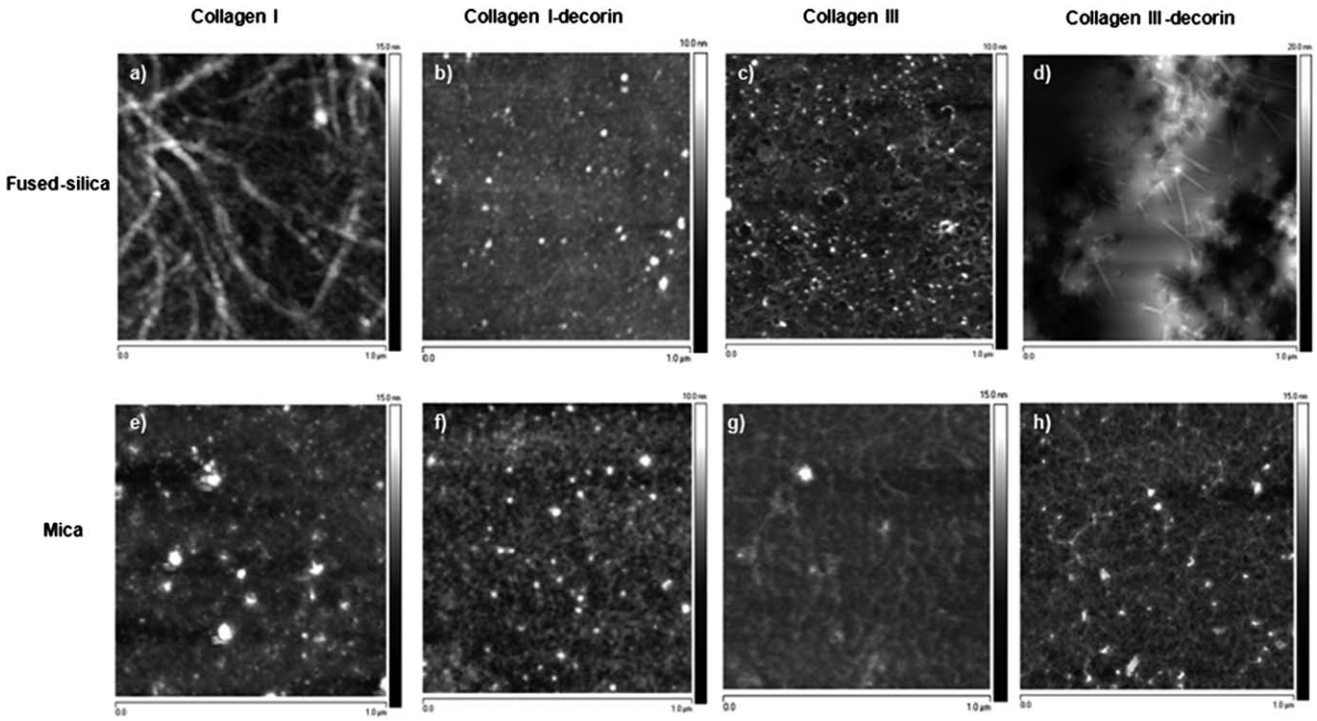

Fig. 1 AFM images of (a) collagen I, (b) collagen I–decorin, (c) collagen III, and (d) collagen III–decorin coatings adsorbed on fused-silica surface, and (e) collagen I, (f) collagen I–decorin, (g) collagen III, and (h) collagen III–decorin coatings adsorbed on mica. Represented area 1 μm × 1 μm.

Collagen height profile calculations

Height profiles were obtained by plotting the distribution of heights in each image, with the median height of each image set as 0. Typical height profiles displayed a large central peak, corresponding to the actual collagen coating, and a smaller peak centered on a higher value of height. These smaller peaks were caused either by small islands of a second layer of coating or by larger impurities or agglomerations that may have formed.

A smooth and narrow central peak in the height profile, with a low value for FWHM, is typical of a fairly homogeneous and evenly distributed surface coating. Coatings like these are generally the result of a strong interaction between the collagen and the underlying substrate. A broader central peak will occur if single fibrils within the coating are larger in size. This is not necessarily an indication of lower binding strength but may be only a consequence of the greater roughness of a coating composed of larger constituents. If the coatings are uneven due to lower binding strength between the collagen and the underlying substrate, this will usually be detected as a set of smaller peaks superimposed on the larger central peak of the height profile. These smaller peaks are an indication of the preferential agglomeration of collagen into larger particles, as opposed to evenly distributed fibrils tightly bound to the substrate. Such agglomeration was evident for collagen III–decorin on the fused-silica surface (Fig. 2a), for which the central peak of the height distribution was both broad and jagged due to the superimposed smaller peaks of agglomerated fibrils. The bimodal nature of the central peak in the height profile of collagen I on mica (Fig. 2b) was evidence of the formation of a second layer of coating on an even and homogeneous first layer, or of a fairly even coverage of agglomerates of two distinct sizes.

Interaction between apolipoprotein B-100 peptide fragment and different collagen surfaces

To investigate the interaction characteristics between apolipoprotein B-100, the major protein of LDL, and collagen surfaces, we introduced peptide residue of apoB-100 that is responsible for the binding of LDL with proteoglycans[25,26] at increasing concentrations over collagen surfaces immobilized on the silicon dioxide sensor chip. As can be seen from the sensorgrams in Fig. 3, the maximum frequency shift monitored as a result of the binding of the apoB-100 fragment on each collagen surface is concentration dependent. Moreover the biphasic binding response suggested a more complex interaction than a bimolecular interaction. In the view of earlier findings, the positively

Table 1 The full width at half maximum ± SD for each central peak in each individual image

	FWHM/nm			
Material	Collagen I	Collagen I–decorin	Collagen III	Collagen III–decorin
Fused-silica	7.72 ± 0.75	8.14 ± 0.86	7.05 ± 0.97	13.80 ± 1.62
Mica	5.50 ± 0.97	7.81 ± 0.43	8.10 ± 0.65	8.12 ± 1.08

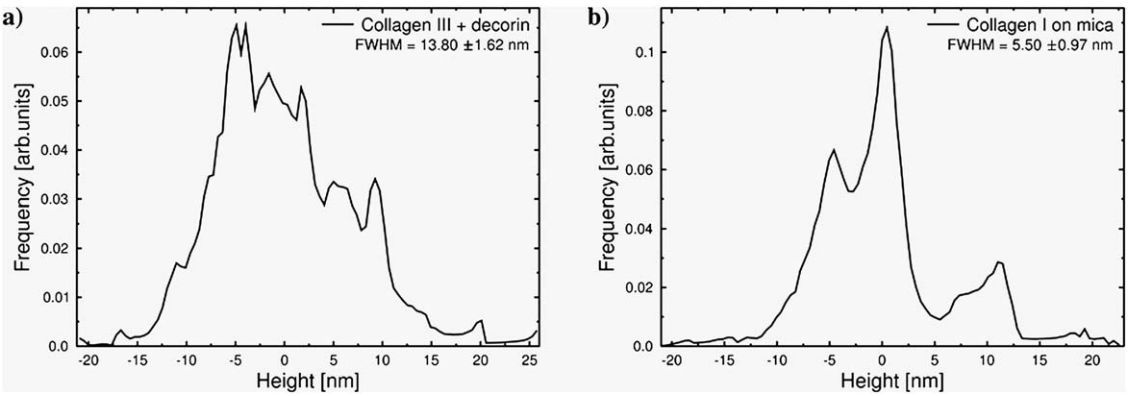

Fig. 2 Height profiles of (a) collagen III–decorin coating adsorbed on fused-silica surface and (b) collagen I coating adsorbed on mica surface.

charged groups of the apoB-100 peptide fragment, lysine and arginine, play a significant role also in the binding with collagen molecule and interact with negatively charged residues of the collagen surface.[27] In our earlier preliminary studies carried out by QCM on collagen I surfaces immobilized *via* amine-coupling to the carboxyl sensor chip, the complexity of the interaction was explained by the fact that collagen I exposed several negative residues that acted as potential binding sites for peptide residue 3359–3377.[28] Based on our results (Fig. 3a and b and Table 2) the interaction between the positive apoB-100 fragment and collagen I directly immobilized on the silicon dioxide sensor surface is much weaker than that with collagen III. The reason of the differences in the binding patterns and values of dissociation constants can be attributed to the differences in the structures of collagen I and collagen III, the latter being more hydrophilic with more negative charges due to its hydroxyproline groups.[1] Moreover, the affinity of collagen surfaces immobilized with premixed collagen–decorin solution toward apoB-100 peptide fragment was stronger due to the more negative collagen surface (Fig. 3c and Table 2). However, as previously mentioned, when decorin was premixed with collagen III, the collagen aggregated (Fig. 1d) resulting in an uneven surface. In that case the K_D value for the interaction between the apoB-100 peptide fragment and collagen III–decorin surface could not be calculated because of the problems in the regeneration of the collagen III–decorin surface after the introduction of apoB-100 peptide that was most probably very strongly adsorbed on bare silicon dioxide surface spots present due to the collagen aggregation mentioned (Fig. 4). This finding is supported by AFM images of the collagen III–decorin coating (Fig. 1d). As can be seen in Fig. 4, the binding curves of apoB-100 peptide at 20 μg mL^{-1} on the collagen I, collagen III and collagen I–decorin surfaces showed similar binding patterns, although binding capacities varied probably due to variation in surface preparation, while no or very slow dissociation was observed for the interaction between the very negatively charged collagen III–decorin coating and

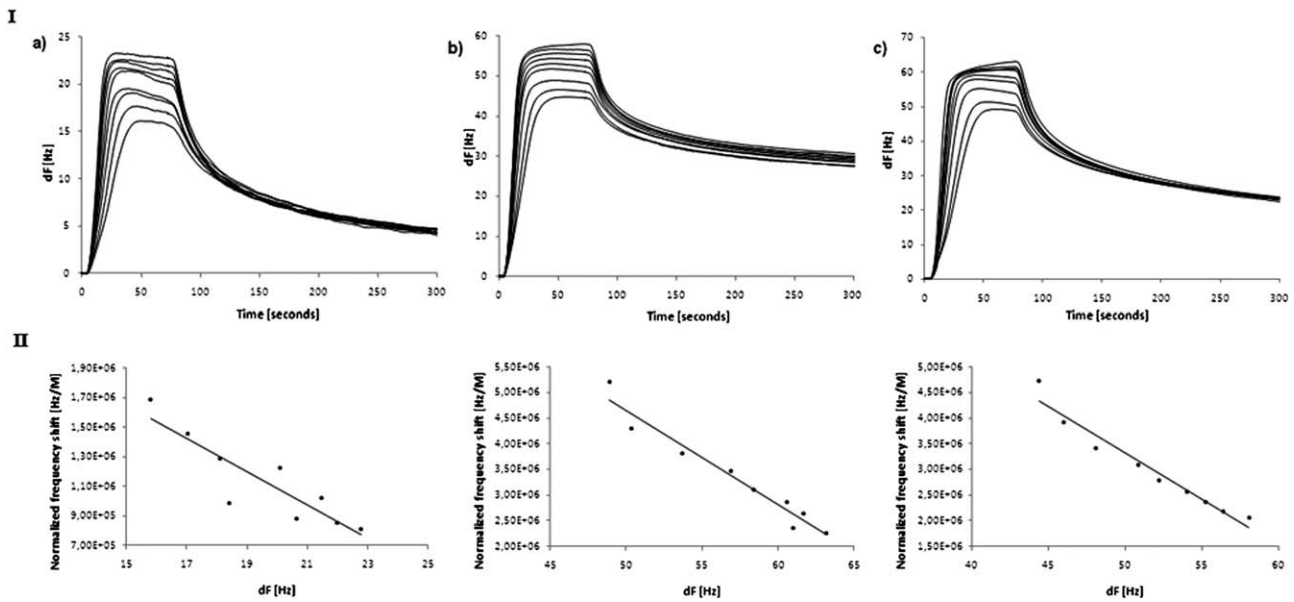

Fig. 3 (I) Binding pattern of interaction between positive apolipoprotein B-100 peptide (3359–3377) and (a) collagen I, (b) collagen III, and (c) collagen I–decorin surfaces immobilized on silicon dioxide sensor chip. (II) Scatchard plot for equilibrium analysis of data at the end of the association phase. Running conditions: flow rate, 25 μL min^{-1}; 25 °C, 20 mM BGE at pH 7.4 (phosphate buffer in the case of collagen I and collagen I–decorin, and HEPES in the case of collagen III surface). ApoB-100 concentrations ranged from 20 to 60 μg mL^{-1}.

Table 2 Calculated K_D values ± RSD% of interaction between positive apoB-100 peptide (3359–3377) and collagen-coated silicon dioxide sensor chip

Ligand	Analyte	K_D/μM
Collagen I	ApoB-100 peptide	8.9 ± 0.2
Collagen III	ApoB-100 peptide	5.5 ± 0.1
Collagen I–decorin	ApoB-100 peptide	5.4 ± 0.1
Collagen III–decorin	ApoB-100 peptide	Very strong interaction

apoB-100 peptide and proved the strongest interaction from all the collagen surfaces studied. Our results clearly demonstrate that the apoB-100 peptide fragment has strong affinity toward extracellular matrix component collagen and this interaction is facilitated by decorin.

Experimental

Materials

Collagen type I, collagen type III and decorin were purchased from Sigma-Aldrich (C8919; C3511; D8428). The apoB-100 peptide residue (3359–3377) with 19 amino acids and a net charge of +6 was synthesized at the Meilahti Protein Chemistry Facility and analyzed at the Protein Chemistry Core Facility (Biomedicum, University of Helsinki, Finland).[10] The membrane for dialysis (molecular weight cut-off 12 to 14 kDa) was obtained from Spectrator (VWR International Oy, Espoo, Finland).

Sample and buffer preparation

The ionic strength of the background electrolyte solutions (BGE; phosphate or HEPES buffer) was 20 mM with pH 7.4 adjusted to the desired value with 1.0 M sodium hydroxide or 1.0 M hydrochloric acid. Before use, the BGE was filtered through 0.45 μm Millipore filters using a Millipore vacuum system. The pK_a value of 7.2 for phosphoric acid and 7.5 for *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] were used for calculations of ionic strength of the BGE solutions.

The apoB-100 peptide stock solution was prepared in MilliQ water with a concentration of 1.5 mg mL^{-1} and stored at +4 °C.

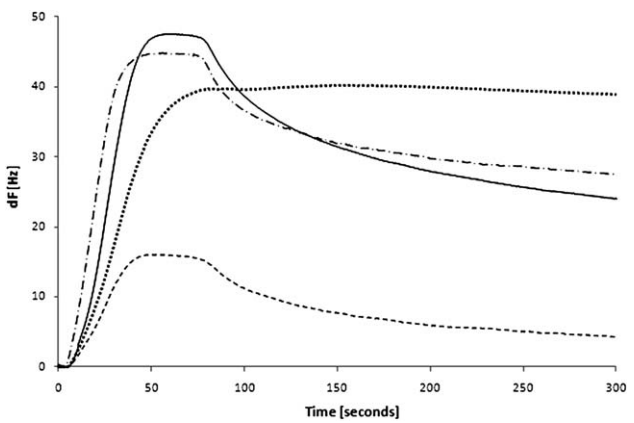

Fig. 4 Comparison of binding pattern of interaction between positive apolipoprotein B-100 peptide (3359–3377) at concentration 20 μg mL^{-1} and (**---**) collagen I, (**-·-**) collagen III, (—) collagen I–decorin, and (···) collagen III–decorin films immobilized on silicon dioxide sensor chip.

Before injection stock solution was diluted in running buffer (phosphate or HEPES) to obtain concentrations ranging from 20 to 60 μg mL^{-1}. Sodium chloride was prepared in MilliQ water and used as the regeneration solution.

Preparation of coating solutions

Solution for collagen I coating. Stock solution of collagen I (1 mg mL^{-1} in acetic acid 0.1 M) was dialysed for 3 h against phosphate buffer pH 7.4 I 20 mM at 4 °C. After dialysis, collagen I was diluted in phosphate buffer pH 7.4 I 20 mM to obtain 0.5 mg mL^{-1} as final collagen I concentration and then immediately submitted to the coating procedure.

Solution for collagen I–decorin coating. Stock solution of collagen I (1 mg mL^{-1} in acetic acid 0.1 M) was dialysed against phosphate buffer pH 7.4 I 20 mM containing 135 mM NaCl, for 3 h at 4 °C. After dialysis, collagen I was mixed with decorin and diluted in 20 mM phosphate buffer at pH 7.4 in the presence of 135 mM NaCl to obtain 0.5 mg mL^{-1} as final collagen I concentration and 0.025 mg mL^{-1} as final decorin concentration. Then, the solution was employed immediately for the coating of the substrate.

Solution for collagen III coating. Stock solution of collagen III (1 mg mL^{-1} in acetic acid 0.1 M) was dialysed against HEPES buffer pH 7.4 I 20 mM containing 135 mM NaCl, overnight at 4 °C. After dialysis, collagen III was diluted in HEPES buffer pH 7.4 I 20 mM in the presence of 135 mM NaCl to obtain 0.3 mg mL^{-1} as final collagen III concentration being then ready for the coating.

Solution for collagen III–decorin coating. Stock solution of collagen III (1 mg mL^{-1} in acetic acid 0.1 M) was dialysed against HEPES buffer pH 7.4 I 20 mM containing 135 mM NaCl, overnight at 4 °C. After dialysis, collagen III was mixed with decorin and diluted in 20 mM HEPES buffer at pH 7.4 in the presence of 135 mM NaCl to obtain 0.3 mg mL^{-1} as the final collagen III concentration and 0.015 mg mL^{-1} as the final decorin concentration. Then, the solution was immediately submitted to the coating procedure.

Preparation of the coating substrates for both AFM and QCM studies

Fused-silica capillary (Optronis GmbH, Kehl, Germany), muscovite mica (YA-Kemia, Sigma-Aldrich Finland Oy, Helsinki, Finland) and silicon dioxide sensor chip (Attana AB, Stockholm, Sweden) were used as coating surfaces. The polyimide layer of the outside wall of the fused-silica was first burned off and cleaned with methanol. To remove all impurities, all types of surface were pretreated for 15 min with 1 M HCl, 15 min with MilliQ water, 15 min with 1 M HCl, and 15 min with MilliQ water. After pretreatment, the substrates were exposed to the coating solutions for 160 min, and then treated for 30 min with BGE solution at pH 7.4 (phosphate buffer in the case of collagen I and collagen I–decorin, and HEPES in the case of collagen III and collagen III–decorin coatings). All coatings were performed *ex situ* at 37 °C. Coated fused-silica and mica substrates were stored in appropriate BGE at pH 7.4 until submission to AFM

measurement. The AFM measurements were conducted in various and random fragments of coated substrate to verify the presence of the coating and to select the representative sample for the further studies. Each selected coated substrate was then measured with AFM at least two times.

The collagen-coated silicon dioxide sensor chips were stabilized overnight at 25 °C under continuous flow (10 μL min^{-1}) of phosphate or HEPES buffer in the Attana A100 QCM biosensor (Attana AB, Stockholm, Sweden). Equilibration was complete when the baseline drift yielded <5 Hz min^{-1}.

Instrumentation

The AFM experiments were performed with Veeco Instruments Multimode V with Nanoscope V controller. Intermittent-contact (tapping) mode in air was employed at ambient temperature and humidity ($T = 21 \pm 1$ °C; RH = 25%) using Veeco RTESP rectangular phosphorus-doped Si cantilevers ($k = 3$ N m^{-1}), with a nominal resonance frequency of 75 kHz and a nominal tip radius of 10 nm. Measurements were carried out in different parts of the samples with a scanning frequency of 0.5 Hz. All images were captured from a scanning area of 1 μm × 1 μm. SPIP image processing software (Image Metrology, Denmark) was used to determine the height profile for each image. The FWHM and standard deviations of the height profiles were obtained from fitting algorithms in the software.

The QCM experiments were performed with an Attana A100 QCM biosensor (Attana AB, Stockholm, Sweden). All studies were carried out under continuous flow (25 μL min^{-1}) of running buffer (phosphate or HEPES buffer at pH 7.4) at 25 °C.

To clarify the characteristics of interaction between immobilized collagen films and apoB-100, increasing concentrations of apoB-100 peptide fragment were injected for 84 s over the various collagen films previously described and dissociation was recorded for 216 s prior to surface regeneration. All experiments were performed two times. The equilibrium dissociation constant (K_D) was then determined by Scatchard plot analysis of the QCM data (Attana Evaluation software).

A MeterLab PHM220 pH meter (Radiometer, Copenhagen, Denmark) and a Jenway 3030 pH meter (Jenway, Felsted, UK) were used to adjust the pH of the electrolyte solutions. Distilled water was further purified with a Millipore water purification system (Millipore, Molsheim, France).

Conclusions

Atomic force microscopy was used to elucidate the structure and molecular properties of collagen I and III immobilized on fused-silica capillary and mica surfaces. The effect of another ECM component, decorin, which promotes the binding of low-density lipoprotein particles with collagen, was clarified. Our results showed the addition of decorin to affect the size and shape of collagen type I and III fibrils. Decorin not only effected the reduction of fibril diameter but caused collagen films to become more even and uniform. Furthermore, our studies indicated the type of substrate affected the collagen adsorption pattern. In addition, quartz crystal microbalance was employed to specify the interaction between the same collagens immobilized on a silicon dioxide biosensor chip and positive apoB-100 peptide (3359–3377). The estimation of dissociation constants confirmed that bindings occur *via* the positively charged residues lysine and arginine and negative charges of collagen I and collagen III surfaces. Moreover, the achieved results followed the order of the interaction strength that could be expected.

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