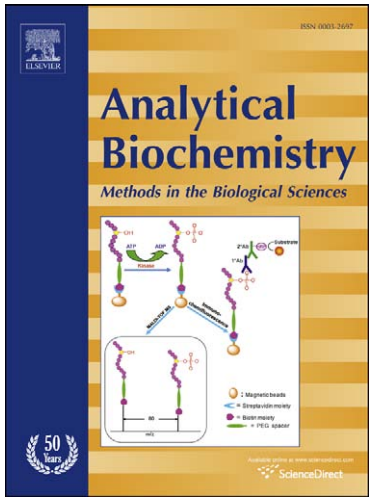

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Capillary electrochromatography and quartz crystal microbalance, valuable techniques in the study of heparin - lipoprotein interactions

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Abstract

Atherosclerosis is initiated when lipoproteins bind to proteoglycans (PGs) in arterial wall. The binding is mediated by apolipoprotein apoB-100 and/or apoE, both of which have binding affinity toward heparin. We developed covalently bound heparin coatings for APTES-modified silica capillary and SiO_2 chips and carried out capillary electrochromatography (CEC) and quartz crystal microbalance (QCM) studies on the interactions of heparin with selected peptide fragments of apoB-100 and apoE and, for CEC, also with low and high density lipoproteins (LDL and HDL), the latter with and without apoE. The peptides are known to mediate interactions of HDL and LDL with arterial PGs. Interactions and affinities were expressed in CEC as retention factors and reduced mobilities and in continuous flow QCM technique as affinity constants. Both techniques showed heparin interactions to be stronger with apoB-100 peptide than with apoE peptide fragment, and they confirmed that the sulfate groups in heparin play an especially important role in interactions with apoB-100 peptide fragment. In addition, CEC confirmed the importance of sulfate groups of heparin in interactions between heparin and LDL and between heparin and apoE-containing HDL. CEC and QCM acted as excellent platforms to mimic these biologically important interactions, with small sample and reagent consumption.

Keywords: Open tubular capillary electrochromatography; Quartz crystal microbalance; Heparin; Low-density lipoproteins; High-density lipoproteins; Interactions

1. Introduction

The specific interactions between human plasma lipoproteins and proteoglycans (PGs), which are structural components of the extracellular matrix (ECM) of the arterial wall, play an important role in the development of atherosclerosis [1-5]. PGs contain glycosaminoglycans (GAGs), which are unbranched carbohydrate polymers composed of repeating disaccharide units and which are covalently attached to a protein core (by a glycosidic linkage). The GAGs bear negative charges due to sulfate and carboxylic groups. The most common GAGs are heparin, chondroitin sulfate, dermatan sulfate, heparan sulfate, and hyaluronic acid.

Lipoproteins are nanometer-sized particles (between 11 and 80 nm), which carry lipids in circulation. High concentrations of low-density lipoprotein (LDL) particles are considered as a main cause of human atherosclerosis. Indeed, there is strong experimental evidence that LDL particles may initiate the development of atherosclerotic lesions [4]. In this scenario, LDL enters the subendothelial space of the arterial wall, where it is retained, via ionic interactions, when the positively charged lysine and arginine residues of apolipoprotein (apo) B-100 interact with the negatively charged sulfate and carboxyl groups of the glycosaminoglycan (GAG) chains of the proteoglycans. High density lipoprotein (HDL) particles, containing apoA lipoproteins, are considered antiatherogenic since they do not interact with GAGs and, accordingly, are not retained by arterial wall proteoglycans. However, a fraction of HDL particles contain apoE, which, like apoB-100, has domains of positively charged residues that have been shown to mediate the binding of HDL to GAGs [6].

In this study, we analyzed the heparin binding of LDL and of HDL (containing or not containing apoE), and of two positively charged peptides corresponding to relevant sequences of apoB-100 and apoE. The first peptide was a 19 amino acid peptide of apoB-100 (3359-3377) with net charge of +6, and the second a 15 amino acid peptide of apolipoprotein E (136-150) with net charge of +7. Because both peptides are rich in

lysine and arginine residues, they can selectively interact with the GAG chains of the proteoglycans. ApoE has proved to be a strong competitor of apoB-100 for interaction with arterial PGs [7]. The residues from 3359 to 3369 in apoB-100 have been shown to be the major proteoglycan-binding site in LDL particles [8], and the residues from 136 to 150 in apoE have been identified by Weisgraber et al. [9] as the heparin-binding site of this protein. The helical wheel corresponding to the sequence leu-arg-lys-leu-arg-lys-arg-leu-leu-arg (residues 141-150) of apoE is similar in charge distribution to that of residues 3359-3369 of apoB-100 [9].

Many techniques, including quantitative frontal elution affinity chromatography [7], homogeneous enzyme-based binding assay [10], and surface plasmon resonance [11], have been applied for the interaction studies between GAGs and lipoproteins. Capillary electrochromatography (CEC) not only offers high resolution separations but also great flexibility and the potential for elucidation of the interactions of nanodomains of biological importance [12,13].

The quartz crystal microbalance (QCM) is a mass-sensing device belonging to the family of acoustic biosensors, whose signal transduction mechanism depends on the piezoelectric effect of the quartz crystal. The QCM has been successfully applied to the study of a number of biochemical processes, and also to the development of biomimetic biosensors and systems [14-16]. Molecules of interest (ligands) are directly immobilized on the gold surface, which can be modified for interaction studies with specific analytes. The main advantages of the QCM are low mass sensitivity and the possibility to follow the kinetics of the binding process.

Our recent demonstration [16] that QCM can be successfully applied for the elucidation of interactions between chondroitin-6-sulfate (C6S) and apolipoprotein fragments, where C6S is covalently attached to carboxyl surface with amine coupling,

persuaded us to employ QCM with a SiO_2 chip as a complementary technique in heparin interaction studies carried out by open tubular CEC. In CEC, heparin was both directly immobilized and covalently bound onto a capillary inner wall modified with 3-aminopropyltriethoxysilane (APTES). The stability of the coating was estimated by measuring the electroosmotic flow (EOF) mobilities. The APTES–heparin covalent coating procedure was also applied to SiO_2 chip surfaces for QCM studies. The interactions of heparin with the selected peptide fragments of apoB-100 and apoE, and with apoB-100- and apoE-containing human plasma lipoproteins, were examined by CEC in terms of reduced mobilities and retention factors and by QCM in terms of affinity constants.

2. Materials and methods

2.1. Materials

Heparin (H4784), 3-aminopropyltriethoxysilane (APTES), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, E 1769) were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO), sodium hydroxide (1.0 M), and hydrochloric acid (1.0 M) were from Oy FF-Chemical Ab (Yli Ii, Finland), phosphoric and acetic acids were purchased from Merck, and NaCl was from JT Baker. Sodium acetate was obtained from Fluka (Buchs, Switzerland) and acetone was from Mallinckrodt Baker (Deventer, The Netherlands). Peptide fragments of apoB and apoE were synthesized at the Meilahti Protein Chemistry Facility and analyzed at the Protein Chemistry Core Facility, both at Biomedicum, University of Helsinki, Finland.

2.2. Instrumentation

A Hewlett-Packard ^{3D}CE system (Agilent, Waldbronn, Germany) equipped with a diode array detector (detection at 200, 214 and 254 nm) and an air-cooling device for the capillary cassette was used with a data acquisition rate of 100 Hz and detector

response time of 0.1 s. Bare fused silica capillaries were from Composite Metal Services Ltd. (Worcestershire, U.K.). Dimensions were 50 µm i.d. and 375 µm o.d. The effective length of the capillary was 30.0 cm with a total length of 38.5 cm. A Lauda Ecoline Re-104 water bath (Lauda-Königshofen, Germany) was used to control the temperature of the autosampler. QCM experiments were carried out with an Attana A100 instrument QCM biosensor (Attana AB, Stockholm, Sweden). A Jenway 3030 pH meter (Jenway, Felsted, UK) and a MeterLab PHM 220 pH meter (Radiometer, Copenhagen, Denmark) were used in the pH measurements. Distilled water was further purified with a Millipore water purification system (Millipore S.A., Molsheim, France). Millipore filters (Bedford, MA, USA) were used for filtering background electrolytes (BGEs).

2.3. Methods

2.3.1. Isolation of LDL particles from human plasma

Human LDL (d = 1.019-1.050 g/ml) was isolated from the plasma of healthy volunteers (Finnish Red Cross) by sequential ultracentrifugation in the presence of 3 mM ethylenediaminetetraacetic acid (EDTA) [17]. Briefly, solid KBr was added to plasma to adjust the density to 1.019 g/ml. The floated VLDL and IDL fractions were removed, and the density of the supernatant solution was adjusted to 1.050 g/ml with solid KBr. After ultracentrifugation for 72 h at 35,000 rpm in a Beckman Coulter type 50.2 Ti rotor (148,000g_{max}), LDL was recovered from the top of the centrifuge tubes, recentrifuged (d=1.060 g/ml) for 24 h at 35,000 rpm in a Beckman Coulter-type 80 Ti rotor (115,000g_{max}), and dialyzed extensively against LDL buffer (1 mM EDTA and 150 mM sodium chloride in water, pH 7.4, adjusted with sodium hydroxide). The amount of LDL is expressed in terms of its protein concentration, which was determined by the method of Lowry *et al.* [18] with bovine serum albumin (BSA) as standard.

2.3.2. Isolation of spherical HDL_2 particles from human plasma

Human HDL_2 (d = 1.063–1.125 g/ml) particles were isolated by sequential density ultracentrifugation [19]. Solid KBr was used to adjust the densities in each centrifugation step. The HDL subclasses were washed by reflotation at their upper density (1.125 g/ml for HDL_2). All of the centrifugation steps were carried out at 50,000 rpm in a Beckman Ti 50.2 rotor (302,000 g_{max}) for 40 h at +5 . HDL and its subclasses were dialyzed against sodium phosphate buffer (20 mM phosphate-buffered saline, pH 7.4) containing 140 mM NaCl to remove KBr. The dialyzed HDL subclasses were stored at +4˚ C. Protein and lipid compositions of the isolated HDL particles (%, w/w) were determined by standard methods [20].

2.3.3. Purification of HDL with and without apoE

HDL with and without apoE was purified by heparin-affinity chromatography by a modification of the reported method [21]. Briefly, HiTrap Heparin-Sepharose column (1 mL, Amersham Biosciences AB, Uppsala, Sweden) was equilibrated with 5 mM Tris-HCl / 50 mM NaCl, pH 7.4, with a flow rate of 0.5 mL/min. Just before application to the column, solid $MnCl_2$ was added to the HDL solution to give a final Mn^{2+} concentration of 25 mM, and the column was equilibrated with 5 mM Tris-HCl / 50 mM NaCl, pH 7.4, containing 25 mM $MnCl_2$. Next, HDL (1-2 mg as protein, to which $MnCl_2$ had been added) was applied to the column, and the column was washed with 5 mM Tris-HCL / 50 mM NaCl / 25 mM $MnCl_2$, pH 7.4, until the A_{280} dropped to the baseline. The apoE-free HDL particles not bound to heparin could then be collected. The heparin-bound apoE-containing HDL was detached from heparin and eluted with 5 mM Tris-HCl / 100 mM NaCl, pH 7.4, and collected in 1 mL fractions. The non-bound fractions (representing apoE-free HDL) and the bound fractions (representing apoE-containing HDL) were pooled, dialyzed separately against PBS, and stored at +4^{o}C until use. ApoE was analyzed in isolated HDL pools by immunoblotting and ELISA.

2.3.4. Sample and buffer preparation

Phosphate buffers (I=20 mM) at pH 7.4, 6.5 and 5.5; phosphate buffers (I=20 mM) at pH 7.4 and 5.5 containing 25 mM NaCl, and acetate buffers (I=20 mM) at pH 4.0, 4.5 and 5.0 were prepared, and the pH was adjusted with 1.0 M NaOH, 1.0 M HCl or 100% acetic acid, respectively. Before use, the buffers were filtered through 0.45-µm Millipore filters using a Millipore vacuum system. The stock solutions of DMSO (1% v/v) and EDC (4 mg/mL) were prepared in Milli-Q water. The solutions of heparin (0.1 – 20 mg/mL) used for the non-covalent coating of the capillary were diluted in water, and the heparin solution (3 mg/mL) containing EDC (0.125 mg/mL) that was used for the covalent coating was prepared in phosphate buffer (I=20 mM), pH 7.4. The heparin-EDC mixture was prepared 16 h before use. APTES solution (4%, v/v) was prepared in acetone. The stock solutions of peptide fragments (1 and 2 mg/mL) were prepared by dissolving their powders in MilliQ water. Before injection, the peptide stock solutions were diluted in the phosphate buffer (I=20 mM) at pH 7.4 for CEC and in phosphate buffer (I=20 mM) pH 7.4 containing 25 mM NaCl for QCM to give final concentrations of 0.25-0.5 mg/mL for CEC and 8-50 mg/mL for QCM. The regeneration solution for QCM (0.4 M NaCl) was prepared in Milli-Q water. The DMSO and peptide solutions were stored at + 4 ^{o}C and – 20 ^{o}C, respectively.

2.3.5. Coating procedures

2.3.5.1. Treatment of fused silica capillaries

The capillary was flushed with 1 M HCl for 10 min and water for 5 min, and then with 1 M NaOH for 15 min and water for 5 min. The capillary was equilibrated with BGE for 10 min. Between runs, the capillary was rinsed with 0.1 M NaOH, water and BGE for 2 min each.

2.3.5.2. Coating of capillary with polyacrylamide

The procedure was modified from the method described by Cifuentes *et al.* [22]. The first step was rinsing the capillary with 5 M HCl for 30 min, water for 30 min, 5 M NaOH for 30 min. and water for 30 min. The capillary was purged with N_2 gas at 265 ˚C during 2 h and then rinsed for 1 h with a solution containing 5 µL of acetic acid and 5 µL of 3-(trimethoxysilyl)propyl methacrylate in 1 mL of methanol. Afterwards, the capillary was washed with methanol during 30 min. In a final step the capillary was rinsed with a solution of polyacrylamide containing N,N,N',N'-tetra methylethylendiamine (TEMED) and ammonium persulfate (APS), and immediately afterwards it was filled with the acrylamide+APS+TEMED solution. The capillary was sealed at both ends and left to polymerize over night. After polymerization was compleated the capillary was washed with water and buffer.

2.3.5.3. Coating of capillary and SiO_2 chip with APTES

The coating with APTES was optimized for fused silica capillaries, and the optimized coating procedure was then applied to the SiO_2 chip surfaces used in QCM measurements.

Before coating, the fresh capillary was pretreated-treated in the following steps: 15 min capillary flush with 1 M NaOH, 15 min flush with deionized water, and 15 min flush with acetone at pressure of 940 mbar. After preconditioning, the APTES coating was applied to the capillary inner surface as follows: the capillary was flushed with 4% (v/v) APTES solution in acetone at 50 mbar for 30 min and left to stand filled with the silanization solution for 16 h. Then the capillary was flushed with acetone for 10 min, with water for 5 min, and with phosphate buffer at pH 5.5 for 60 min to eliminate the unbound APTES.

SiO_2-coated chips were purchased from Attana AB (Stockholm, Sweden). Coating of the chip with APTES was done *ex situ* at 37 °C, with the temperature controlled with

a water bath. Coating was performed in a small vial. APTES coating of the SiO_2 surface was starting with preconditioning. The chip was rinsed with 1 M NaOH for 20 min, twice with Milli-Q water for 15+15 min, and with acetone for 15 min. After preconditioning, the chip was left to react with 4% (v/v) APTES solution for 16 h. Postconditioning consisted of rinsing with acetone for 20 min, with Milli-Q water for 15 min, and finally with phosphate buffer (I=20 mM), pH 5.5, and 25 mM NaCl for 15 min.

2.3.5.4. Direct immobilization of heparin onto APTES-modified capillary surface

After preconditioning and coating of the capillary with APTES, the amino-silanized capillaries were treated with heparin. Briefly, the capillaries were flushed with heparin solutions at 50 mbar for 40 min, left to stand filled with the coating solutions for 15-60 min, and flushed with phosphate buffer at pH 7.4 for 15 min.

2.3.5.5. Covalent binding of heparin onto APTES-modified capillary surface and SiO_2 chip

APTES–heparin covalent coating was done by a modification of the method described by Stege *et al.* [23]. After APTES coating of the capillary, a solution of heparin (3 mg/mL) dissolved in 2 ml phosphate buffer, pH 7.4 (I: 20 mM), containing 0.25 mg of EDC was passed through the capillary for 1 h. The APTES-coated SiO_2 chip was left to react with the same solution for 2 h. Finally, the capillary and the chip were rinsed with phosphate buffer at pH 7.4 to remove the unbound heparin.

2.3.6. Electroosmotic flow (EOF) measurements

EOF marker DMSO (0.05%) was used as an indicator of changes in surface charge during the coating procedure. The electroosmotic flow (EOF) mobilities were employed for estimation of the stability of the coatings. Owing to the very low EOF in polyacrylamide-coated capillaries, the EOF and electrophoretic mobilities of selected

peptide fragments were measured by the method of Williams and Vigh [24]. Before each run, the capillary was flushed with 20 mM BGE at pH 7.4 for 2 min. The running conditions were the following: sample injection by pressure at 50 mbar, injection time 2 s, voltage +20 kV, and temperature 37 ºC.

2.3.7. Quartz crystal microbalance analysis

All the QCM experiments were conducted with an Attana A100 QCM biosensor (Attana AB, Stockholm, Sweden). The chip consisted of a 0.4-mm thick crystal sandwiched between two gold-plated electrodes. An acoustic wave was generated in the crystal by applying an oscillating potential through the electrodes. The c-fast program was used for sample injection, and frequency shifts due to binding of the analyte to the surface were recorded with Attester®. The sample was injected by loading the injection loop and then switching the valve so that the sample was transported to the sensor surface. After the injection the valve was switched back and the running buffer was transported to the surface.

The APTES–heparin-coated chip was inserted to the instrument and left to stabilize in phosphate buffer (I=20 mM), pH 7.4 + 25 mM NaCl, flow rate 25 µl/min. Constant flow was generated with a piston pump that transported the buffer to the surface. The same phosphate buffer was used as running buffer in all experiments. Peptide fragments of apoB-100 and apoE were used as analytes and the interactions were studied at 25 and 37 °C. The flow rate and injection volume in all experiments were 25 µl/min and 35 µl. The chip was regenerated after every analyte injection with 0.4 M NaCl, injection volume 30 µl.

3. Results and discussion

Glycosaminoglycans play important biological roles through their interactions with other macromolecules. They specifically interact with proteins such as apoE and apoB-100, lipoprotein lipase (LPL), and ECM proteins to modulate their functions

[25]. About fifty years ago, it was suggested that the binding of circulating LDL with GAGs in the inner wall of arteries is a key process in the development of atherosclerosis [26,27]. ApoE, a protein component of a small fraction of HDL, acts as a ligand for the LDL receptor. Further, by interacting with the heparan sulfate on the cell surface, apoE, like apoB-100, plays an important role in regulating the lipoprotein metabolism and in transporting and redistributing lipids among cells and tissues [28,29]. Physiologically, apoE is also a crucial ligand in the clearing of chylomicron remnants from the circulation via interaction of apoE with the hepatic LRP-1 receptor. The binding sites of apoB-100 and apoE with GAGs have been identified and characterized [7]. In both, an abundance of positively charged amino acid residues of lysine and arginine are present in the domains responsible for the binding. The present study will shed light on the interactions of heparin with two carefully selected peptide fragments of apoB-100 and apoE, human low-density and high-density lipoproteins, and full-length apoE.

3.1. Development of APTES–heparin coating procedures for fused silica capillaries and SiO_2 chips

In previous studies, we have developed various protocols for the immobilization of human lipoproteins [30-32] and collagens [13] on the inner surface of fused silica capillaries via positively charged amino acid residues and for the immobilization of chondroitin-6-sulfate and collagen on carboxyl surface via amine coupling [15,16]. The first ones were done to allow interaction studies by capillary electromigration techniques and the second ones to allow studies with a quartz crystal microbalance. In the present investigation, heparin was covalently and non-covalently coated on fused silica capillaries and covalently coated on SiO_2 chip surfaces. We have noted in previous studies that the immobilization technique may greatly affect the interaction strength depending on which functional groups are wasted during the immobilization step. Accordingly, in the present study the effects of covalent and non-covalent

coating on the interactions were clarified by CEC. Different functionalities are lost in the covalent and non-covalent coating procedures, allowing more detailed information to be obtained about the role of these functionalities in heparin and peptide interactions. Because heparin, silica capillary and SiO_2 chip surfaces were negatively charged, the capillary and chip surfaces were first modified with APTES, which through covalent and ionic bonding provided an excellent, positively charged platform for the binding of heparin. Coating of the capillary with APTES reversed the direction of EOF and resulted in a stable surface, as confirmed by a repeatable EOF mobility (RSD 3.05%) (n=6).

3.1.1. APTES–heparin non-covalent coating for CEC

The non-covalent immobilization of heparin on APTES-coated capillary was based on ionic interactions between the positively charged amino groups of APTES and negatively charged sulfate and/or carboxylic groups of heparin. We studied the effects on the coating of the incubation time employed in the heparin coating, the concentration of the heparin, and the pH of the BGE solution. The stability of the coating was evaluated by measuring the EOF mobility (n=6).

The stability of the coating improved when the incubation time was increased from 15 min (RSD of EOF 32.4%, n=6) to 60 min (RSD of EOF 4.5% , n=6). The stability was not improved with more than 60 min incubation time, and 60 min incubation time was selected for the further studies.

The EOF mobility was increased with heparin concentrations from 0.1 to 3.0 mg/mL. Increase in the concentration from 3.0 to 20.0 mg/mL did not improve the EOF mobility or stability of the coating further, and since the RSD values of the EOF mobilities were lowest with 3.0 mg/mL, this was selected for further experiments.

The effect of pH on the stability of the coating was studied from pH 4.5 to 7.4 by using phosphate buffer in pH range 6.0-7.4 and acetate buffer in pH range 4.5-5.5. The coatings were stable over the whole range. Physiological pH 7.4 was selected for the further studies since this is the most relevant value for interactions between lipoproteins and glycosaminoglycans during initiation of atherosclerosis.

The stability of the coating, which is important not only for practical reasons but also to minimize the amount of heparin required, was tested by measuring the EOF mobilities at pH 7.4 and 25 and 37 °C (n=6) on ten successive days. At the end of each day, the capillary was rinsed with phosphate buffer (I=20 mM) at pH 7.4 and left filled with the same BGE overnight. The EOF did not change significantly over the ten-day period, confirming that the coating was stable at 25 and 37 °C for at least ten days. The reliability of the measurements was determined by evaluating the reproducibility of different, individually coated capillaries. The results demonstrated that the capillary-to-capillary reproducibility was good at both 25 and 37 °C.

3.1.2. APTES–heparin covalent coating for CEC capillary and SiO_2 chip

APTES–heparin covalent coating was used to coat both the inner surface of the capillary and the SiO_2 surface of the QCM chip. In covalent coating, heparin was attached to APTES with EDC linker, which reacts with the carboxyl groups in heparin and free amine groups on the APTES surface. A stable amide bond is formed, and the sulfate groups in heparin are free to interact with analytes.

The stability and the coating-to-coating reproducibility of the APTES–heparin covalent coating were evaluated by measuring the mobility of the EOF during successive runs. The coating was stable at 37 ˚C for at least ten days, and the RSD of 0.6% to 1.2% of the EOF mobilities (n=6) in three different capillaries demonstrated that the capillary-to-capillary reproducibility was good (Table 1). The higher EOF

mobilities at 37 °C in capillaries with covalently bound heparin demonstrated the availability, for interaction, of a greater amount of negatively charged groups on the heparin surface than on the capillary with electrostatically attached heparin.

The covalent ligand coating, which minimizes heparin losses during the regeneration step, was chosen for the QCM experiments. In addition, as noted in the CEC studies, fewer negatively charged groups important for interactions were lost when heparin was covalently coated onto the chip surface. The APTES–heparin covalent coating was stable for one week, after which the binding capacity of the surface weakened rapidly and the repeatability of the frequency shifts decreased.

3.2. Affinity of heparin with peptide fragments expressed as retention factors and affinity constants

The 19 amino acid peptide of apoB-100 and the 15 amino acid peptide of apoE were selected as analytes for the interaction studies because they are present in the sequences involved in the PG interactions of LDL and HDL, respectively. These peptides highlight the crucial role of the positively charged amino acids arginine and lysine in the interactions. Retention factors and affinity constants (K_a) were calculated to describe the interactions between the peptide fragments used as analytes and heparin immobilized on the surface of the capillary or SiO_2 chip.

The retention factor *k* can be used to describe the interactions between the analytes and the coatings in capillary electrochromatography:

$$k = \frac{t_m - t_{eo}}{t_{eo}} \quad (1)$$

where migration times of the analyte (t_m) and an unretained component (t_{eo}) describe the ratio of the time the peptide stays in the stationary phase to the time it is in the mobile phase. Equation 1 is suitable for calculating the retention factor of uncharged compounds. For charged analytes, which bind reversibly to the open tubular stationary phase, Rathore and Horvath [33] have introduced k′ as a measure of retention. Equation 2 can be used for both uncharged and charged analytes:

$$k' = \frac{t_m(1+k'_e)-t_{eo}}{t_{eo}} \quad (2)$$

where the velocity factor k'_e is u_{ep}/u_{eo} (u_{ep} is the electrophoretic mobility of the analyte in the uncoated capillary and u_{eo} is the EOF in the coated capillary). Wiedmer et al. [34] transformed Eq. 2 into the more convenient form (3)

$$k'' = t_m\left(\frac{1}{t_{eo}} + \frac{1}{t_{m'}} - \frac{1}{t_{eo'}}\right) - 1 \quad (3)$$

where $t_{m'}$ and $t_{eo'}$ are migration times of the analyte and the EOF in the uncoated capillary, respectively. We used Equation 3 for measurement of the retention factors for peptide fragments. Because the retention factors obtained for the peptide fragments in uncoated capillaries by CEC were negative, we employed polyacrylamide-coated capillary for the determination of the migration times of peptide fragments (t_m′) and EOF (t_{eo}′). From Figure 1 it can be seen that sulfate groups, all still present in the covalently bonded heparin coating, play an important role especially in the heparin interaction with the 19 amino acid peptide.

QCM technique has been successfully applied for the elucidation of various biological interactions, especially interactions between antibodies and antigens. In the present

study, we employed QCM as a complementary technique to clarify the affinity between heparin and the 19 amino acid peptide of apoB-100 and the 15 amino acid peptide of apoE. Investigations were done at 25 °C and 37 °C. The frequency shifts recorded are presented in Figure 2. Scatchard plots were linear, and the Scatchard plot analysis exploited for calculation of the affinity constants (K_a) showed the repeatability of the technique to decrease at physiological temperature 37 °C (see Figure 3 and the 19 amino acid peptide). The slope of the plot of $\Delta f/C$ *vs* Δf gives the K_a value for the system, where Δf is the frequency shift and C is the concentration of the injected analyte. The concentration of the analytes ranged from 13 mg/mL to 50 mg/mL for the 15 amino acid peptide of apoE and from 8 mg/mL to 40 mg/mL for the 19 amino acid peptide of apoB-100. Each concentration point was run three times and RSD values were calculated (Table 2). The interaction between the positive peptides and heparin was described as log K_a values (Table 2). Surprisingly, the interaction of the 19 amino acid peptide of Apo B with charge of +6 was stronger than that of the 15 amino acid peptide of ApoE with charge of +7. It could be that, if heparin does not totally cover the APTES–coated silica chip surface, positive charges of APTES also take part in the interaction.

The retention factors obtained for peptide fragments by CEC showed the interactions for the 15 amino acid peptide of apoE with heparin to be at the same level in the APTES–heparin non-covalently coated and the APTES–heparin covalently coated capillaries. In contrast, the interactions of the 19 amino acid peptide of apoB-100 with heparin in the APTES–heparin covalently coated capillary were about twice as strong as those in the APTES–heparin non-covalently coated capillary. The log K_a values of 5.22 and 5.02 at physiological temperature 37 °C and 5.54 and 5.20 at 25 °C obtained by QCM for the 19 amino acid peptide of apoB-100 and the 15 amino acid peptide of apoE, respectively, showed the interaction of the 19 amino acid peptide with heparin to be slightly stronger than that of the 15 amino acid peptide.

In the case of the APTES–heparin covalent coating, carboxylic groups were covalently bound to amino groups of APTES, while sulfate groups were free on the surface available for interaction. On the APTES–heparin non-covalent coating, in turn, both carboxylic and sulfate group took part in the electrostatic interaction between heparin and APTES, resulting in fewer sulfate and more carboxylic groups on the heparin surface. The stronger affinity of 19 amino acid peptide of apoB-100 to covalently attached heparin suggests that sulfate groups play a more important role than carboxylic groups. The weaker affinity of the 15 amino acid peptide of apoE towards heparin was seemingly not affected by the number of carboxylic and sulfate groups. Our results for the 15 amino acid peptide of apoE, with log K_a 5.20 at 25 ^{o}C and 5.02 at 37 ^{o}C, are in good agreement with those obtained by Futamura et al. [35], who report log K_a-value of 5.59 for the 22 kDa peptide fragment of apoE3 allele, 5.80 for the 10 kDa peptide fragment of apoE and 7.66 for apoE. Our log K_a values were slightly higher, probably due to differences in the heparin immobilization technique and the shorter peptide we used. When Futamura et al. [35] used biotinylated heparin, no carboxylic groups were lost. Moreover, our previous studies, carried out by partial filling technique in affinity capillary electrophoresis, showed that when both negative sulfate groups and carboxyl groups of chodroitin-6-sulfate are available in the interaction, both peptides interact fairly strongly with C6S, and the shorter 15 amino acid peptide slightly more strongly[16].

3.3. Heparin interactions with human plasma lipoproteins and apoE studied by CEC

The suitability of capillary electrochromatography for interaction studies of heparin with biomacromolecules was investigated with capillaries coated with heparin covalently bound on APTES-modified surfaces. In addition, because we wanted to evaluate the importance of apoE in HDL interactions, HDL with and without apoE

and pure full-length apoE were investigated as well. Physiological conditions were mimicked by carrying out the experiments at pH 7.4 and 37˚C. The reduced mobilities that Kenndler [36] introduced to express the effect of the EOF on all separation relevant parameters for CE were used to clarify the relative interactions. The reduced mobility μ_i is given by

$$\mu_i = \frac{\mu_{eff,i}}{\mu_{eff,i} + \mu_{eo}} \quad (4)$$

where μ_{eff} is the effective electrophoretic mobility of the analyte and μ_{eo} is the EOF in the coated capillary.

Figure 4 demonstrates that all the biomolecules, except for HDL without apoE, interact more strongly with heparin-coated capillaries than with uncoated capillaries. Since the affinity towards heparin decreased in the order apoE-containing HDL > apoE > HDL without apoE, it is clear that apoE plays an important role in the interactions between HDL and heparin. This is in agreement with previous studies [6]. Multiple binding sites in HDL, or the presence of a more favourable lipid-induced conformation of apoE on HDL, may be responsible for the stronger heparin interactions of apoE when it is located on the HDL particle surface than when it is free in solution [7].

Sulfate groups played a more important role than carboxylic groups in the interaction of heparin with LDL, as was also the case in the interaction of heparin with 19 amino acid peptide of apoB-100 described above. This was evident in the reduced mobility of LDL: the mobility of LDL in capillaries with APTES–heparin non-covalent coating, where most of the carboxylic and sulfate groups were lost through electrostatic interactions with APTES, was about one half that in capillaries with APTES–heparin covalent coating, where sulfate groups were on the surface, freely available for interaction.

4. Conclusions

Heparin coatings were applied to fused silica capillary modified with APTES. Both APTES–heparin non-covalent and APTES–heparin covalent coatings were successfully used for interaction studies between heparin and selected peptide fragments of apolipoproteins, human plasma lipoproteins, and apoE. The protocol for the coating where heparin was covalently attached to the APTES-modified silica capillary was also employed in immobilization of a SiO_2 chip surface used in QCM studies. The differences between the covalent and non-covalent coatings revealed the important role of the sulfate groups in the interactions between heparin and peptide fragments. The interactions and affinity were expressed in CEC as retention factors and reduced mobilities and in QCM studies as affinity constants. The methods developed allowed highly efficient and easy characterization of the interactions under different conditions. The results were in a good agreement with earlier findings in regard to the importance of apoE in the interactions of HDL with proteoglycans. The CEC method developed was not only simple but it enabled studies with very low sample and reagent consumption. The QCM technique acted as a reliable complementary tool for CEC, with drawbacks, however, of relatively low heparin surface stability on expensive SiO_2 chips.

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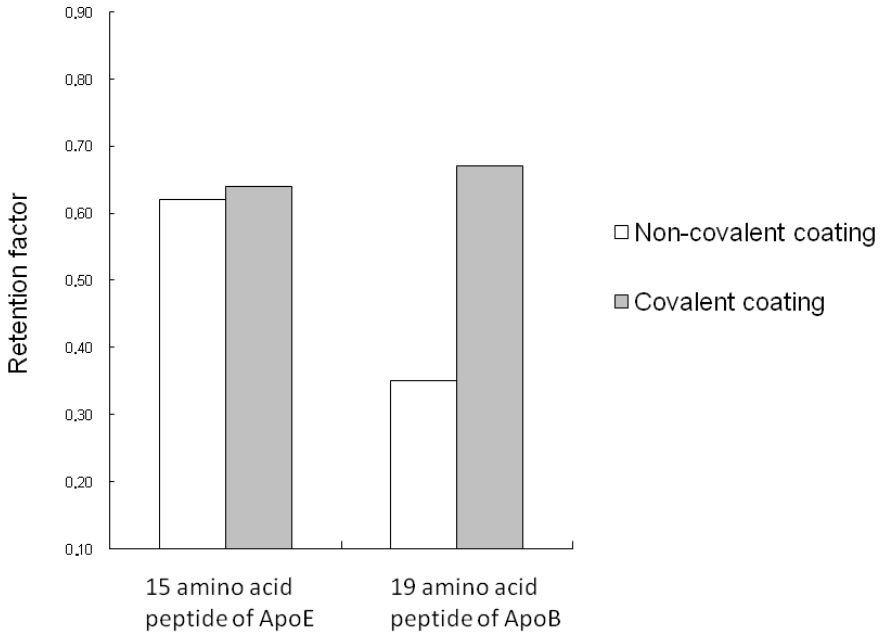
Figure Legends

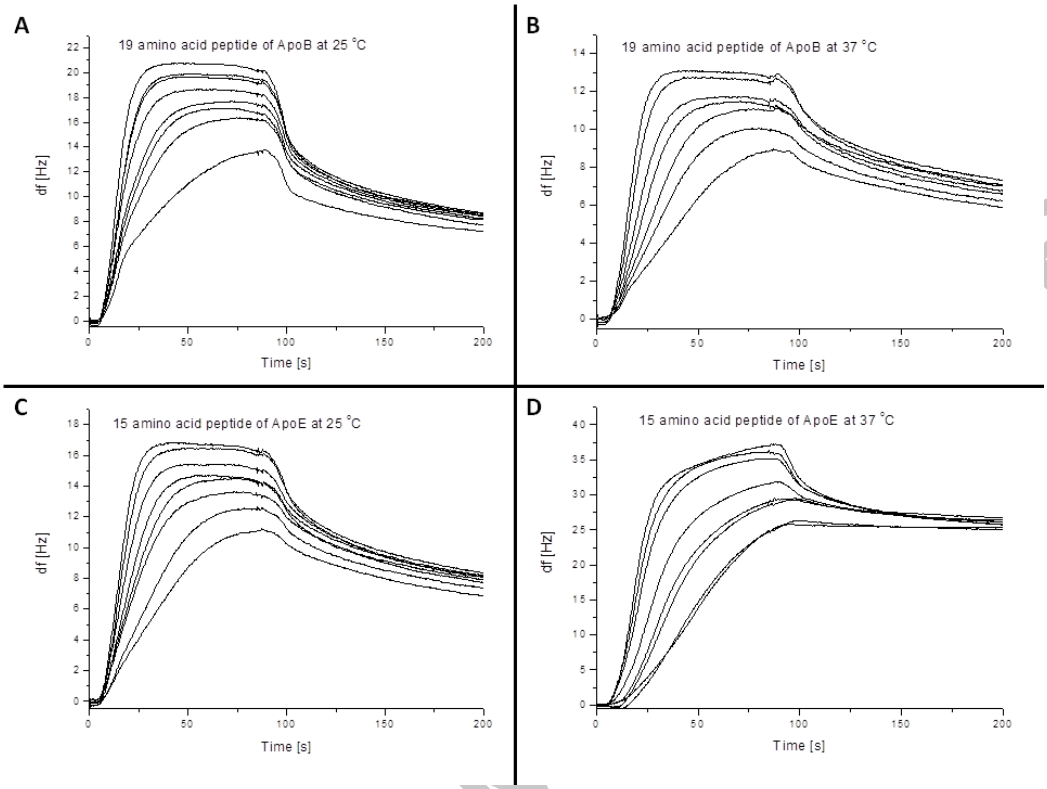
Figure 1. Retention factors of peptide fragments. ▪ : APTES–heparin covalently coated capillary; ⌴ : APTES–heparin non-covalently coated capillary.

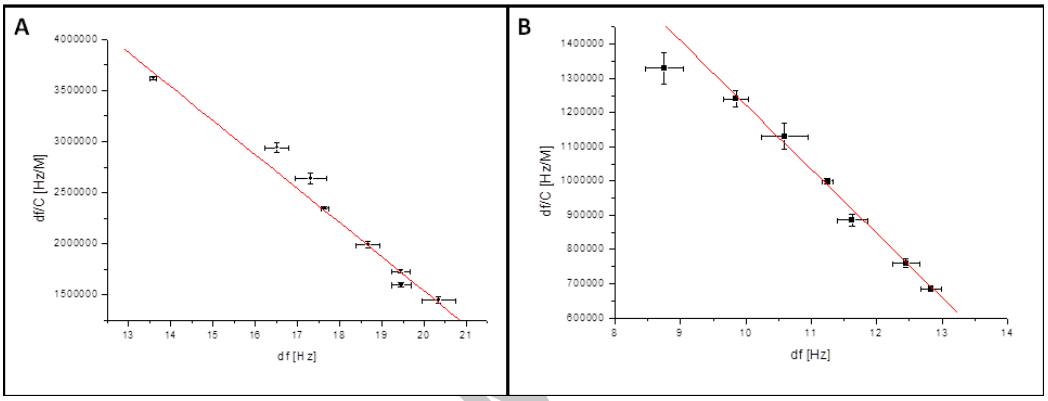
Figure 2. Frequency shifts recorded with Attester© for apoB-100 A) at 25 °C and B) at 37 °C and for 15 amino acid peptide of apoE C) at 25 °C and D) at 37 °C.

Figure 3. Scatchard plots for 19 amino acid peptide of apoB-100 A) at 25 °C and B) at 37 °C.

Figure 4. Reduced mobilities of macromolecules. ■: uncoated capillary; ▪ : APTES–heparin covalently coated capillary; ⌴ : APTES–heparin non-covalently coated capillary.







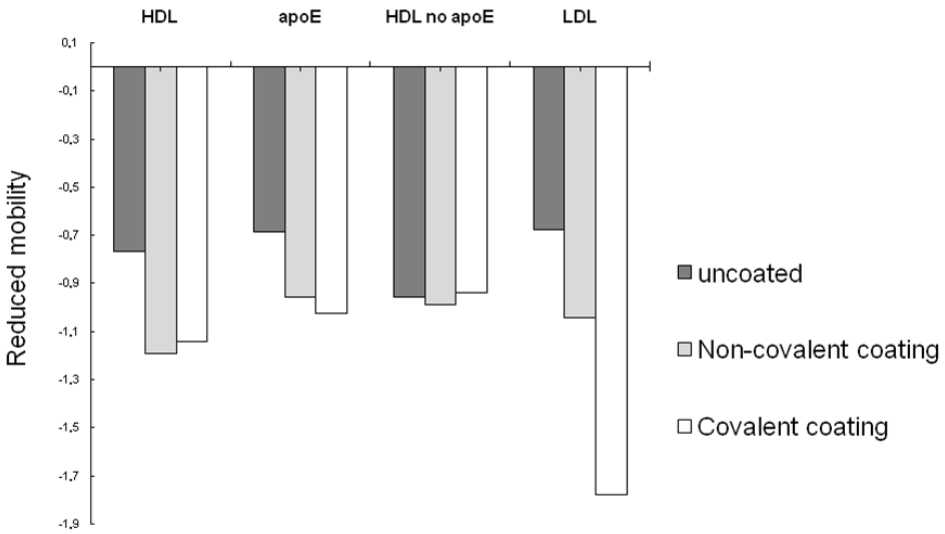


Table 1. Coating to coating reproducibility (n=3) measured at 25˚C and 37˚C for three different capillaries with APTES-heparin non-covalent coatings, and at 37˚C with APTES-heparin covalent coating. RSD values (n= 6) are given in parentheses.

	APTES-heparin non-covalent coating 25˚C	APTES-heparin non-covalent coating 37˚C	APTES-heparin covalent coating 37˚C
EOF mobility[10^{-4}cm^2/Vs]			
Capillary 1	5.58 (0.4%)	5.88 (4.5%)	6.16 (1.2%)
Capillary 2	5.18 (3.9%)	5.95 (2.2%)	6.18 (0.6%)
Capillary 3	5.40 (3.3%)	5.82 (2.4%)	6.22 (1.2%)

Table 2. Affinity constants expressed as log K_a obtained by QCM technique for 19 amino acid peptide of apoB and 15 amino acid peptide of apoE at 25 °C and 37 °C. RSD values between different concentration points are in parenthesis.

Temperature [°C]	ApoB 19 aa	ApoE 15 aa
25	5.54	5.20
	[0.48-2.17%]	[0.71-3.41%]
37	5.22	5.02
	[0.31-1.42%]	[6.52-13.09%]