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Affinity membranes for hormone removal from aqueous solutions

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Abstract

A novel affinity membrane was prepared by covalent binding of antibodies (against 17-β-estradiol) to a micro-porous poly(ethylene vinyl alcohol) (EVAL) membrane, taking benefit from the high surface area of EVAL membranes and the large number of reactive groups available for further surface modification. The covalent coupling of the antibody occurred via its non-specific chain to maximize the number of available binding sites for hormones. To achieve this site-oriented coupling first poly(ethylene glycol) bis-hydrazide (PEG-Hz) was reacted with glutaraldehyde-modified EVAL membrane, followed by coupling of oxidized antibody to the PEG-Hz spacer arm. Confocal microscopy demonstrated that most of the antibodies were grafted on the outer surface, rather than inside the membrane. In dynamic filtration, 17-β-estradiol was retained for 99%. The specificity of the membrane was demonstrated by its capability to distinguish 17-β-estradiol from the nearly identical compound estrone.

Keywords: Affinity membrane; Antibody immobilization; Hormone removal

1. Introduction

Separation or removal of diluted biological compounds from aqueous solutions may be achieved by affinity binding separation materials. A frequently used method, separation chromatography, is sometimes inconvenient because of the required large pressure drop that hampers the treatment of large feed volumes [1]. Affinity membranes are a good alternative to separation chromatography, because in these membranes the adsorbent (the ligand) is covalently attached to the membrane surface along the flow path of the soluble target (the ligate). This minimizes the diffusional and accessibility problems associated with the gel beds used in chromatography.

The interaction between a ligand and ligate is expected to have high specificity when it is based on molecular recognition. Since one of the most specific molecular recognition processes is the antibody–ligate reaction, we focused on the possibility of using antibodies as affinity binders. The purpose of our study was to prepare and characterize a novel affinity membrane that combines the advantages of a hydrophilic polymeric membrane with the affinity properties of antibodies. We have investigated the immobilization of antibodies onto the surface of poly(ethylene vinyl alcohol) (EVAL) membrane. To prove the principle, we chose, as model compounds, an antibody against 17-β-estradiol, an estrogenic hormone and we studied the application of the functionalised membranes as affinity binders for estrogenic hormones. The choice of the antibody against 17-β-estradiol was based on (a) possible applications in pharmaceutical industry or drinking water production (removal of 17-ethynylestradiol) or (b) as an analytical tool for quantification of estrogenic compounds in wastewater streams. At this moment, chemical analysis for quantification of estrogenic compounds in the complex mixtures employs a large number of separation steps. Affinity separation techniques could, in principle, simplify the analytical procedures for hormone quantification. EVAL membranes have already been used for affinity protein separation [2,3] and bio-molecule binding [4,5] but, to our knowledge, have not yet been employed for

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site-oriented antibody immobilization. EVAL micro-porous membranes have the advantage of combining high water fluxes with high surface areas and a large number of reactive groups available for further surface modifications, allowing antibody immobilization.

Analogous to the preparation of affinity stationary phases for packed columns, affinity membranes are generally obtained via three steps: (1) preparation of the basic membranes, (2) surface activation of the base membrane, and (3) coupling of affinity ligands to the activated membranes [6].

The base membrane should fulfill a number of conditions: (1) proper pore structures and mechanical strength for use at high flow rates and low back pressure in rapid processing [7], (2) availability of reactive groups such as –OH, –NH, –SH, –COOH for the further coupling of spacer arms or ligands, (3) chemical and physical stability under harsh conditions, and (4) hydrophilic surface in order to avoid any unspecific interaction between bio-molecules and the membrane surface [4].

Activation of the basic membrane comprises several chemical reactions to obtain reactive groups for further coupling of the ligand. The methods used for various affinity columns can be directly applied to membrane activation [6,8]. Immobilization of ligands, and we refer here more specifically to antibodies, onto surfaces is well documented [9–11]. The two major requirements for the immobilization of ligands onto insoluble matrices for affinity separation are a stable linkage between the matrix and the ligand, and maintaining of specific binding characteristics of the immobilized ligand. The last requirement implies avoiding chemical modifications that may lead to major changes of the ligand conformation. Moreover, in the case of antibodies, site-oriented coupling of the antibody is preferred since this results in optimal availability of the ligate-binding sites.

Antibodies have a typical immunoglobulin structure with two ligate-binding sites (Fab fragments) and a non-specific chain (Fc fragment). Amino groups present on the outside of the antibody molecule could, in principle, be used to covalently bind antibodies to supports. However, this leads to random coupling of the immunoglobulin molecules with eventually only very limited availability of the ligate-binding sites. In contrast, site-oriented coupling of antibodies is possible by using the oligosaccharide moieties present on the Fc fragment (sialic acid residues) for immobilization. Oxidation of these residues with meta-periodate results in formation of aldehyde groups [12], which can subsequently be used for binding the immunoglobulin molecules through their Fc fragments to amino group-containing surfaces.

Biomolecules such as antibodies are known to retain their functionality when attached to a surface via a hydrophilic spacer arm [13]. The extra length of the spacer arm provides less sterical hindrance to conjugation and offers more active complexes. A spacer molecule may also provide greater mobility, allowing the immobilized antibody to orient into the correct position for optimal binding of the target molecule. For various surfaces, polyethylene glycol has proven to be a suitable spacer molecule for immobilizing antibodies [13].

In this study, the site-oriented anti-17-β-estradiol antibody immobilization on EVAL membrane using a hydrophilic spacer molecule was examined. In addition, the affinity membrane was characterized in static adsorption and dynamic filtration conditions for 17-β-estradiol removal from aqueous solutions.

2. Experimental

2.1. Materials and methods

All chemicals were used as supplied unless stated otherwise. EVAL-copolymer (Aldrich) with an average of 44 mol% ethylene groups was used for membrane preparation without any further modification. EVAL membranes were prepared by an immersion precipitation technique using dimethyl sulfoxide (DMSO) as solvent and 1-octanol (Aldrich) as additive [14]. EVAL membranes were used as a support material for binding of anti-17-β-estradiol antibodies (MP Biomedicals). The surface modification of the membranes followed the reaction scheme shown in Fig. 1.

Static adsorption experiments and adsorption isotherms were carried out using 17-β-estradiol (E2, Sigma) as a model compound. A second hormone, estrone (E1, MP Biomedicals), with a very similar molecular structure was tested separately as well as in a mixture with E2. From both hormones stock solutions of 53.2 mg/L in methanol were prepared and stored in a refrigerator at 4 °C. All glassware was washed with a strongly alkaline (NaOH) detergent and subsequently rinsed extensively with de-mineralised and distilled water. E2 and E1 adsorption were quantified using an ELISA kits (MP Biochemicals and Japan EnviroChemicals Ltd.), respectively.

2.2. Membrane preparation (EVAL)

Fourteen percent (w/w) polymer was dissolved in DMSO containing 14% (w/w) 1-octanol at 50 °C. After de-aeration, the polymeric solution was cast onto a glass plate with a doctor blade knife at room temperature and then immersed in a hot (40–45 °C) water bath. The membranes were subsequently washed with water to remove the 1-octanol.

2.3. Membrane characterization

Membrane morphology was imaged with a Jeol JSM-5600LV scanning electron microscope. The cross-section of the membranes was obtained by freeze fracturing the sample under liquid nitrogen. The specimen was platinum coated with a Jeol JFC-1300 Auto fine coater.

BET measurements to determine internal surface area were carried out with an ASAP-2400 nitrogen adsorption apparatus (Micromeritics). A sample tube was filled with a known amount of EVAL membrane and degassed over-night at 150 °C. Higher temperatures are not recommended because of the physical characteristics (T_g and melting point) of the EVAL copolymer.

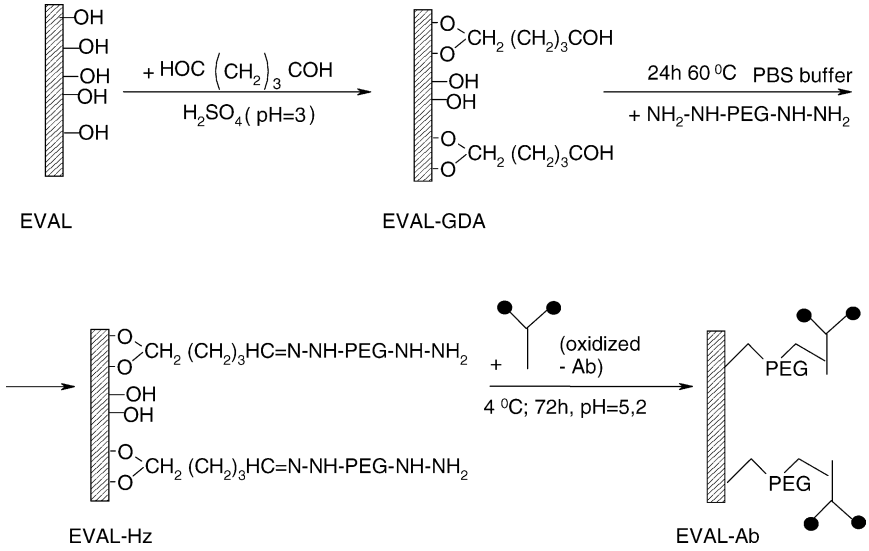


Fig. 1. Reaction scheme for EVAL monoclonal antibody membrane-preparation.

The *pure water flux* of unmodified and modified membranes was determined at room temperature using a dead-end ultrafiltration cell connected to a gas cylinder of compressed nitrogen to achieve transmembrane pressures ranging from (0.1 to 0.5) $\times 10^5$ Pa (0.1 to 0.5 bar). The pure water flux was determined after steady-state conditions were reached.

Pore size distribution of the initial membrane was determined using a Coulter^R Porometer II, which records the pressure needed to expel liquid from a membrane impregnated with Porofil (Aldrich).

Porosity of the membrane was determined by immersing a membrane at room temperature for 24 h in a 50 mL vial containing distilled water. The porosity $P = [(V_s - V_0)/V_s] \times 100\%$, is calculated from the difference of the volume occupied by the polymer (equal to the volume of dry polymer V_0) and the volume of the membrane equilibrated in water V_s. The volume of the polymer can be calculated as ratio between the dry membrane weight and the polymer density.

Swelling degree (*Swelling ratio* (%) SD): The membrane was immersed for 24 h in a 50 mL vial containing distilled water. The swollen membrane was removed from the water, padded using a filter paper. The volume of wet and dry membranes were measured and used to calculate the swelling degree.

X-Ray photoelectron spectroscopy (*XPS*): The XPS spectra were obtained with a Physical Electronics Quantera Scanning X-ray Microprobe. A monochromatic Al K^{α} X-ray beam with a diameter of 100 μm was scanned over an area of 700 μm × 300 μm. Low-energy electrons and low-energy ions were used for charge neutralization of the non-conducting samples. The binding energy (BE) scales for the spectra were referenced by setting the CHx peak maxima in the C 1s spectra to 284.8 eV. Survey scans (0–1100 eV) were performed for detection of elements on the surface, detailed spectra (C 1s, O 1s, N 1s, F 1s) were analysed and quantified using the software package MultiPak (Physical Electronics). The data were obtained at a takeoff angle of 45° corresponding to an analysis depth of about 5 nm.

2.4. Glutaraldehyde derivatization of the surface (EVAL-GDA)

The modification of membranes with glutaraldehyde (10% supplied by Electron Microscopy Science) was done following [17]. EVAL membranes cut in circular pieces with a diameter of 2.5 cm were immersed in a 1% glutaradehyde solution at pH 3 for approximately 24 h at room temperature and then subsequently thoroughly washed with distilled water and PBS 0.15 M phosphate buffered saline solution. The dried membranes were characterized by FTIR for the presence of the absorption peak at 1720 cm^{-1} attributed to the aldehyde groups.

2.5. Surface modification with PEG bis-hydrazide (EVAL-Hz)

Glutaraldehyde modified membranes were reacted with an excess of poly(ethylene glycol) bis-hydrazide (PEG-Hz) (M_w = 3400, Necktar) 20 mg/mL PBS buffer solution for 24 h at 60 °C [13]. After carefully rinsing with distilled water, the quantity of hydrazine groups was determined qualitatively by XPS and quantitatively using *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, Pierce) reaction as described elsewhere [15].

2.6. Quantitative determination of available hydrazide groups

Seventy five milligrams dried circular membranes (EVAL-Hz) 2.5 cm in diameter, was soaked in 1.5 mL dry ethanol prepared according to standard purification methods [16]

and reacted at room temperature with 0.5 mL SPDP solution (12 mg/mL) and 3 mg 4-methylaminopyridine as catalyst. After 30 min, the membranes were removed from the reaction mixture and subsequently washed with ethanol, demi-water, 1 M NaCl, 1 M $NaHCO_3$ and finally soaked in 4 mL 0.1 M $NaHCO_3$ for 30 min. Next, the membranes were immersed in 4 mL of 50 mM 1,4-dimercapto-2,3-butanediol (DTT) for about 15 min at room temperature to release pyridine-2-thione. The liquid was then diluted 4 times with 0.1 M $NaHCO_3$ followed by absorbance measurements at 343 nm against diluted 50 mM DTT as blank. The molar absorption coefficient for the thione is 8080 M^{-1} cm^{-1}. From the pyridine-2-thione concentrations, the quantity of SPDP on the surface was calculated giving the number of hydrazide groups.

2.7. Reaction of the hydrazide groups from the spacer molecule with fluorescein isothiocyanate (EVAL-Hz-FITC)

Three 2.5 cm circular EVAL-Hz membranes were soaked in carbonate buffer pH 9.5 to which an excess of fluorescein isothiocyanate (FITC, Aldrich) in DMSO was added drop wise, at room temperature and left to react with the NH_2 groups for 2 h. The membranes were washed extensively with distilled water to remove all un-reacted FITC from the membrane. The hydrazide groups distribution onto the membrane surface was determined by confocal microscopy (Zeiss LSM510). All the emission measurements were done at the same conditions: pinhole 104 μm; filter 500–550; stack size *x* 921; *y* 921; scan zoom1; wave length 10%.

2.8. Reaction with ethanolamine

Free aldehyde groups on the membrane surface, which had not been reacted with PEG bis-hydrazide were blocked by reaction with a 0.2 M ethanolamine solution at room temperature for 30 min. Afterwards the membranes were rinsed with distilled water, and PBS for storage purposes. Before using them in immobilization reactions, the membranes were washed several times with distilled water and acetate buffer (see below).

2.9. Oxidation of the antibody

To a solution of 18 mg antibody in 1.5 mL 0.15 M acetate buffer pH 5.2, 1.5 mL of 50 mM $NaIO_4$ was added. The mixture was stirred at room temperature for 1 h. The mixture was dialyzed against acetate buffer at 4 °C for 48 h. The final concentration of oxidized antibody was about 2 mg/mL acetate buffer.

2.10. Immobilization of the antibody (EVAL-Ab)

The 18 mg-oxidized antibody was immobilized onto the membrane in pH 5.2 acetate buffer solution at 4 °C. After 72 h, the excess of oxidized antibody was removed; the membranes were rinsed with PBS buffer. The membranes were stored for several weeks at 4 °C. The concentration of the antibody can be estimated using an extinction of 1.4 of the antibody solution at 280 nm for 1 mg/mL in a cuvet of 1 cm. The quantity of immobilized antibody was determined by the difference in concentration of the antibody in solution before and after reaction.

2.11. Hormone binding activity of the oxidized antibody

2.2 mg antibody and oxidized antibody in solution were reacted with 12.5 mL 1 μg/L 17-β-estradiol salt solution. After 24 h, the un-reacted 17-β-estradiol was separated from the solution by centrifugation at 50 Hz (3000 rpm) for 2 h using Microsep centrifugal tubes 10 K (Pall Life Science). The concentration of un-reacted 17-β-estradiol from the supernatant was quantitatively determined by ELISA method.

2.12. Fluorescent labeling of the antibody for confocal microscopy

Five milligrams per millilitres antibody in carbonate buffer pH 9.5 was dialyzed against the same buffer for about 2 h at room temperature to remove any low molecular weight impurities. To the dialyzed solution was added drop wise, at room temperature under gentle mixing, a FITC solution in DMSO, in a molar ratio FITC/antibody = 6 and subsequently let to react for 2 h. The labeled antibody was purified by dialysis against acetate buffer (pH 5.2). The FITC labeled antibody was oxidized, purified by dialysis and immobilized onto the membrane according to the procedure mentioned before. Two different starting concentrations 0.1 and 1 mg/mL, respectively, of labeled antibody were used to determine the antibody distribution inside and onto the surface. The presence of the antibody was investigated by confocal microscopy as described in Section 2.7.

2.13. Adsorption isotherms

A known weight of membranes (EVAL; EVAL-GDA; EVAL-Hz; EVAL-Ab) was contacted for 24 h at constant temperature (25 °C) with different concentrations of 17-β-estradiol dissolved in a salt solution with a composition similar to that of urine: 22.59 g/L, NH_4HCO_3; 0.68 g/L, Na_2HPO_4; 0.14 g/L, $CaCl_2$; 0.84 g/L, K_2SO_4; 4.68 g/L, NaCl; 2.24 g/L, KCl. The E2 concentration at equilibrium was determined using ELISA. The amount of solute adsorbed per unit membrane mass at the equilibrium state follows from mass balance:

$$E_2 = \frac{V_{s0}(C_0 - C_e)}{W_0} \times 10^{-6} \quad (1)$$

where E_{2ads} is the quantity of 17-β-estradiol adsorbed onto membrane (mg/mg), W_0 the weight of the dry membrane placed into the solution (mg), C_0 the initial concentration of

17-β-estradiol (ng/L), C_e the equilibrium concentration of 17-β-estradiol (ng/L) and V_{s0} is the volume of solution used for the experiments (L).

2.14. Selectivity determination of the EVAL-Ab

A known quantity of membranes was contacted for 24 h at constant temperature (25 °C) with a solution of 1.5 μg/L E2 to which various quantities of E1 stock solution in methanol were added to reach E1 concentrations of 1.5, 2, 2.5, 3, and 3.5 μg/L, respectively, dissolved in a salt mixture similar as in urine (see Section 2.12). The E1 and E2 concentrations at equilibrium were determined using ELISA. The selectivity of the membrane was calculated according to:

$$\alpha_{E2/E1} = \frac{[E2]_{ads}/[E1]_{ads}}{[E2]_0/[E1]_0} \quad (2)$$

where $[E1]_{ads}$ is the quantity of adsorbed estrone (mg ads/mg membrane), $[E2]_{ads}$ the quantity of adsorbed of 17-β-estradiol (mg ads/mg membrane), $[E1]_0$ the initial concentration of estrone [M] and $[E2]_0$ is the initial concentration of 17-β-estradiol [M].

2.15. Static measurements of kinetic binding of 17-β-estradiol to the immobilized antibody

The measurements were performed in glass bottles in which a fixed volume (100 mL) of 1 μg/L 17-β-estradiol solution in different buffer solutions (acetate, pH 5.2; phosphate, pH 7; phosphate, pH 8) or salt solutions (see Section 2.12) was stirred gently with 10 modified membranes (2.5 cm diameter) for 24 h. In time, fixed sample volumes were taken out.

2.16. Dynamic adsorption filtration performance

A stack of nine membranes was placed in a dead-end filtration cell and the dynamic adsorption capacity was evaluated at constant flow-rate by determining the E2 concentration in fractionated fixed volumes of permeate. The flow rate was 120×10^{-5} L/(h Pa m^2) (120 L/(h bar m^2)) membrane frontal area.

2.17. Membrane regeneration

The used membranes were soaked in 3 mL methanol for the removal of bound 17-β-estradiol. After 20 min, the membranes were washed with demi-water and re-used in static or dynamic adsorption experiments [18].

3. Results and discussion

3.1. Membrane preparation and characterization

EVAL membranes appear to be suitable support material for our purposes because of the hydrophilicity of the polymer, the presence of active groups that can be modified by (a sequence of) chemical reactions; stability of the material during these chemical modification, low non-specific adsorption and large internal surface area. In addition, extensive information is available about the preparation of EVAL micro-porous membranes [14].

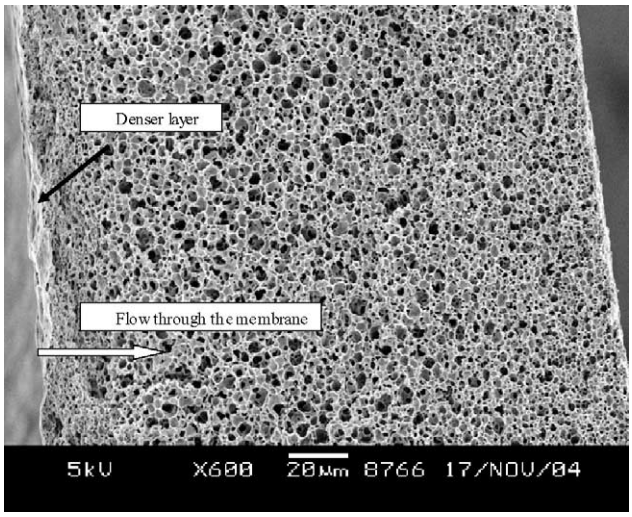


Fig. 2. SEM picture of EVAL membrane cross-section.

Membrane characterization: SEM micrographs reveal an asymmetric membrane structures with a skin layer and a porous support having a pore size gradient (Fig. 2). Chemical modification of the membranes does not change the morphology of the membrane. The *internal surface area* of the EVAL membrane is 9.7 m^2/g as measured by BET (minimum and maximum pore size are 17 Å and 3 μm, respectively). The prepared membranes have pore sizes in the range of 0.179–0.3 μm, an average porosity of 80% and a swelling degree of 8%. *Pure water permeability* of unmodified membranes is in the range $(1650–1750) \times 10^{-5}$ L/(h Pa m^2) (1650–1750 L/(h bar m^2)). The presence of antibody substantially reduces pure water fluxes to about 670×10^{-5} L/(h Pa m^2) (670 L/(h bar m^2)), which is probably due to the narrowing of pores by the presence of polyethylene glycol chains and antibodies and/or by blocking the access of water to the pores because of a high density of antibodies onto the surface of the membrane. The later hypothesis was confirmed also by confocal microscopy (see below).

Introduction of the spacer molecule with hydrazide end groups has the advantage that the terminal amine of the hydrazide group has a pK of 2.6 [6]. Therefore, this group differs significantly from the primary aliphatic amine, for which p$K \cong$ 9–10, depending on its environment. Primary amino groups are also present in the antibody molecule. At pH 5.2, the oxidized antibody is preferably bound to the hydrazide end groups thus avoiding intermolecular antibody reactions.

XPS measurements on unmodified and modified membranes surface reveal a slight difference in atomic concentrations between the glass and airside of the membrane (see

Table 1
Atomic % concentration table

	C (%)	O (%)	N (%)	O/C (%)	N/C (%)
EVAL theoretical	78.1	21.9	–	0.28	
EVAL[a]	79 ± 0.8	20.9 ± 0.1	–	0.26 ± 0.01	
EVAL[b]	76.2 ± 0.2	23.8 ± 0.1		0.31 ± 0.01	
EVAL-Hz theoretical	66.95	31.45	1.6	46.9	0.024
EVAL-Hz[a]	72.9 ± 1.7	25.5 ± 1.6	1.6 ± 0.3	0.4 ± 0.01	0.02 ± 0.005
EVAL-Hz[b]	74.0 ± 1.1	23.6 ± 0.5	2.4 ± 0.6	0.32 ± 0.01	0.03 ± 0.008

[a] Smooth side, glass side.
[b] Rough side, air side.

Table 1). This difference may be attributed to a hydrophilic gradient along the cross-section of the membrane or the irregularities on the surface. Many investigations on various enzyme immobilizations show a similar hydrofilicity difference between membrane surfaces [19]. Ethylene vinyl alcohol copolymer contains a number of hydrophobic parts –(CH_2–CH_2)– and also hydrophilic parts –(CH_2–CHOH)–. During the coagulation step in water, the hydrophilic part will orient towards the waterside possibly leading to a degree of hydroxyl group orientation into the surface of the pores. It can be expected that the slight asymmetry of the hydroxyl group distribution can be related to the asymmetry in membrane's morphology. XPS measurements (see Table 1) performed on the two membrane sides reveal a modest increase of the number of OH groups on the surface from O/C = 0.26 for the glass side, to 0.31 for the waterside. A similar difference is observed for *N* content (0.02 ± 0.005 for glass side to 0.03 ± 0.008 for the water side).

It can be observed from Table 1 that the *N* concentration of the smooth side is almost identical with the theoretical value clearly indicating that every available OH group has reacted. The higher *N* value indicated by XPS for the rough surface agrees with the higher O values determined for the same side of the membrane.

Hydrazide quantification with SPDP yields a surface amino group concentration of $1.9 \pm 0.2 \times 10^{-10}$ mol/cm^2 based on the internal surface area from BET measurements. The surface concentration corresponds to an average distance of about 9.6 Å between the attachment points. In our calculations, we use BET measurements performed on the dry membranes. Considering the swelling degree, the coverage distance between the attachment points becomes approximately 12 Å. This value is close to the radius of gyration of the polyethylene glycol molecule $R_g \cong N^{3/5}$ where *N* is equivalent to the number of polymer segments for the dense brush regime (R_g value for PEG $M_w = 3400$ calculated according to the assumption that water is a good solvent for PEG bis-hydrazide) [20]. Our results imply that the EVAL membrane is fully covered with a PEG brush distributed uniformly inside the membrane as determined qualitatively by confocal microscopy (see Fig. 3A).

The antibody up-take calculated from the experimental UV–vis data is about 4.67×10^{-12} mol/cm^2. The antibody volumetric capture is about 0.4% from the antibody up-take and therefore is negligible. The quantity of immobilized antibody is significantly less than the number of active sites available for antibody coupling, which is presumably due to the fact that antibodies have a relatively large dimension about 15 nm [10]. The coupling reaction between the surface and antibody depends on the antibody concentration. The antibody is preferentially attached to the surface of the membrane where the first available reaction sites are present. Modification of membranes with two different starting concentrations of antibody revealed that the highest coverage of the membrane surface is to be found on the surface itself and less in the cross-section (Fig. 3B and C), despite of the rather uniform distribution of spacer molecules. The antibody is reacting with the first available hydrazide groups on the membrane surface. When comparing the emission densities from the two sides of the membrane (81.034 mg Ab-FITC/g membrane; Fig. 3C) we can clearly distinguish a difference between the skin layer and the micro-porous porous side of the membrane: close to the porous side of the membrane the intensity inside the membrane is higher. This suggests that the pore size plays a role in the antibody distribution inside the membrane. Activity measurements show that oxidation of soluble antibody reduces the activity of the antibody with about 25%. Moreover, the density of the antibody on the surface will greatly influence the activity of the antibody for the ligate. If a large amount of antibody is immobilized, neighbouring antibodies may obstruct each other and restrict access of the ligate to potential binding sites deeper in the pores of the support [21]. Furthermore, the structure of the antibody may be distorted by attachment on the surface of the support to such extend that its affinity for ligate is lost or diminished. To achieve a more homogeneous distribution of antibody immobilization, one may want to permeate the antibody solution through the membrane instead of simple incubation.

3.2. 17-β-Estradiol adsorption measurements

To develop a suitable separation process in which the E2 concentration is reduced to acceptable levels, it is necessary to have some insight in the interaction between the 17-β-estradiol and the immobilized antibody. Furthermore, it should also be established that indeed the antibody is the responsible ligand for the removal of 17-β-estradiol from aqueous solutions. Therefore, we measured adsorption isotherms

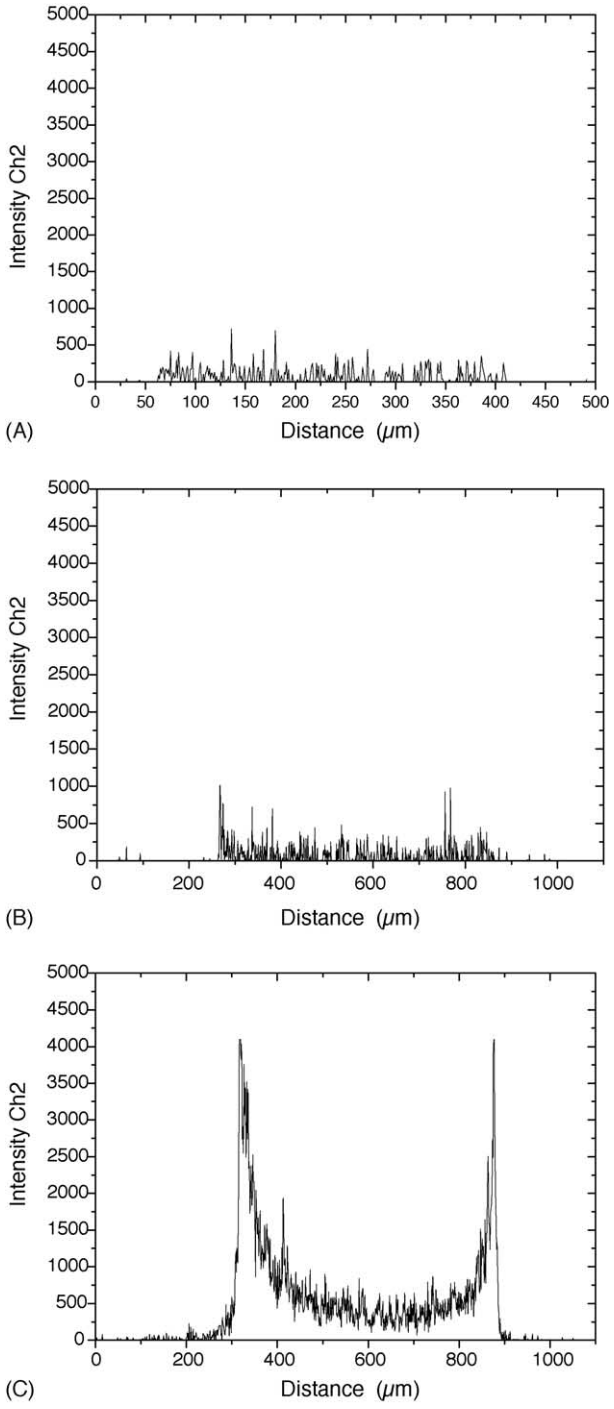


Fig. 3. Intensity profiles for EVAL-HZ (A) and membranes with two different surface concentrations of antibody (B) EVAL-Ab-FITC1 and (C) EVAL Ab-FITC2.

for the various types of membranes involved in the surface modification. As it can be seen from Fig. 4, unspecific adsorption of 17-β-estradiol onto the EVAL, EVAL-GDA and EVAL-Hz only takes place in low extent (less then 10% of total binding). The non-specific binding can be attributed to some hydrogen bonding interaction between the OH groups present in E2 and the groups available on the membrane

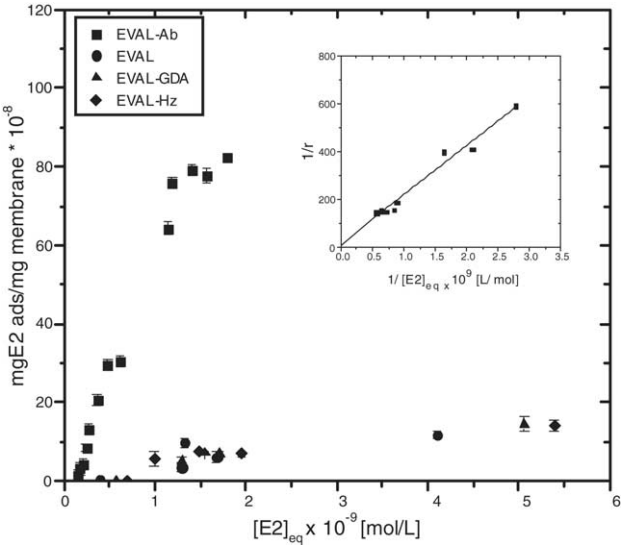


Fig. 4. Adsorption isotherm of 17-β-estradiol from synthetic urine. The insert shows a comparison to the Scatchard Eq. (4) only for higher estradiol concentrations the data seem to obey the linear behaviour for Eq. (4).

surface. Comparing membranes with and without antibody (Fig. 4), it can be concluded that the antibody strongly enhances the adsorption of E2 from solution by a factor of 10 for concentrations $[E2] \geq 10^{-9}$ mol/L, and even several orders of magnitude for lower E2 concentrations.

In literature it is assumed that the binding interaction is similar to that in true solutions and can therefore be described by a reversible equilibrium [21], characterized by an equilibrium constant K. The antibody–hormone interaction may be a multivalent binding and the adsorption cannot always be described by the Langmuir isotherm. The assumption of the Langmuir model is that the adsorption onto the surface is homogeneous. Furthermore, occupation of a binding site by an adsorbed molecule is assumed not to affect adsorption of newly adsorbed molecules onto other sites [22,23]. For an immobilized macromolecule or biological molecule (antibody) with n binding sites and a heterogonous surface, the adsorption isotherm is better analysed by the Scatchard plot [21,24]. To interpret the adsorption isotherm from Fig. 4, we first briefly recapitulate the Scatchard equation and its assumptions, applied to the equilibrium between E2 molecules bound to an antibody and free in solutions:

$$E_{2,\text{free}} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_{2,\text{ads}}; \quad K = \frac{k_1}{k_{-1}} \tag{3}$$

Consider a total surface concentration C of antibodies on a membrane, each having (on average) n binding sites, σ is the total surface concentration of sites occupied by E2 molecules. The rate at which E2 adsorbs onto the membrane is assumed to be proportional to the free E2 concentration and the number of free binding sites:

$$\frac{\mathrm{d}[E_2]_{\text{free}}}{\mathrm{d}t} = k_1[E_{2,\text{free}}](Cn - \sigma), \tag{4}$$

On the other hand, E2 may desorb from the membrane, at a rate that is proportional to the number of occupied sites:

$$\frac{d[E_2]_{free}}{dt} = k_{-1}\sigma, \quad (5)$$

When equilibrium is reached, the net change in time of the free E2 concentration is zero. Hence, from Eqs. (4) and (5) we find the equilibrium constant:

$$K = \frac{\sigma}{[E_2](Cn - \sigma)} = \frac{r}{(n - r)[E_2]}, \quad (6)$$

in which $r = \sigma/C$ is the ratio of the surface concentration of bound E2 to the surface concentration of antibody molecules placed in the system; and [E2] is the equilibrium concentration of free E2. Eq. (6), also known as Scatchard equation, is equivalent to:

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nK}\frac{1}{[E_2]}, \quad (7)$$

predicting that a plot of r^{-1} versus $[E_2]^{-1}$ provides the average valence n, as well as the equilibrium constant K, in case the Scatchard plot holds. Fig. 4, however, allows a linear fit according to Eq. (7), only for the higher concentrations while at the lower concentrations the results clearly deviate from the Scatchard plot suggesting that the binding sites are not identical and independent [25]. From the linear part of the model in Fig. 4 we obtain,

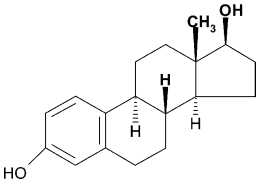
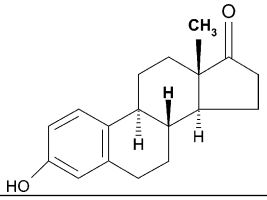
$$n \approx 0.07, \quad K \approx 13.1 \times 10^6\,M^{-1}$$

which is an order of magnitude estimate in view of the limited number of data in the linear region in Fig. 4. Nevertheless, it is within the range of reported values in literature [21]. In our calculation, we corrected the antibody surface concentration for a 25% activity loss that was determined for the oxidized antibody. In addition, one assumption in the model is that all Cn binding sites are equally accessible for the 17-β-estradiol molecules. It is highly likely that upon immobilization, a substantial proportion of ligand may be in an environment or configuration where the ability to bind a ligate is either impaired or prevented thus altering the value of n and K. Sterical hindrance of E2 binding influences strongly the membrane adsorption properties. The high antibody concentration on the external surface of the membrane proved by confocal microscopy (see Section 3.1) has a large contribution to the sterical hindrance phenomena. Adsorption characteristics of the membrane are less influenced by the loss of antibody activity due to immobilization step [10]. In consequence, the membrane E2 adsorption capacity ($\approx 90 \times 10^{-8}$ mg E2/mg membrane) is less then the calculated theoretical values (10.9×10^{-6} mg E2/mg membrane, considering $n = 2$). The experimental value is according to the literature where in some affinity chromatography systems, only 0,1% of immobilized ligand was involved in binding the ligate [21]. One could suspect that the Schatchard model also fails when the binding of a molecule hinders the neighboring binding sites such that only a certain fraction α of the binding sites can actually be occupied. This limited occupation as such, however, does not change the linearity in Eq. (4); it merely modifies the valency n into an effective, lower valency αn. Fig. 4 suggests that there are actually two regimes in the 17-β-estradiol adsorption process: a regime at higher concentration that at least qualitatively is in accordance with the Scatchard plot and a regime at low 17-β-estradiol concentration where the model clearly fails. Further work will be needed to explain this phenomenon in more detail.

3.3. Membrane specificity

Selective separation is our primary reason for using antibodies. However, an antibody can bind one or more ligats with a structure similar to the molecule that induces the immune response. This phenomenon, the so-called cross-reactivity of antibodies could hamper, or even obstruct the selective adsorption of E2. In our experiments, we determined the selectivity of EVAL-Ab membrane towards E2 as follows. Estrone and 17-β-estradiol are both estrogenic compounds with very similar chemical structure and physico–chemical properties (see Table 2). Note also in Table 2 that molecular weights differ only with two units. Taking in account all these similarities, we expect that the antibody will interact to a certain extent with both compounds. Indeed, if either only E1 or E2 is present, the antibody will just interact with the available estrogenic compound in the solution (see Fig. 5). The concentration dependence of the adsorption, however, clearly differs for the two compounds. While the antibody will react with 17-β-estrdiol at low concentrations, for estrone concentrations of at least 1.5–2 μg/L are needed (Fig. 5). Further, when both compounds are present the antibody is discriminating between the two, clearly preferring the 17-β-estradiol (Fig. 6). Even so, the overall concentration is also favoring a certain interaction between estrone and the antibody. Upon increasing the concentration of estrone, a competition between the two compounds takes place that reduces the quantity of adsorbed

Table 2
Chemical structure and physical–chemical properties of estrone and 17-β-estradiol

Compound	Chemical structure	MW (Da)	pK_a	Dimension (nm)
17-β-Estradiol		272	10.4	0.8
Estrone		270	10.4	0.8

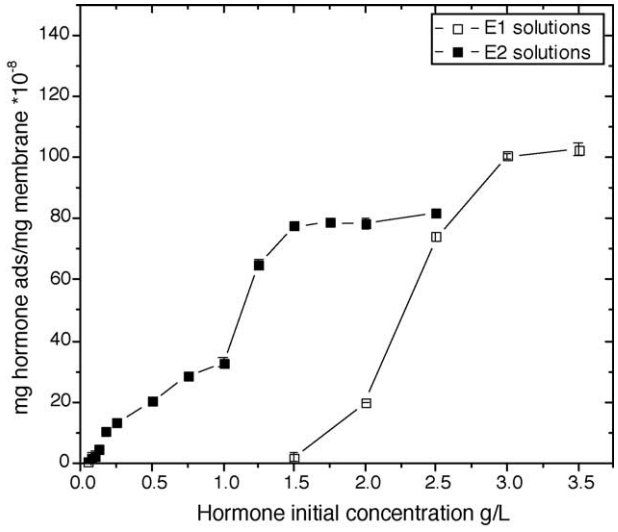


Fig. 5. Comparison between adsorption isotherms for E2 and E1 using EVAL-Ab membranes.

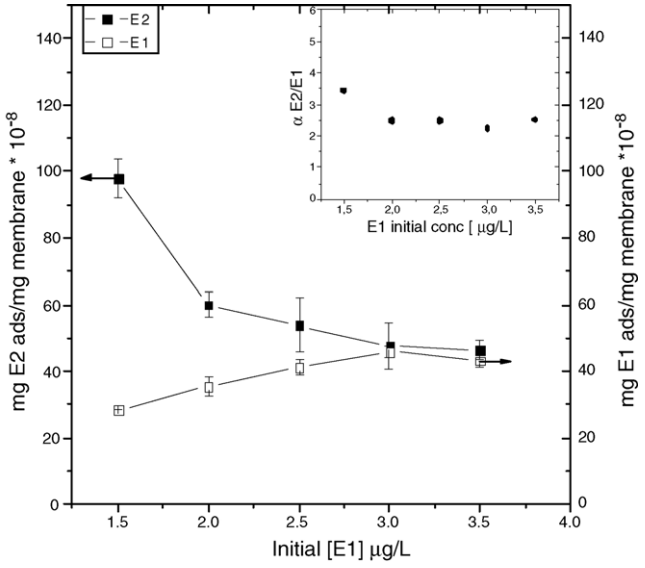


Fig. 6. Adsorption of hormones from E1 and E2 mixture solution. The insert shows the membrane selectivity as function of E1 initial concentration.

17-β-estradiol. We may expect that since the membrane is even able to discriminate between 17-β-estradiol and estrone, it will indeed act as a specific binding material for 17-β-estradiol in quite a variety of solutions.

3.4. Interaction kinetics between E2 and EVAL-Ab membrane

The removal process takes place under non-equilibrium conditions, so it is important to study the kinetic behaviour of the interaction between E2 by antibody-immobilized membrane EVAL-Ab (Fig. 7). The rate at which the E2 binds to the membrane in the static adsorption experiments depends on the transport of the hormone from the bulk solution through possible boundary layer onto the membrane, on surface transport and on interaction with the active sites on the membrane. Mixing the liquid with a magnetic stirrer avoids the presence of a hydrodynamic boundary layer resulting in a homogeneous concentration distribution of the ligate. Consequently, the removal rate of E2 has two components: (a) the contribution of the diffusion controlled transport of the hormone in the liquid present in the membrane pores from the boundary layer to the active sites onto the membrane surface and (b) the kinetic of the hormone–antibody binding. We neglect here the influence of the other occupied neighbouring sites on antibody–hormone interaction. As an approximation, in concordance with the literature date [21,26,27], we assume that the binding reaction can be described by a single overall rate constant k_1. Since the overall association rate (that includes the mass transfer and the binding kinetics) is governing the process until the equilibrium is reached, using Eq. (4) we approximate $k_1 = 1.4 \times 10^9\ \mathrm{cm^2\ M^{-1}\ s^{-1}}$ and from K we then estimate $k_{-1} = 1.29 \times 10^2\ \mathrm{s^{-1}}$. As expected, the overall association rate is several orders of magnitude higher than the overall dissociation rate (that includes the mass transfer and the dissociation kinetics) meaning that most of the hormone will react with the bound antibody.

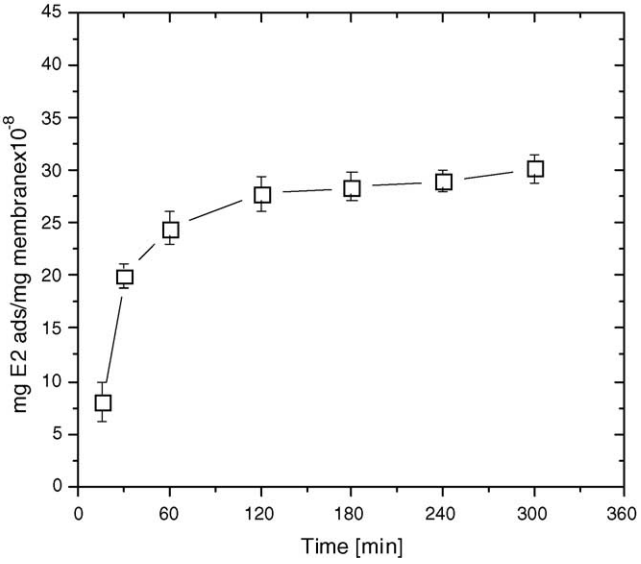


Fig. 7. Adsorption of E2 from urine like solution (1 µg/L E2) as function of time.

pH, organic solvents and salts present in the aqueous solution may affect the interaction between the antibody and the hormone. The binding site of the antibody can be totally or partly covered by ionizable groups that are stabilized by charges on the protein surface. Changes in pH can furthermore induce conformational changes in the antibody that could interfere with the correct positioning of amino acid residues participating in ligat binding. Such conformational changes may be restricted especially at extremely high or low pH, affecting the overall structure, permanently or temporally. Such changes are becoming more evident for the interaction between antibodies and charged molecules such as antigens. E2 is a hydrophobic compound and its structure is

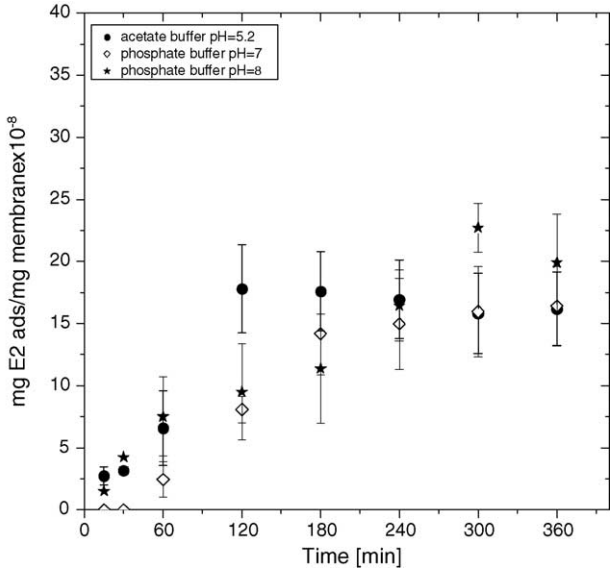


Fig. 8. Influence of the E2 starting solution composition on the adsorption properties of EVAL-Ab.

less affected by pH and salt concentrations. Nevertheless, the solution composition can affect antibody conformation and in consequence the binding process. Indeed from Fig. 8, we can clearly see that pH is influencing the binding rate between the hormone and the antibody. Adsorption experiments were carried out at different pH maintaining constant the number of active sites available on the membrane and 17-β-estradiol concentration. As it can be seen from Fig. 8 increasing the pH from 5.2 to 8 is directly decreasing the binding rate between the EVAL-Ab and E2. At pH 5.2, the time to reach equilibrium is 120 min and for pH 8 becomes 300 min. Comparing the results from Fig. 8 with the ones from Fig. 7, we can conclude that a high concentration of salts is beneficial for the binding rate as well for the binding capacity of the prepared membranes.

3.5. Dynamic adsorption filtration experiments

In membrane chromatography, removal of compounds from complex mixtures is done in practice in a dynamic non-equilibrium regime. The requirement is to have no hormone, or very little, present in permeate after contact of the hormone solution with the membrane. When the binding sites available for hormone–antibody interaction are occupied, the concentration of the hormone in permeate should equal the feed concentration. Fig. 9 shows a representation of the dynamic filtration experiment with the adsorbed amount of hormone on the membrane as a function of the permeated volume. Theoretically, the membranes can adsorb about 90×10^{-8} mg E2 per mg of membrane and the loading should increase linearly with the amount of permeated volume for a rapid enough hormone–antibody coupling reaction. This is shown as the solid line in Fig. 9. In order to fulfil the above-mentioned requirement however, the hormone solution and

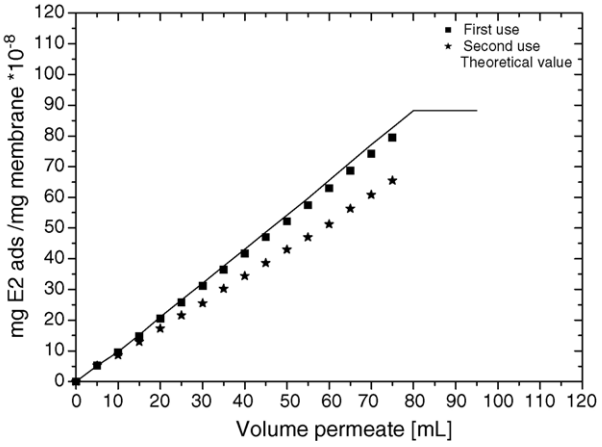


Fig. 9. Adsorption of E2 in dynamic filtration conditions. The line represents the theoretical calculated value.

the antibody-immobilized membrane have to be sufficient time in contact, allowing local equilibrium. In consequence, in dynamic filtration operation, the removal of hormones is depending on flow conditions. The presence of the antibody is reducing considerable the water fluxes as mentioned earlier in Section 3.1. Using a stack of nine membranes reduces even more the flux of the hormone solution reaching the value of 120×10^{-5} L/(Pa h m^2) (120 L/(bar h m^2)). Considering the total adsorption capacity as determined by adsorption isotherms, one can simple predict the amount of hormone adsorbed based on the assumption of complete hormone removal from the solution to the adsorptive interface. As it can be observed from Fig. 9, the 17-β-estradiol removal (99% of E2) from hormone solutions is close to the theoretical value.

Finally, for possible applications the re-use of the membrane is critical. Elution requires the complete dissociation of the hormone–antibody complex and preferably the hormone should be eluted at high concentration in a small volume of solution. Most frequently used method in immuno-adsorbant elution, is alteration the physical and chemical properties of the solution such that the interaction of antibody and ligates is reduced. The principle behind this method is to modify the properties of the solution such that three-dimensional change in the antibody and/or ligate structure will take place reducing significantly the bio-molecular recognition between them [21]. A possible candidate as an eluent is methanol. In addition to the fact that methanol can interfere with the geometry of the antibody–hormone complex by changing the polarity of the solution, it also is a good solvent for E2. Using methanol as eluent we recovered E2 completely from the membrane, obtaining a concentrate solution of the hormone. The starting adsorption results of the antibody-immobilized membrane and the re-used membrane are almost identical as it can be clearly seen from Fig. 9. Nevertheless, in time a loss in removal performance can be observed and can be attributed to a loss of antibody activity (loss of about 10% after one re-use) possibly due to the methanol use. Our study has clearly shown that in dynamic operation is possible to

remove 99% from hormone and regenerate the membrane. More work has to be done to find out the optimum removal and elution conditions in dynamic filtration conditions.

4. Conclusions

A new affinity membrane was designed and prepared starting from poly(ethylene vinyl alcohol) (EVAL) membrane. The surface of the membrane was modified to achieve a site-oriented coupling of the antibody. The spacer molecule, poly(ethylene glycol) bis-hydrazide, was reacted with gluataraldehyde-modified EVAL membrane. The surface modification yielded a fully covered surface with the spacer molecule uniformly distributed inside the membrane. During oxidation of the antibody the activity drops with 25%. Oxidized antibody immobilization led to a preferential distribution on the membrane surface rather than inside the membrane and in consequence high surface densities of the antibody inducing probably a loss in active sites. This inconvenience can be overcome by improving the antibody immobilization conditions and can be the objective of future work. The specificity of the membrane was demonstrated by its capacity to discriminate between two compounds with similar structure, 17-β-estradiol and estrone. The dynamic adsorption experiments showed that a stack of at least nine membranes lowers the 17-β-estradiol concentration in urine like solutions from 2.5 μg/L to values below 50 ng/L in a single step.

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Nomenclature

C_0	initial concentration of 17-β-estradiol (ng/L)
C_e	equilibrium concentration of 17-β-estradiol (ng/L)
E_{2ads}	quantity of E2 adsorbed onto membrane (mg/mg)
$[E1]_{ads}$	quantity of adsorbed estrone (mg ads/mg membrane)
$[E2]_{ads}$	quantity of adsorbed of 17-β-estradiol (mg ads/mg membrane)
$[E1]_0$	initial concentration of estrone (M)
$[E2]_0$	initial concentration of 17-β-estradiol (M)
P	porosity
SD	swelling degree
V_0	initial membrane volume
V_{s0}	volume of solution used for the experiments (L)
V_s	volume of the swollen membrane
W_0	weight of the dry membrane placed into the solution (mg)

Greek letter

$\alpha_{E2/E1}$	selectivity

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