Evaluation of an anion-exchange hollow-fiber membrane adsorber containing γ-ray grafted glycidyl methacrylate chains

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ABSTRACT

It is widely recognized that membrane adsorbers are powerful tools for the purification of biopharmaceutical protein products and for this reason a novel hollow-fiber AEX type membrane adsorber has been developed. The membrane is characterized by grafted chains including DEA ligands affixed to the pore surfaces of the membrane. In order to estimate the membrane performance, (1) dynamic binding capacities for pure BSA and DNA over a range of solution conductivity and pH, (2) virus reduction by flow-through process, and (3) HCP and DNA removal from cell culture, are evaluated and compared with several other anion-exchange membranes. The novel hollow-fiber membrane is tolerant of high salt concentration when adsorbing BSA and DNA. When challenged with a solution containing IgG the membrane has high impurity removal further indicating this hollow-fiber based membrane adsorber is an effective tool for purification of biopharmaceutical protein products including IgG.

1. Introduction

In the 1990s numerous studies on membrane chromatography [1–6] expected functionalized membranes to have many applications based on the possibility of high flow rates and the lack of column packing required of traditional resin based chromatography. The use of membrane chromatography in biopharmaceutical manufacturing processes was, however, not widely accepted due to the relatively low binding capacity of membrane adsorbers and low resolution during elution [7]. Eventually, it was recognized that membrane chromatography is feasible when operated not in bind-and-elute mode, but rather in flow-through mode where the molecule of interest remains unbound and impurities are adsorbed.

In 2001, Knudsen et al. [8] reported that anion-exchange membranes in flow-through mode may provide a reasonable alternative to packed bed columns for the removal of low levels of impurities such as DNA, host cell protein (HCP) and virus in process-scale antibody purification. They also suggested that, due to economic and process restrictions, cation-exchange membranes may not be advantageous for process-scale antibody purification in a bind-and-elute mode. In 2006, Zhou and co-workers [9,10] thoroughly examined the advantages and disadvantages of using anion-exchange membrane chromatography as a purification unit operation and showed that anion-exchange membrane chromatography was a viable alternative to Q column chromatography as a polishing step in process-scale antibody production when operated in flow-through mode. Zhou also showed that using anion-exchange membranes in antibody manufacturing processes was cost effective. After their work, the use of anion-exchange membranes in the purification process became more common in bio-pharmaceutical production.

Although all the market leading anion-exchange adsorbers are flat-sheet membranes, hollow-fiber membrane adsorbers are widely thought of as potentially advantageous because of their high membrane area to housing volume ratio. Preparation and application of hollow-fiber type anion-exchange membranes have been studied rigorously [11–18]. The characteristic property of this novel membrane is the addition of grafted chains fixed on the pore surface to which the ligands are attached. The advantage is that the grafted chain enhances the accessibility of the ligand to the binding site on the protein or contaminant thus increasing the binding capacity. A similar three-dimensional adsorption is reported by Janzen et al. [19] and Muller and Klein [20].

The grafted chain hollow-fiber membrane was developed by Asahi Kasei Medical under the name QyuSpeed[TM] D (hereafter "QSD"). In order to estimate the membrane performance, (1) dynamic binding capacities for pure BSA and DNA over a range of solution conductivity and pH, (2) virus reduction by flow-through process, and (3) HCP and DNA removal from cell culture, were evaluated and compared with several other anion-exchange membranes.

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2. Materials and methods

2.1. Materials

2.1.1. Materials for the anion-exchange hollow-fiber membrane

A porous polyethylene (PE) hollow-fiber membrane is the base material for grafting. The hollow-fibers have an inner and outer diameter of 2.0 and 3.0 mm, respectively. The average maximum pore diameter is 0.3 μm, determined by bubble point method [21]. The porosity is approximately 70%. The monomer material for the graft chains is technical grade glycidyl methacrylate (GMA, $CH_2=CCH_3COOCH_2CHOCH_2$) purchased from Tokyo Kasei (Tokyo, Japan) and used without further purification. The ligands are comprised of diethylamine (DEA, $NH((CH_2CH_3)_2)$) purchased from Wako Pure Chemical Industry (Tokyo, Japan).

2.1.2. Materials for dynamic binding capacity evaluation and impurity removal test

Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (Albumin, from bovine serum >98% (agarose gel electrophoresis) powder) for the evaluation of the protein dynamic binding capacity (DBC). DNA was purchased from Invitrogen (Salmon Sperm DNA Solution, 10 mg/mL, <2000 bp). Serum free CHO cell culture with pH 7.5 and conductivity of 9.8 mS/cm was kindly provided by Asahi Kasei Pharma Co., Ltd. γ-Globulins (Sigma, γ-globulins, Human: From Cohn Fraction II, III Approx. 99% (electrophoresis)) was added to the CHO cell culture as antibody protein.

2.2. Preparation

2.2.1. Preparation of anion-exchange porous hollow-fiber membrane

The porous hollow-fiber membrane containing grafted DEA ligands is prepared by a γ-ray grafting technique and subsequent chemical modification as shown in Fig. 1. This preparation process is based on the method reported by Saito and co-workers [11]. A vinyl monomer containing an epoxy group (GMA) is grafted onto the membrane after radicals have been generated by γ-ray irradiation. In this case, the intensity of γ-ray radiated from Cobalt 60 equipment was ca. 10 kGy/h and irradiation time was 20 h, i.e., the total dose of γ-ray was ca. 200 kGy. After the irradiation, the hollow-fiber membranes are immersed in a GMA solution (GMA: methanol = 5:95 in volume) for 10 h at 313 K. The amount of GMA graft polymerized (degree of grafting) is defined as [(weight gain)/(weight of PE membrane) × 100 (%)] and averaged 70%. The DEA group is added by exposing the epoxy group to diethylamine solution [diethylamine:H_2O = 1:1 in volume (pH 13.2)] for 12 h at a temperature of 303 K. The degree of substitution was 86–96%, determined from the weight change before and after exposure to diethylamine solution. A schematic illustration of a micropore of the membrane with grafted chains, and the chemical structure of the grafted chains are shown in Fig. 2. Unreacted epoxy groups turn to diol groups (from –CH–CH$_2$O to –CH(OH)–CH$_2$OH) under alkaline conditions for 12 h. FTIR and solid state NMR methods confirmed no unreacted epoxy groups remained after substitution. After grafting the hollow-fibers were found to have expanded to 3.6 mm OD and 2.2 mm ID. Scanning electron microscopy (SEM) images of the cross-section for the hollow-fiber membrane are shown in Fig. 3.

The length and the density of GMA graft chains are considered to be controlled by irradiation dose and degree of grafting [22]. The length and density were not evaluated directed since any chemical or physical methods would not separate the chain from the base polymer. Alternatively, Lee et al. measured the density of free radicals generated by the irradiation by electron spin resonance (ESR) and evaluated the length and density of GMA graft chains from radical density and degree of grafting [23]. Applying this principle to QSD, where the degree of grafting was 70% and 200 kGy of total irradiation was dosed, the length and density of GMA graft chains was estimated as ca. Mn = 3×10^5 and 2×10^{19} (brush/g-BP), respectively. Where, brush/g-BP is the number of graft chains per 1 g of PE base polymer.

In order to evaluate the change in permeability due to the addition of the grafted chain and ligand, pure water was pumped through the hollow-fibers (effective length: 9 cm, inner diameter: 2.0 mm for unreacted PE hollow-fiber and 2.2 mm for grafted QSD) from inside to outside at the flow rate of 2 mL/min. The trans membrane pressure at 2 mL/min flow was 0.015 MPa for the PE hollow-fiber and 0.013 MPa for QSD indicating that the permeability was unchanged as a result of the grafting process. Additionally, the pressure at 2 mL/min flow through QSD was 0.02 MPa for both buffer alone (20 mM Tris–HCl pH 8.0) and with buffer including 1 M NaCl. Although it was observed that the filter pressure increased from 0.025 MPa to 0.045 MPa when pure water was filtered after a solution of 20 mM Tris–HCl pH 8.0, 1 M NaCl. When 20 mM Tris–HCl pH 8.0 was used instead of pure water the pressure remained constant 0.025 MPa. The pressure increase is likely due to the swelling of the membrane by high concentration salt solution.

2.2.2. Fabrication of the hollow-fiber membrane filter

A schematic illustration of the hollow-fiber membrane filter is shown in Fig. 4. One hollow-fiber (outer diameter of 3.6 mm) is housed in the cartridge with inner diameter of 5.0 mm and potted by sealant at both ends using epoxy resin. The cartridge material is polysulfone. The effective length of the hollow-fiber between both sealant surfaces is 9.3 cm. Four openings are present in the cartridge, i.e., two inlets to the fiber (A and D) and two outlets (B and C) on the shell side. For dead-end filtration use, flow direction is arranged from A to C as permeating from the inside of the hollow-fiber to the outside. The effective membrane area, defined by the inner surface area of the fiber is 6.3 cm^2. The effective membrane volume is 0.6 mL.

2.3. Instrument and membranes

All the measurements for the evaluations of the dynamic binding capacity and impurity removal test from cell culture are carried out using an ÄKTA Explorer 100 (GE Healthcare). The adsorption performance of the membrane is evaluated in dead-end mode at a defined flow rate normalized for each filters membrane volume. The normalized flow rate is given in the units mL/min/mL-ad where the flow rate (mL/min) divided by the membrane volume (mL-ad). In order to also normalize the volume of fluids used including load volume, membrane volumes (MV) are used where the volume applied (mL) is divided by the membrane volume (mL-ad). For example, in the case of QSD: 100 MV = 60 mL applied volume/0.6 mL-ad membrane volume.

Four different types of membranes were used: QSD (DEA group, membrane volume = 0.6 mL-ad), membrane A (Sartorius stedim, Sartobind Q15, Q amine group, 0.41 mL-ad), membrane B (Pall Corporation, Mustang Q Acrodisc 25 mm, Q amine group, 0.18 mL-ad) and membrane C (CUNO, Zeta Plus 90ZA, Zeta potential type, >5 mL-ad). QSD is a hollow-fiber membrane with long graft chains, membranes A and B are flat sheet type membranes, and membrane C is depth filter with an inorganic filter aid, cellulose and a proprietary positive charge.

2.4. Dynamic binding capacity

In order to compare the flow rate dependence of membranes and packed bed column, dynamic binding capacity (DBC) for QSD,
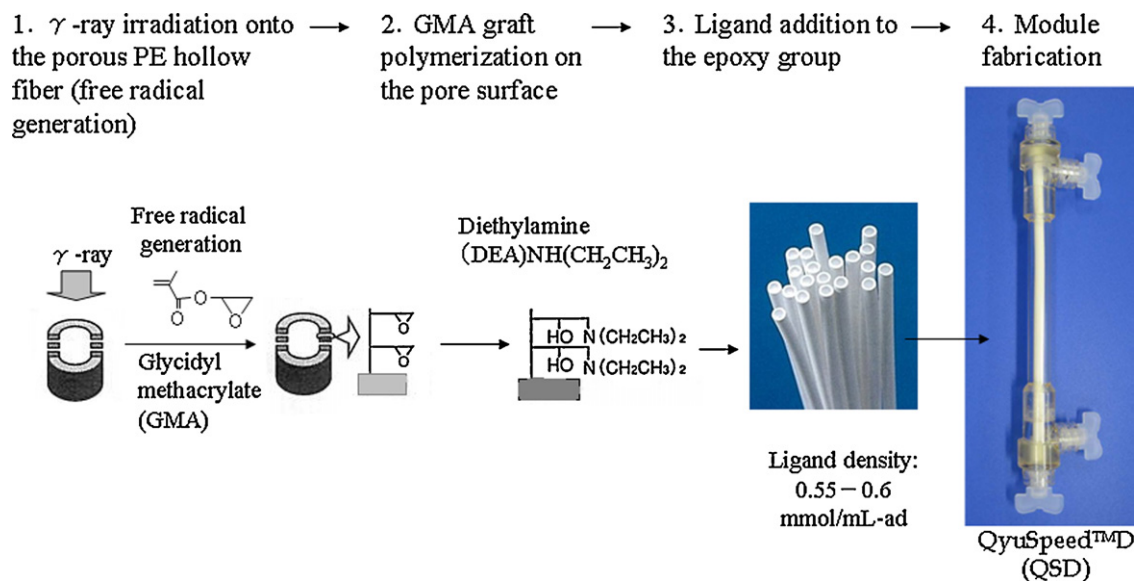
Fig. 1. Procedure for introduction of grafted chains to the porous polyethylene hollow-fiber, immobilizing DEA anion-exchange groups and module fabrication.

membrane A and HiTrap DEAE 1 mL column (GE Healthcare) were evaluated at the flow rate from 1 mL/min to 10 mL/min. Each adsorbent was equilibrated with 20 mM Tris–HCl pH 8.0 before loading the 1 g/L BSA solution in the same buffer.

Dynamic binding capacity of BSA at pH 7.0 and pH 8.0 with varying amounts of sodium chloride were evaluated in a 20 mM Tris–HCl buffer system. Similarly DNA was evaluated at pH 8.0 only. The concentration of BSA and DNA in each solution was 1 g/L and 0.1 g/L, respectively. The DBC was measured by loading sample solution at a defined normalized flow rate (5 mL/min/mL-ad) through the anion-exchange membrane and measuring the UV signal (280 nm for BSA and 260 nm for DNA) at the outlet of the membrane. DBC was calculated at 10% of the breakthrough. In the case of BSA DBC, the membranes were reused after a 20 MV elution with 20 mM Tris–HCl, 1 M NaCl and a 15 MV of 1 M NaOH regeneration.

2.5. Virus clearance

A 0.5 vol% serum free-PPV (PPV, pI = 5.0–5.5 [24], icosahedron with diameter of 18–24 nm [25]) spike (ca. 10^6/mL) with 10 g/L human-IgG in 0.1 M NaCl pH 7.9 was used to evaluate virus removal. Under this condition virus is expected to bind to the adsorber while product flows through unbound. The log reduction of virus as a function of filter throughput was determined by hemagglutination assay TCID50 method.

2.6. Impurity removal from cell culture

γ-Globulin powder was added to the serum free CHO cell culture (pH 7.5, 9.8 mS/cm) to make an IgG solution of 0.5 g/L concentration. The IgG cell culture was filtered using 0.45 μm polysulfone hollow-fiber membrane (Asahi Kasei Medical, BioOptimal^{TM} MF-SL). The
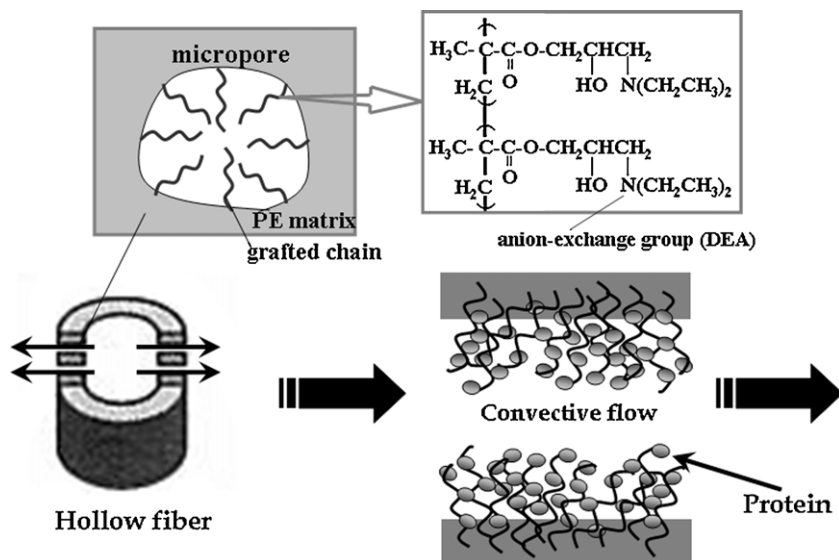

Fig. 2. Schematic representation of the membrane with grafted chains, chemical structure of grafted chains with DEA anion-exchange group, convective flow in the porous hollow-fiber and of proteins captured by grafted chains.
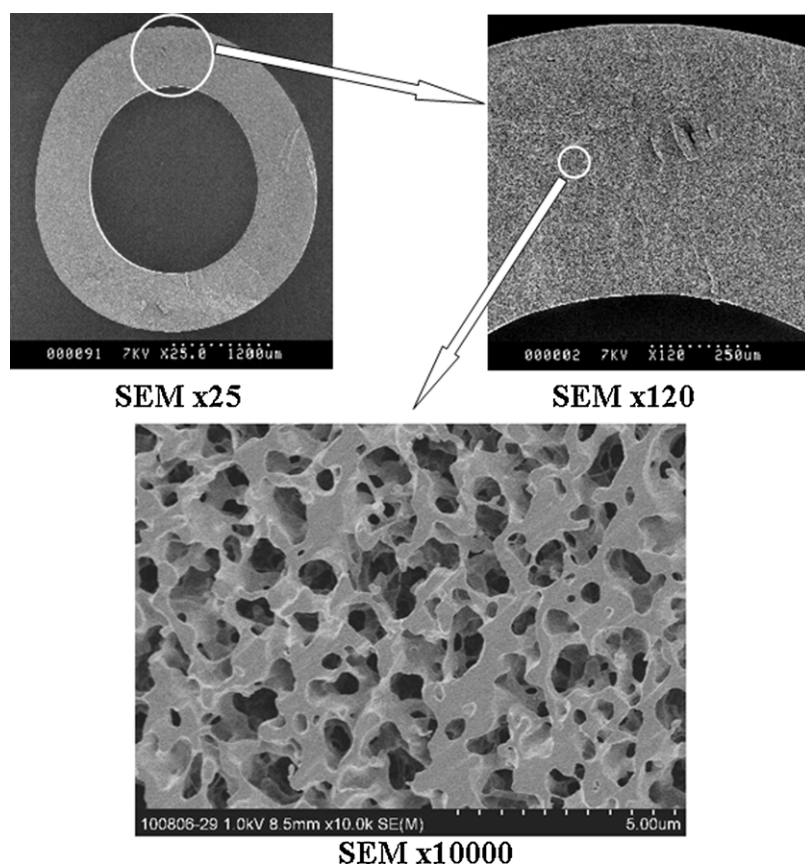
Fig. 3. SEM images of the cross-section for the hollow-fiber membrane with grafted chains (QSD).

0.45 μm filtered material (MF material) was applied to the four types of anion-exchange membranes to obtain the flow-through pool for each membrane. The volume applied to each membrane was normalized to 100 MV and the flow rate was 5 mL/min/mL-ad.

The flow-through pool of QSD and membranes A, B and C was then applied to a protein-A column (GE Healthcare, HiTrap Protein-A HP 1 mL) to obtain the respective elution. The column was loaded to 15 mg/mL-resin (30 mL volume) at a flow rate recommended by the manufacturer, 1 mL/min. MF material not filtered by a membrane adsorber was also protein-A purified. The column was eluted using 10 mL of 0.1 M citrate buffer (pH 3.0) after washing the column with 20 mL of 20 mM sodium phosphate buffer (pH 7.0). The column was regenerated by 0.1 M HCl and reused.

The flow-through pools were analyzed by SDS-PAGE (reduced, silver stain, using precast gel: Cosmo Bio, Multi Gel mini II 8/16 (8–16%)). For both the flow-through pools and the protein-A eluates, HCP concentration was determined by an in-house CHO protein ELISA assay (Asahi Kasei Pharma). DNA concentration was determined by the fluorometry method (Invitrogen, Qubit Fluorometer with Quant-iT dsDNA BR assay kit).

3. Results and discussion

3.1. BSA dynamic binding capacity

Bovine serum albumin is widely used to evaluate the binding capacity of both membrane adsorbers and chromatography resins. The relationships between BSA dynamic binding capacity at 10% break through and flow rate are plotted in Fig. 5. Although the capacity for QSD decreases slightly as the flow rate increases, the DBC for the membrane adsorbers show very little dependence on the normalized flow rate. On the other hand, the packed bed column has a significant dependence on flow rate. The difference between the membrane adsorber and packed bed column is due to each hav-
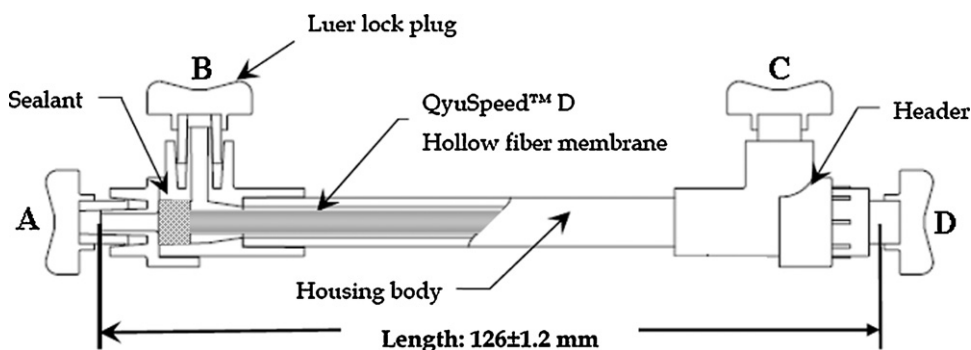

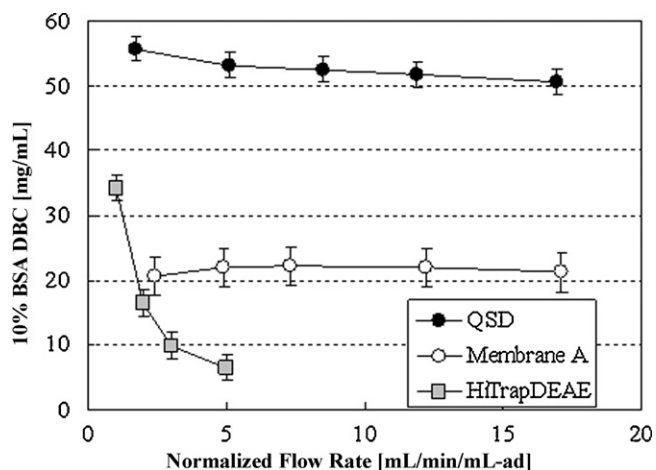**Fig. 4.** A schematic illustration of a hollow-fiber membrane filter (QSD).

Fig. 5. The normalized flow rate dependence of BSA at 10% DBC for two types of membrane adsorbers (QSD and membrane A) and a packed bed column (HiTrap DEAE FF 1 mL).

Fig. 7. 10% DBC of DNA for QSD, membranes A and B. Equilibrium buffer: 20 mM Tris–HCl (pH 8.0) with NaCl 0–1.2 M. Load solution: 0.1 g/L DNA in equilibrium buffer. Flow rate: 5 mL/min/mL-ad.

ing their own different mechanism of mass transfer. As shown in Fig. 2, mass transfer between proteins and binding sites is governed by convective flow through the pore structure of the membrane [13], whereas, for the packed bed column the contact based on diffusive flow into the porous structure of the beads. Additionally the DBC for QSD is almost twice as high as membrane A and is likely due to be the multi-layer adsorption of proteins by the grafted chains.

The effect of salt concentration and pH on 10% DBC of BSA is summarized in Fig. 6 at constant flow rate of 5 mL/min/mL-ad. The DBC for ion-exchange adsorbents usually decreases as the salt concentration increases due to the inhibition effect by ions in the solution. Accordingly, for all the membranes as salt concentration increases capacity decreases. The decrease of DBC for QSD, however, is less drastic than that for membranes A and B. The DBC decrease is especially significant for membrane B. For example, at pH 7.0 and 0 M NaCl DBC for QSD, membranes A and B were 57 mg/mL, 37 mg/mL and 91 mg/mL, respectively. The addition of 0.15 M NaCl causes the DBC of membranes A and B to decrease to 1.2 mg/mL and 3.3 mg/mL, respectively, while the DBC of QSD is 12.4 mg/mL at the same condition. This tolerance for salt concentration is considered to be an effect of protein adsorption by grafted chains perhaps by allowing more or stronger binding sites to be accessible to the ligand.
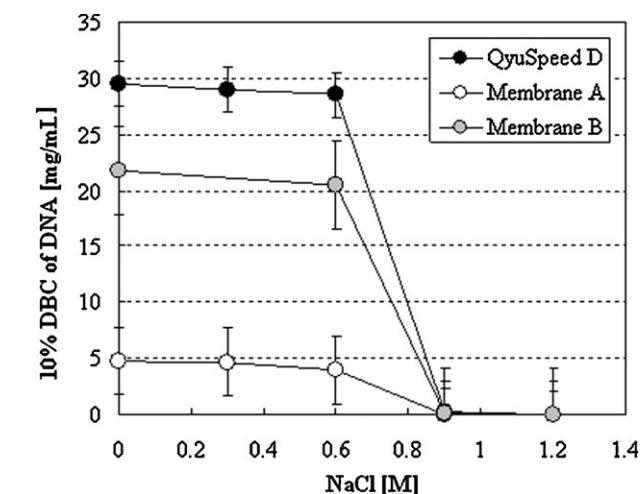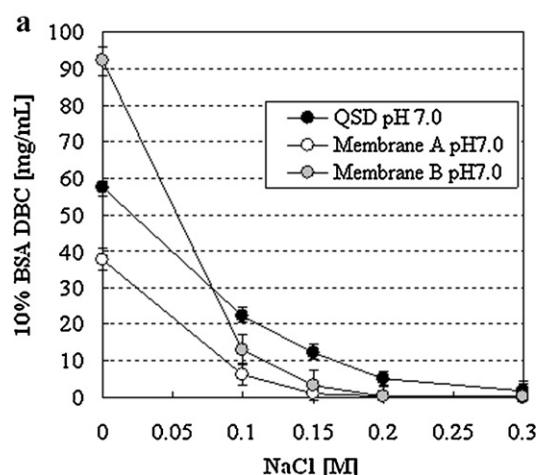
3.2. DNA dynamic binding capacity

In bio-pharmaceutical manufacturing process such as mAb purification, DNA is one of the important impurities to be removed from the cell culture. The characteristics of DNA are large molecular weight and low pI value compared to other impurities such as HCP. These properties of DNA suggest that anion-exchange membrane adsorbers are ideal to remove DNA from solutions because of their convective flow mechanism and larger pore size than traditional beads [26]. Fig. 7 shows the dynamic binding capacity at 10% breakthrough of DNA at pH 8.0 using a flow rate of 5 mL/min/mL-ad. In contrast to the capacity for BSA, DBC of DNA is almost independent of salt concentration up to 0.6 M of NaCl for all the membranes tested. While membrane B showed higher BSA DBC in the 0 M NaCl condition than QSD, for DNA the DBC is smaller than that of QSD at the same condition. The DNA DBC for QSD is more than 5 times higher than that of membrane A. Furthermore, for the buffer solution of 20 mM Tris–HCl pH 8.0 without salt, the DBC values of DNA were much smaller than that of BSA for all membranes. Yang et al. [26] explained that the larger size of protein leads to the higher DBC value on anion-exchange membranes. On the other hand, in this study the comparison between BSA and DNA shows that the DBC of DNA is significantly smaller than that of BSA, even though DNA has much larger molecular size than BSA. This result suggests
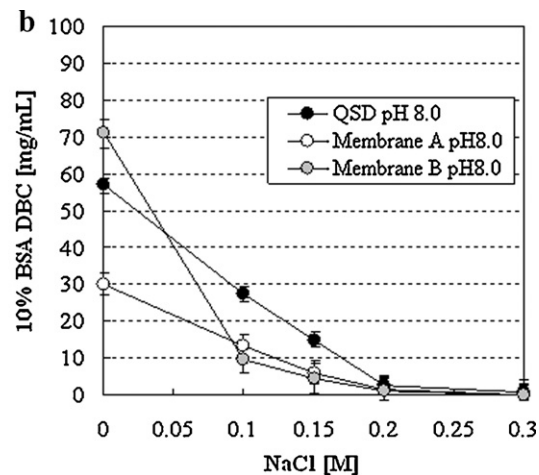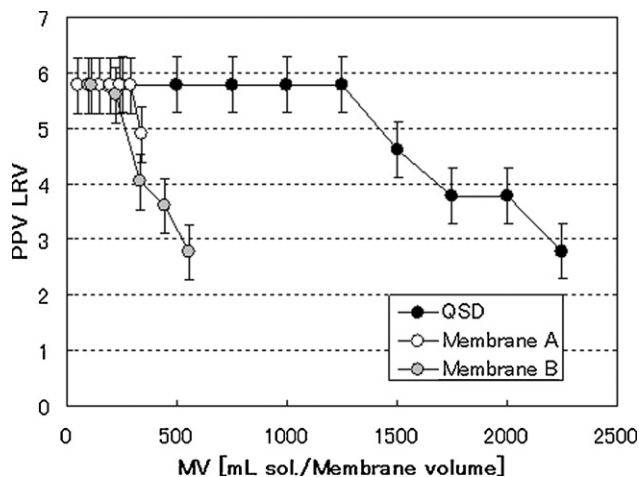

Fig. 6. The effect of NaCl concentration on 10% DBC of BSA (a) pH 7.0 and (b) pH 8.0. Load solution: BSA 1 g/L in 20 mM Tris–HCl. Flow rate: 5 mL/min/mL-ad.

Fig. 8. PPV clearance for flow-through fractions of QSD, membranes A and B. Load solution: 0.5 vol% serum free-PPV spike (ca. ~10^6/mL) with 10 g/L human-IgG, 0.1 M NaCl (pH 7.9). LRV evaluation: hemagglutination assay TCID50 method.

that the adsorption property of DNA might be different from that of proteins. The DNA DBC for QSD is about a half of the BSA DBC, is about 17% for membrane A and about 30% for membrane B suggesting that for the larger size molecules, adsorption by grafted chains is more effective than surface adsorption.

3.3. Virus clearance

Flow-through virus clearance results at a flow rate of 5 mL/min/mL-ad are summarized in Fig. 8 as a function of normalized load volume. At pH 7.9 QSD showed complete PPV clearance (LRV >5) up to the load volume of 1250 MV (750 mL or 12.5 g/mL-adsorbent). Complete PPV clearance was achieved up to 291 MV for membrane A, and 222 MV for membrane B. These results again suggest that adsorption by grafted chains is more effective than surface adsorption for large size molecules. Membrane B which had the highest BSA adsorption and higher DNA adsorption than membrane A has the lowest virus clearance. This indicates the difficulty in estimating impurity removal from the BSA and DNA adsorption data obtained at the same condition.

3.4. Comparison of impurity removal from model IgG cell culture

The extent of the impurity removal from harvested cell culture fluid by flow-through was determined by filtering MF material through QSD, membranes A, B and C. The pI of impurities such as DNA and a majority of HCP are less than the pH value (pH 7.5) of the cell culture fluid studied, an AEX membrane operating at the pH of cell culture is expected to remove some of the contaminates. The purification was roughly estimated by the SDS-PAGE gel shown in Fig. 9. The MF material consists of γ-globulins and various impurities from cell culture, some of these impurities are also present, although slightly reduced, in flow through pools for membranes A, B and C indicating little impurity clearance. Alternatively, the flow-through pool for QSD clearly shows that a large amount of impurities were removed from the MF cell culture material under the same conditions. The concentrations of both HCP and DNA are summarized in Fig. 10 and the level of HCP reduction coincides with the qualitative representation in Fig. 9. HCP concentration in the MF material is 346 μg/mL and those in the flow-through pools of membrane A to C are in the range of 248–310 μg/mL. On the other hand, HCP in the QSD flow-through is as 39 μg/mL, therefore 90% HCP was removed by QSD in flow-through mode. DNA concentration in the starting MF material is 7200 ng/mL and in the flow-through pools of
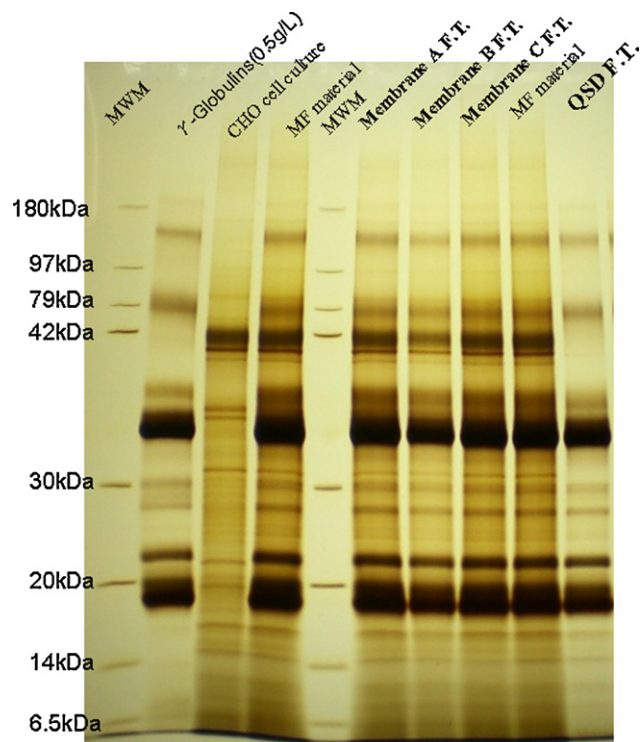
Fig. 9. SDS-PAGE analysis (reduced, silver stain). From left to right: MW marker, γ-globulins, CHO cell culture, MF material (starting material), MW marker, flow-through pools of membranes A, B, C, MF material (starting material), flow-through pool of QSD.

membranes A, B, C and QSD are 492 ng/mL, 3916 ng/mL, 7600 ng/mL and 52 ng/mL, respectively. The discrepancy in DNA concentration of membrane C flow-through pool versus the MF starting material is likely due to the ±10% variability in DNA measurement by the fluorometer. The relatively low impurity reduction of membranes A, B and C is considered to be the effect the high conductivity of cell culture fluid. The HCP and DNA removal for QSD indicate a large amount of impurities in the cell culture are able to be reduced considerably using anion-exchange membrane adsorbers with grafted chains.

Given the high removal rate of impurities from cell culture fluid, the conductivity and pH of used fluid (9.8 mS/cm and pH 7.5) is considered to be within the optimal range for the hollow-fiber membrane. The greater impurity range not retained by membranes A, B and C in Fig. 9 indicate that cell culture conditions may not be optimal for these membranes as suggested by the BSA DBC in the presence of salt (Fig. 6). The better selectivity, especially at high salt, of the QSD membrane for the impurities could be also explained by the increased hydrophobicity of the polyethylene membrane resulting in an intraplanar mixed mode effect.

DNA removal varied greatly between the membranes tested and did not trend consistently with the DNA binding capacity data. The binding capacity of DNA for membrane B, shown in Fig. 7, is higher than membrane A; however DNA removal from the model cell culture for membrane B is lower than membrane A shown in Fig. 10. This may be caused by competition between other impurities such as HCP in the cell culture and the DNA for available binding sites on the membrane.

Protein-A resin used in bind-and-elute mode is recognized as a powerful tool for the purification of antibody proteins from cell culture. Antibodies are typically purified to more than 99% by using a protein-A step. In order to determine if pre filtration using membrane adsorbers increases overall purification, the flow through pool of cell culture material, previously discussed, from
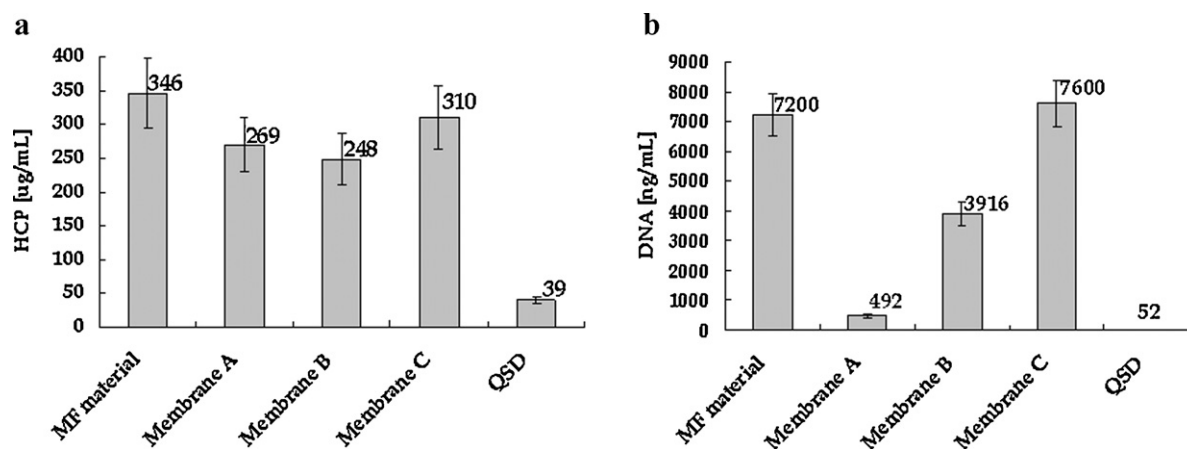
Fig. 10. Concentrations of (a) HCP and (b) DNA in the MF material and flow-through pools of membranes A, B, C and QSD.
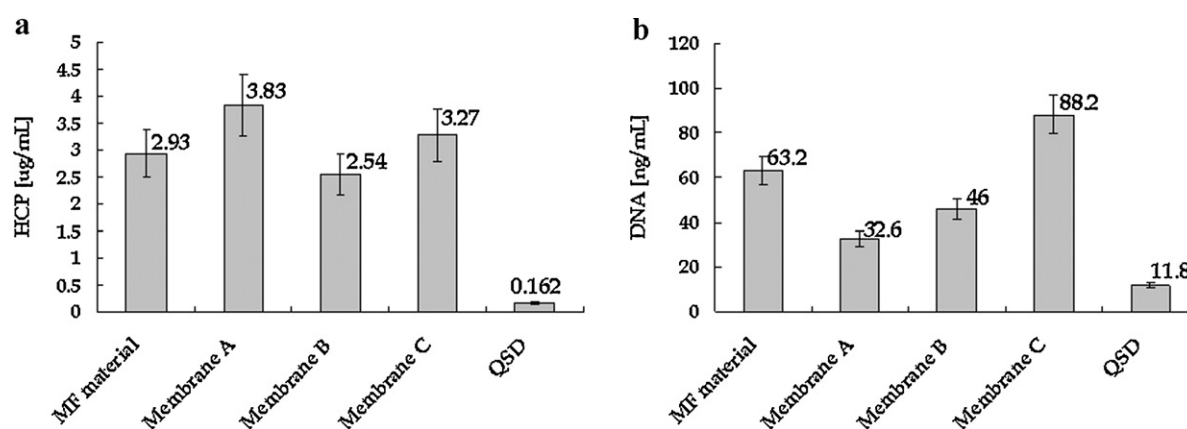

Fig. 11. Concentration of (a) HCP and (b) DNA in the protein-A elution obtained by loading MF material and flow-through pools obtained by membrane A, B, C and QSD.

each membrane adsorber was further purified by identical protein-A methods. The HCP and DNA levels in the protein-A eluates are summarized in Fig. 11. Purification by protein-A alone reduces HCP concentration to 2.9 ug/mL, from 346 ug/mL. Protein-A purification of the QSD flow through reduces the HCP concentration to 0.16 ug/mL. DNA levels are also reduced with the additional filtration using QSD prior to protein-A. Although the protein-A column without pre-filtration is very pure, the additional prefiltration with QSD does increase purity of the elution pool and perhaps more importantly, decreasing the impurity burden prior to the protein-A step decreases the accumulation of contaminates on the protein-A resin which over the lifetime of the resin causes decreased performance [27].

4. Conclusions

Expectedly, BSA binding capacity decreases as the salt concentration increases, however the hollow-fiber membrane with grafted chains (QSD) has relatively higher salt tolerance than the other membranes tested. Impurity removal could not be predicted since not all impurities behave similarly on each adsorbent. Membranes that had high BSA capacity did not necessarily have the same high DNA capacity relative to their competitors. Furthermore, the removal of virus and impurities from cell culture also did not correlate with the trends in BSA and DNA binding capacity. This result suggests it is difficult to estimate purification performance from generic BSA or DNA data without specific experimentation on the impurities of interest.

QyuSpeed D maintained a high dynamic binding capacity for BSA and DNA over a wide range of salt concentrations. In addition to significant virus removal up to 12.5 g/mL-adsorbent, QSD is also able to significantly reduce the level of impurities (HCP and DNA) in harvested cell culture prior to the protein-A step. Pre-column purification contributes to the reduction of impurities in the elution fraction of the affinity column and is expected to decrease the burden on the protein-A column increasing the lifetime of the resin.

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