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High capacity cryogel-type adsorbents for protein purification

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ABSTRACT

Cryogel bodies were modified to obtain epoxy groups by graft-copolymerization using both chemical and gamma irradiation initiation techniques. The free epoxy adsorbents were reacted further to introduce diethylaminoethanol (DEAE) functionalities. The resulting weak anion-exchange cryogel adsorbents showed dynamic binding capacities of *ca.* 27 ± 3 mg/mL, which was significantly higher than previously reported for this type of adsorbent material. Gamma irradiated grafting initiation showed a 4-fold higher capacity for proteins than chemical grafting initiation procedures. The phosphate capacity for these DEAE cryogels was 119 mmol/L and also showed similar column efficiency as compared to commercial adsorbents. The large pores in the cryogel structure ensure convective transport of the molecules to active binding sites located on the polymer-grafted surface of cryogels. However, as cryogels have relatively large pores (10 - 100 μm), the BET area available for surface activation is low, and consequently, the capacity of the cryogels is relatively low for biomolecules, especially when compared to commercial beaded adsorbents. Nevertheless, we have shown that gamma ray mediated surface grafting of cryogel matrices greatly enhance their functional and adsorptive properties..

Keywords: megaporous cryogels; protein chromatography; weak anion-exchange; dynamic binding capacity; monoliths.

1. Introduction

Recent advancement in genetic engineering and recombinant DNA technology has boosted the demand for the recombinant and natural proteins as well as for large biomolecules like plasmid DNA and virus-like particles. The biggest concern for the biopharmaceutical industry is to deliver these new therapeutic products to a highly demanding and regulated market [1] While upstream production of such biomolecules has made significant strides in terms of product yield, the major bottleneck lies in their downstream bioprocessing and purification, which needs to be simplified and made cost-effective [2-4]. Anion-exchange chromatography is one such purification methodology, which is widely used for isolating proteins under neutral or slightly basic pH conditions [5-7]. Commonly used weak anion-exchangers contain diethylaminoethyl (DEAE) or polyethyleneimine (PEI) functionalities, whereas quaternary (Q) amines are used as strong anion-exchangers [8, 9].

As a result of their simple preparation and enhanced mass transfer properties, monolithic cryogels present an attractive alternative to currently used beaded adsorbent media. In this paper, we have prepared monolithic cryogels which offer several advantages over other commercial adsorbents. The structural aspects of these adsorbents allow the separation and purification of biomolecules that is independent of flow rate and while maintaining efficient mass transfer as compared to the limited operating pressures required by conventional particle based resins. In earlier studies, cryogel-based weak anion-exchangers (WAX) were prepared by appending DEAE groups onto their epoxy-functionalized backbone [8]. Such functionalities have been introduced either directly during the preparation of the cryogel backbone [9-11] or by surface grafting, where the epoxy groups are first introduced onto the cryogel and subsequently modified into any desired functionality [8]. For example, Gu *et al.* adopted the former approach and synthesized a series of monoliths using functionalized copolymers with strong cation-exchange properties in a single step [8, 12, 13]. On the other hand, various materials have also been synthesized by adding ionizable groups onto the backbone of monoliths by surface grafting For example, anion-exchangers like poly[2-(dimethylamino)ethyl methacrylate] (pDMAEMA) and poly[2-methacryloxy)ethyl trimethylammonium chloride] (pMETAC) were chemically grafted with functional polymers using potassium diperiodatocuprate as radical initiator, and reported protein-binding capacities of up to 6-12 mg/mL [14].

Various kind of radical initiation techniques, i.e., chemical [11], photo-initiation [15] and gamma irradiation [16], have been employed to graft-polymerize acrylate-based derivatives for adding epoxide groups onto monolithic adsorbents. In the present study, GMA was graft-polymerized onto monolithic cryogels using both chemical as well as gamma irradiation initiation techniques. Compared to previous reports [16, 17], the scope of this work is to compare the effects of using different graft-initiation procedures to develop efficient weak anion-exchange adsorbents. Their physico-chemical properties were characterized by various methods, while their chromatographic properties were assessed in terms of the binding capacities of BSA at various operational flows rates.

2. Material and methods

2.1. Chemicals and reagents

Methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), polyethylene glycol dimethacrylate (PEGDA), glycidyl methacrylate (GMA), diethylamine (DEA 99.5%), dimethylamine (40%), ammonium cerium (IV) nitrate (CAN), N,N-dimethylacrylamide (DMA), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Germany). Acetone, ammonium persulfate (APS), sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride (NaCl), N,N,N',N'-Tetramethylethylenediamine (TEMED), sodium hydroxide (NaOH), nitric acid (HNO_3, 65%) were purchased from AppliChem (Germany). Tris (99.3%, buffer grade) was purchased from Carl Roth (Germany). Ethanol absolute was purchased from Honeywell specialty chemicals Seelze GmbH (Seelze, Germany).

Buffer solutions were filtered through a 0.45 µm cellulose acetate filter (Sartorius, Goettingen, Germany).

2.2. Instrumentation

Tricorn chromatography columns 10/50 (10 mm internal diameter (i.d.) × 4 cm length) and the ÄKTA explorer 100, controlled by Unicorn 4.10 software, were obtained from GE Amersham Bioscience (Uppsala, Sweden). Absorbance was measured at 280 nm for the static capacity determination and was performed using a Shimadzu UV-1700 PharmaSpec spectrophotometer.

Dehydrated monolithic cryogels were coated with gold, and the samples were examined at different magnification using a Joel JSM 5900 (Peabody Inc., USA) scanning

electron microscope (SEM).

2.3. Cryogel synthesis and characterization

2.3.1. Preparation of the cryogel backbone

Megaporous cryogels were prepared as follows: the monomer, MAA (230 mmol), the cross-linkers, PEGDA (95 mmol) and EGDMA (32 mmol), and the catalyst, TEMED (80 mmol), were dissolved in water to give a final volume of 30 mL. This solution was degassed with nitrogen for 20 minutes. Subsequently, APS (27 mmol) was added as an initiator as described previously [16]. The final solution was poured in plastic syringes (16 mm inner diameter, 7 cm height) and kept at −20 °C for 24 hours. The resulting cryogels were thawed at room temperature, washed with 200 mL water, and dried at 60 °C overnight.

2.3.2. Chemical grafting (CG) procedure

A 0.4 g of dried adsorbents was first grafted with 2.5 ml GMA and 50 mg CAN as an initiator containing 31.25 mL of nitrogen purged water (0.1 M nitric acid) for 3 hours at 40 °C. The grafted monolithic cryogels was extensively washed with tap water until a neutral pH was achieved and then dried at 50 °C as described previously [17].

2.3.3. Gamma irradiation (GIR) grafting procedure

A known amount of dried adsorbent was soaked in 50 mL of a nitrogen-purged (20 minutes) monomer solution of GMA (3.2% v/v) and DMA (7.6% v/v) in ethanol/water (1:1 v/v). The mixture was enclosed in a sealed tube to avoid oxygen diffusion during irradiation, which may hamper the grafting. Samples were irradiated with a 10 kGy dose at room temperature (Beta Gamma Service GmbH and Co. KG, Wiehl, Germany). After irradiation, the resulting material was first washed with ethanol/water (1:1 v/v) followed with 96% ethanol and subsequently dried at 50 °C in a vacuum oven [16, 18].

2.3.4. Surface functionalization by DEAE groups

The epoxy groups present on the monolithic cryogels were modified into DEAE groups by reacting them with an aqueous solution of diethylamine (25% v/v), dimethylamine (25% v/v), and ethanol (10% v/v). Finally, the functionalized monolithic cryogels were extensively washed with deionized water until a neutral pH was obtained, and subsequently dried at 50 °C in a vacuum oven [19].

2.3.5. Physico-chemical characterization

The functionalized cryogels were characterized in numerous ways, including SEM (see Figure 2). The degree of grafting (DG) was measured by the elementary weight gain before (W_0) and after (W_1) grafting [17] as shown in Equation 1.

$$\text{DG (g/g)} = \frac{(W_1 - W_0)}{W_0} \times 100 \quad (1)$$

Additionally, the degree of swelling (DS) was measured by weighing the monolithic cryogels in the wet state (m_{wet}) and dried state (m_{dry}) until constant weight was achieved at 60 °C in a vacuum oven [18]. DS was calculated using Equation 2.

$$\text{DS (g/g)} = \frac{(m_{wet} - m_{dry})}{m_{dry}} \quad (2)$$

Finally, the porosity of the monolithic cryogels was estimated by water uptake (Equation 3). A cryogel sample, 3.5 cm in length, was saturated with deionized water. The mass of the wet material saturated with deionized water was denoted as $m_{swollen}$ and the material was squeezed to remove the excess free water present within the large pores as previously reported [18], and the mass of squeezed material was denoted as $m_{squeezed}$. The porosity of the "squeezed" swollen sample was calculated using Equation 3.

$$\text{Porosity (\%)} = \frac{[m_{swollen} - m_{squeezed}]}{m_{swollen}} \times 100 \quad (3)$$

2.4 Column efficiency & ionic capacity

The monolithic cryogels were packed into Tricorn 10/50 columns and swollen in place with 20 mM Tris-HCl buffer (pH 7.4) at very low linear flow velocity (~ 75 cm/h). The column bed height was fixed so that there was no visible headspace in the column. The efficiency of the packed column was evaluated in terms of HETP by using 5% acetone (v/v) at flow velocities ranging from 75 to 600 cm/h.

A measure of ion-exchange capacity of adsorbents can be determined using transient pH measurements from which the number of phosphate ions binding to the adsorbent can be calculated [20]. These "phosphate capacities" were evaluated by using two buffer solutions having same pH, but with different ionic strengths. The functionalized cryogels were equilibrated with 500 mM phosphate buffer at pH 7.4. The mobile phase composition was

then switched to 20 mM phosphate buffer at the same pH. The change in ion concentration induces a change in observed pH as a result of the release of ions that were bound to the matrix in high salt conditions. The time interval of this change, Δt ($|\Delta pH| > 0$), was determined by monitoring the time required in between the switching point of the mobile phase and the point at which 50% of the maximum change in pH value is attained (see Figure 3). The "phosphate capacity" (K) depends upon the volumetric flow rate ($_v$), the column volume (V_c), the concentration of elution buffer (C_2), as well as Δt [18, 21, 22], as shown in Equation 4..

$$K = \frac{[\Delta t(pH)_{50\%} \times \phi_v]}{V_c} \quad (4)$$

2.5. Static and Dynamic Binding Capacities

Static binding capacity (SBC) of the weak anion-exchange adsorbents was determined by batch adsorption experiments with BSA. Cryogels were equilibrated with 20 mM phosphate buffer (pH 7.4) and were subsequently incubated with 10 mg/mL of BSA at room temperature for 3 h. The amount of BSA adsorbed was determined by the differences in the absorbance (OD_{280}) of BSA before and after incubation. Controls experiments revealed negligible non-specific binding of BSA to the non-functionalized adsorbent.

The dynamic binding capacity (DBC) at 10% breakthrough for the BSA with CG- or GIR-DEAE cryogels was determined by frontal studies [23]. The Tricorn 10/50 column was packed and mounted to an ÄKTA explorer 100 system and equilibrated with 20 mM Tris-HCl buffer at pH 7.4. A solution of BSA (2 mg/mL) was loaded at linear flow velocities ranging from 75 - 600 cm/h. The breakthrough volume (V) of the protein solution that was needed to saturate the column was determined by monitoring the UV absorbance at 280 nm [24]. The bound protein was eluted with 1 M NaCl in 20 mM Tris-HCl buffer. The dynamic binding capacities of the adsorbents can be calculated from the initial load concentration (C_0), breakthrough volume (V), column void volume (V_0), as well as either the dry weight of the adsorbent (W_g) or its volume (CV), as shown in Equations 5 and 6 [17].

$$\text{DBC (mg/g)} = \frac{C_0 \times (V - V_0)}{W_g} \quad (5)$$

$$\text{DBC (mg/mL)} = \frac{C_0 \times (V - V_0)}{CV} \quad (6)$$

3. Results and discussion

The cryogel backbone forms at –20 °C by proper mixing of monomers (MAA) and cross-linkers (PEGDA and EGDMA). During the preparation of the backbone, the water that was used as a solvent simultaneously acts as a porogen in the form of ice crystals that are created during the freezing process. This results in pore sizes ranging from 10 - 100 μm [16, 25]. Additonally, the crosslinkers, like PEGDA and EGDMA, provide an excellent mechanical stability to the final backbone of cryogels. They were activated with epoxy groups either by chemical or by gamma irradiation initiation techniques (see Figure 1), where the purpose of using GMA as a graft monomer was two-fold. Firstly, chain elongation in the absence of cross-linkers leads to the formation of a thin film of flexible polymeric tentacles which in turn minimizes unfavorable steric effects that may hinder consequent functionalization as well as product adsorption [26, 27]. Secondly, the epoxide group can be further transformed to impart a range of different functionalities to the material, for example, anion-exchange [27-29], cation-exchange [6, 26, 30], or metal-ion chelating capabilities [17, 31]. The efficiency of grafting depends upon several parameters like monomer concentration, initiator concentration, reaction time as well as temperature, which were optimized for this study.

Water uptake experiments revealed adsorbent porosities of *ca.* 70 ± 5%, which indicates that both grafting procedures did not affect the physical integrity of the pores present within the material. The water content in these sponge-like materials could be easily removed by squeezing. They rapidly regain their original size and shape when re-immersed in water, thereby showing remarkable elasticity [16, 25]. Their degree of swelling was 2.4 ± 0.2 g/g in distilled water at 24 °C, and indicated their remarkably good convective flow properties. The porous structure of these cryogels was further investigated by SEM, as shown in Figure 2. These images suggest that the pores within the cryogels in their dehydrated state are inter-connected and that their pore sizes range from 10 – 100 μm [16, 25] with an average pores size estimated at 50 μm, which is maintained in the hydrated as well (see Supporting Information).

In the same manner as reported by Plieva et al. [32, 33], the cryogel matrices have

been synthesized within the syringes and 're-packed' into chromatography columns by placing the entire cryogel directly into these columns in their dehydrated state. The sponge-like characteristics of polymethacrylate-based cryogels upon swelling ensure uniform and sufficiently tight 'packing' into the column. The advantage of this process over in-column monoliths is in the ease of further functionalization of the matrices, which is necessary for surface grafting. The column efficiency of chromatographic adsorbents are vital for their application in purification systems. The theoretical plate height (HETP) is one such property that determines the overall separation efficiency of a chromatographic column. Using acetone as a non-interacting probe solute, the HETP of the cryogel column was measured (see Figure 3), and the relation between HETP and flow velocity for CG-DEAE and GIR-DEAE was established. In general, desirable HETPs for these materials result from plate heights ranging from 0.5 - 1 mm. In the case of CG-DEAE, these plate heights were unfortunately not realized irrespective of flow rate. However, the plate heights for GIR-DEAE cryogels fell within the sought out range (0.53 - 0.73 mm), thereby highlighting the distinct advantage of the grafted cryogels generated from gamma irradiation as compared to chemical initiation. We therefore decided to focus further adsorption experiments on GIR-DEAE cryogels

The phosphate capacity for GIR-DEAE monolithic cryogels measured by the pH transient method was 119 mmol/L (see Figure 4) [34], and was comparable to the commercially available DEAE sepharose fast flow adsorbents obtained at similar conditions to be 113 mmol/L (0.11-0.16 mmol/mL chloride ion capacity reported by GE Healthcare). Subsequently, more detailed mass transfer studies on the protein binding capacities of the cryogels, in static as well as dynamic modes, were conducted by using BSA a model protein. The effect of different grafting initiator was determined by frontal analysis (see Table 1 and Figure 5), where it was clear that the highest dynamic binding capacity (DBC) was obtained at 75% degree of grafting with a value of 110 ± 10 mg/g. The corresponding static binding capacity, obtained with 10 mg/mL BSA solution, was 180 ± 20 mg/g.

Generally, the degree of grafting is directly correlated with the number of ionic sites on the matrix, and consequently, the binding capacity for the protein. Counterintuitively, however, breakthrough studies revealed that even though the degree of chemically initiated grafting (93%) was higher compared to that for gamma irradiation (75%), its binding capacity for BSA was *ca.* 3 - 4 fold lower (see Figure 5). The results of the breakthrough experiments carried out at different flow rates (at high flow rate) for the adsorbents are expressed by the DBC were compared and contrasted with other reported adsorbents as

shown in Table 1. A clear existence of mass transfer resistance with increasing flow rates is observed. The 10% DBC values for CG-DEAE and GIR-DEAE were measured as 5 ± 2 mg/mL and 27 ± 3 mg/mL, respectively, and these values are higher or comparable to the DBC values reported in the literature for various monoliths, for example, weak anion-exchange (WAX) pAAm cryogels [27], BIA Separations monolith adsorbents (CIM DEAE disks) [11], and PEI modified WAX on a poly(GMA-co-EDMA) [35]. Furthermore, at higher flow rates, GIR-DEAE showed higher binding capacities for BSA than selected traditional commercial beads available in the market (Toyopearl DEAE 650M, MacroPrep DEAE) [36]. DBCs of 42 mg/mL were obtained from our experiments with DEAE Sepharose FF at a flow rate of 300 cm/h. However, DEAE sepharose Fast Flow and DEAE Ceramic Hyper D 20 outperformed the WAX cryogel synthesized in the current report [36]. The lower DBC values compared to particulate chromatographic media are due to lower accessible surface area in cryogels, where it is evident from the SEM images that the monolithic cryogels have pore sizes in the range of 10-100 μm (see Figure 2).

It has been reported in the literature that the particulate chromatographic media has about 2 - 3 times the surface area of highly porous monoliths [37]. The accessible surface area of cryogels, however, may possibly be increased either by reducing the pore sizes during backbone formation or by increasing the length of polymer chains for introduction of epoxy groups. Nevertheless, the dynamic binding capacities of our cryogels for BSA were independent of flow rates of up to 600 cm/h, ensuring convective flow distribution. This was evident from the negligible reduction in binding capacity they displayed (27 ± 3 mg/mL for GIR-DEAE) when compared to commercial available packed bed adsorbents, where DBCs are reduced by almost half upon increase in flow rates. Additionally, the Péclet number for GIR-DEAE decreases with an increase in flow rate suggesting negligible axial mixing (see Figure 5), and the obtained Péclet numbers of 77 and above additionally confirm the favorable plug flow characteristics of these cryogels. The cryogel matrices are scalable by connecting the matrices in series within a column to any production scale without losing its efficiency [38-41].

4. Conclusions

We have illustrated two different ways to efficiently graft GMA onto monolithic cryogels. In the case of gamma irradiation graft-initiation, about 75% grafting was possible. The results of this study also demonstrated that gamma irradiated DEAE monolithic cryogels exhibit a 4-fold higher binding capacity for BSA than their chemically grafted counterparts.

The highest binding capacity of 110 ± 10 mg/g was achievable at 75% degree of grafting. It might even be possible to further increase this capacity by reducing the pore sizes of the cryogels. Since the hydrodynamic properties were preserved, grafted cryogels may become a resin of choice for the downstream processing of various biomolecules. Furthermore, these adsorbent matrices were shown to have relatively higher capacities for protein at high flow rates. Not only are these cryogels considerably cheaper than commercially available adsorbents, they have also be shown to be easily scalable [42].

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421

422 Figure Captions

423 **Figure 1:** Reaction scheme for graft polymerisation and derivatization of the epoxy groups
424 to a DEAE functionality by either cerium ion-initiated or gamma ray-initiated routes.

425 **Figure 2:** SEM photographs of base material (untreated backbone, 2a and 2b), epoxy
426 backbone of monolithic cryogels (2c and 2d), and derivatization of the expoxy groups to a
427 DEAE functionality (2e and 2f).

428 **Figure 3:** Column efficiency versus flow velocity for GIR-DEAE (■) and CG-DEAE (●),
429 using 10 μL pulse of acetone (5% v/v) and a mobile phase of 20 mM Tris-HCl buffer (pH
430 7.4).

431 **Figure 4:** Determination of Δt ($|\Delta pH| > 0$) from pH profile for GIR-DEAE at a flow rate of
432 300 cm/h by switching between buffer A (500 mM phosphate at pH 7.4) and buffer B (20
433 mM phosphate at pH 7.4).

434 **Figure 5:** a) Dynamic binding capacities (mg/mL - solid lines and mg/g - dotted lines) for
435 GIR-DEAE (■) and CG-DEAE (●) at different flow rates using BSA (2 mg/mL) and a
436 mobile phase of 20 mM Tris-HCl buffer (pH 7.4). b) Corresponding Péclet numbers at
437 different flow rates using 10 μL pulse of acetone (5% v/v) and a mobile phase of 20 mM
438 Tris-HCl buffer (pH 7.4). All experiments have been repeated at least 3 times to confirm the
439 results and have an error of 5% in measurement.

440

Tables with Captions

Table 1: Comparison of DBCs of various anion-exchangers. 2 mg/mL solutions of BSA were used for DBC measurements, while 10 mg/mL solutions of BSA were used for static binding experiments (incubation time of 3 h).

Adsorbents	DBC at 10% (300 cm/h)	SBC (mg/mL)	Phosphate Capacity (mmol/L)
GIR-DEAE	26 mg/mL	44 ± 4	119
CG-DEAE	3 mg/mL	8 ± 1	-
8% pAAm Cryogels	1.3 mg/mL[a]	-	-
CIM DEAE	≤ 21 mg/mL[b]	-	1200
WAX poly(GMA-co-PEGDA)	32 mg/mL[c]	-	-
Poly(DEAEMA-co-PEGDA)	24 mg/mL[c]	-	-
PEI poly(GMA-co-EDMA)	14 mg/mL[d]	-	-
Toyopearl DEAE 650 M	20 mg/mL[e]	-	-
MacroPrep DEAE	16 mg/mL[e]	-	-
DEAE Sepharose FF	42 mg/mL	-	113

[a]Taken from reference (295 cm/h) [14]. [b]Taken from reference [11]. [c]Taken from reference (203 cm/h) [8]. [d]Taken from reference (150 cm/h) [36] [e]Taken from reference (600 cm/h) [37]

446 • Highlights

447 • Anion-exchange monolithic adsorbents have BSA binding capacity of 110 mg/mL

448 • Cryogels are grafted with radiation-induced polymerization of methacrylate
449 monomers.

450 • Higher binding capacities for radiation-induced grafting over chemical grafting.

451 • Adsorbent pore sizes range from 10 - 100 μm with elastic sponge-like structure.

452

Figure 1

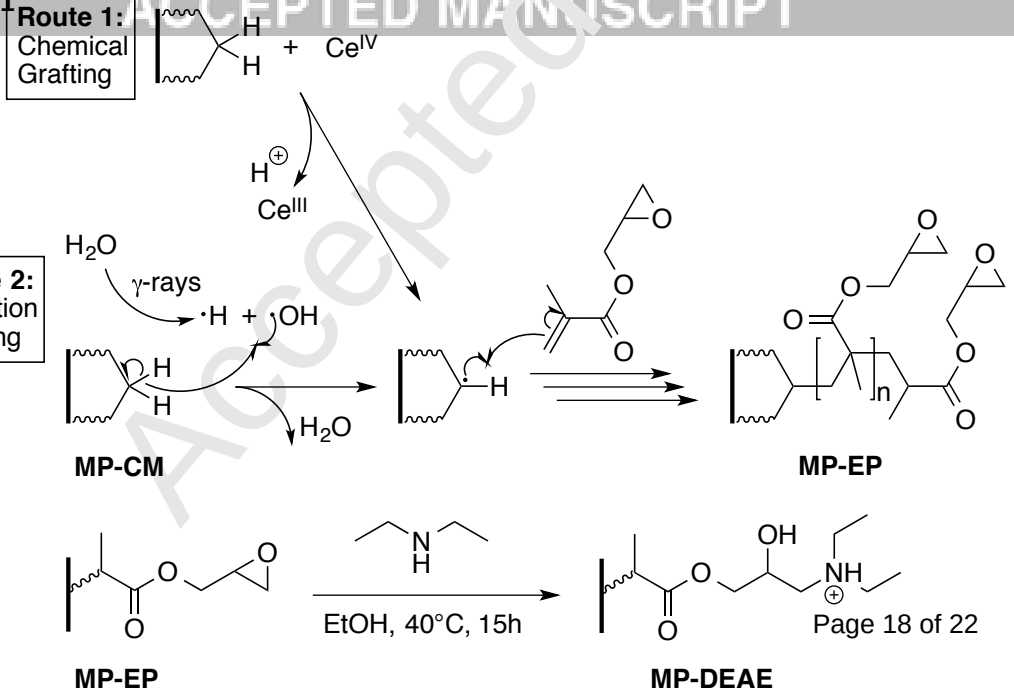
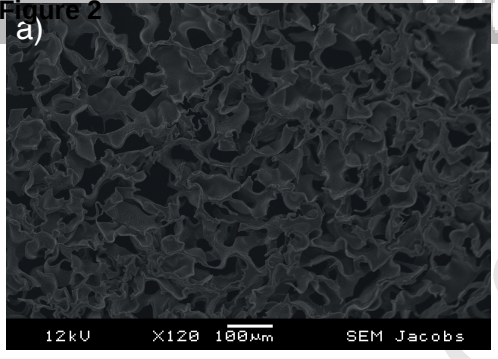
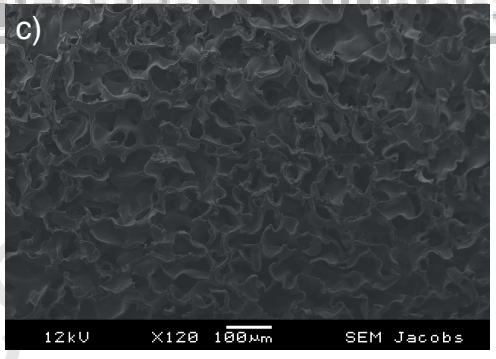


Figure 2


a)
12kV X120 100µm SEM Jacobs


c)
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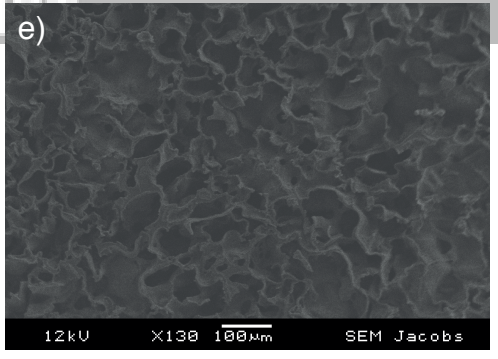
e)
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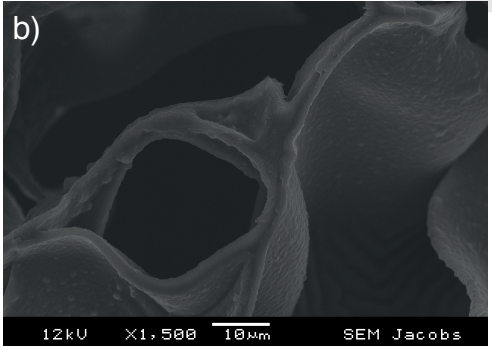
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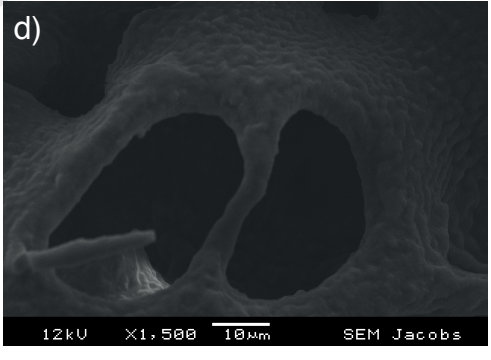
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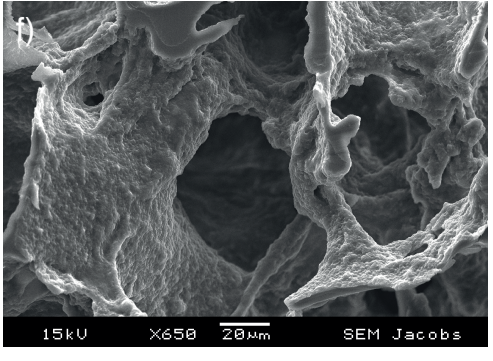
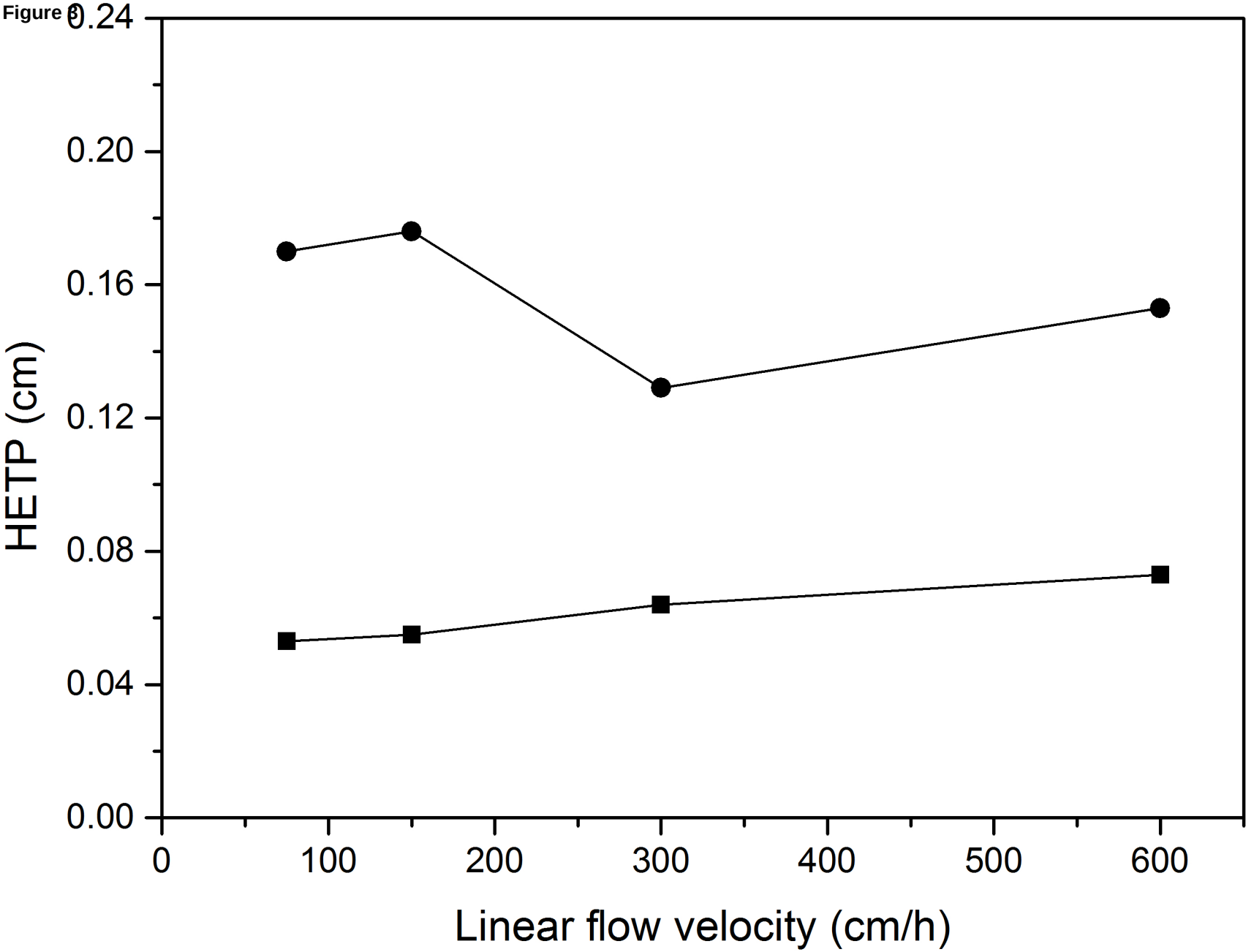
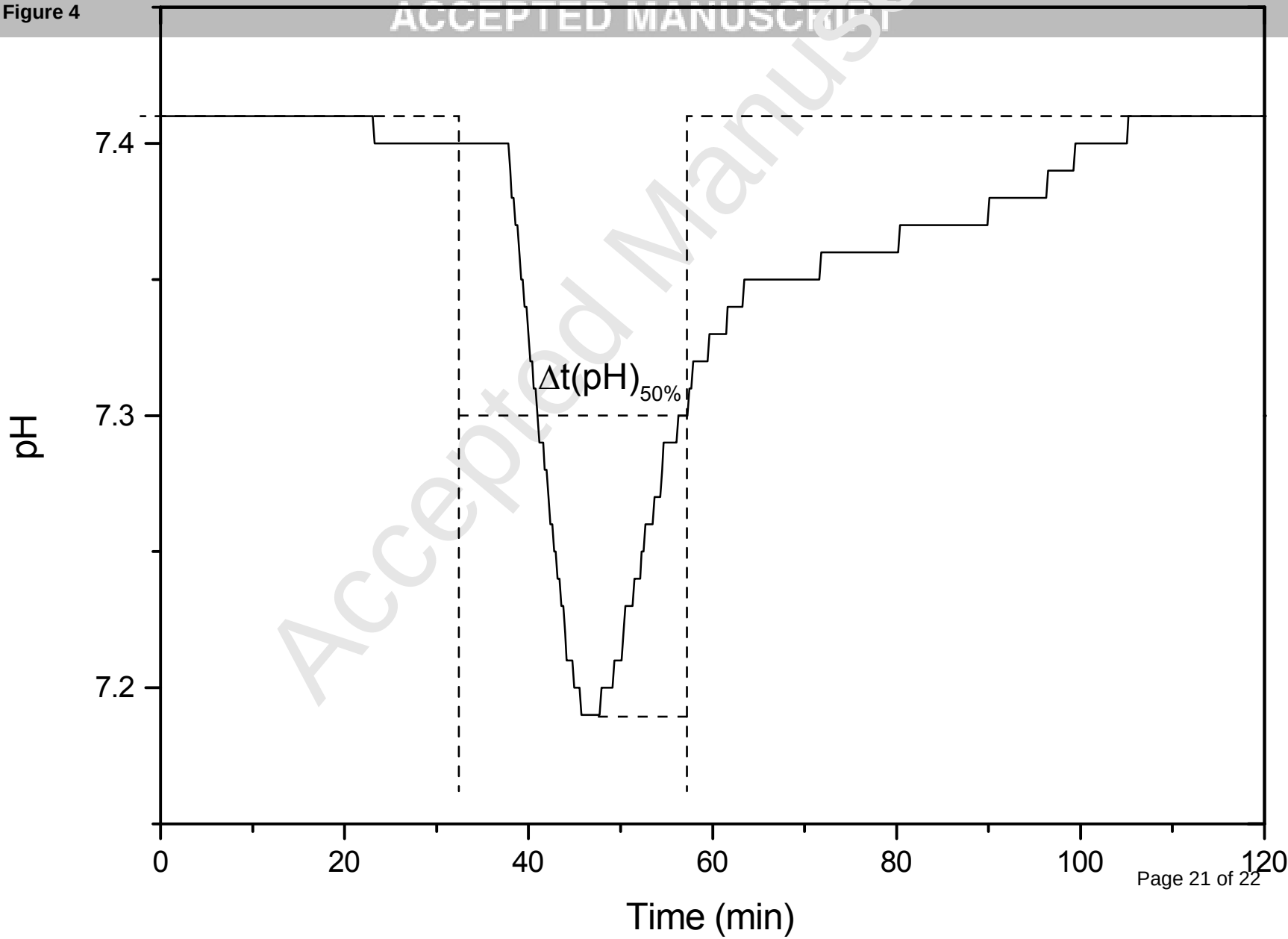
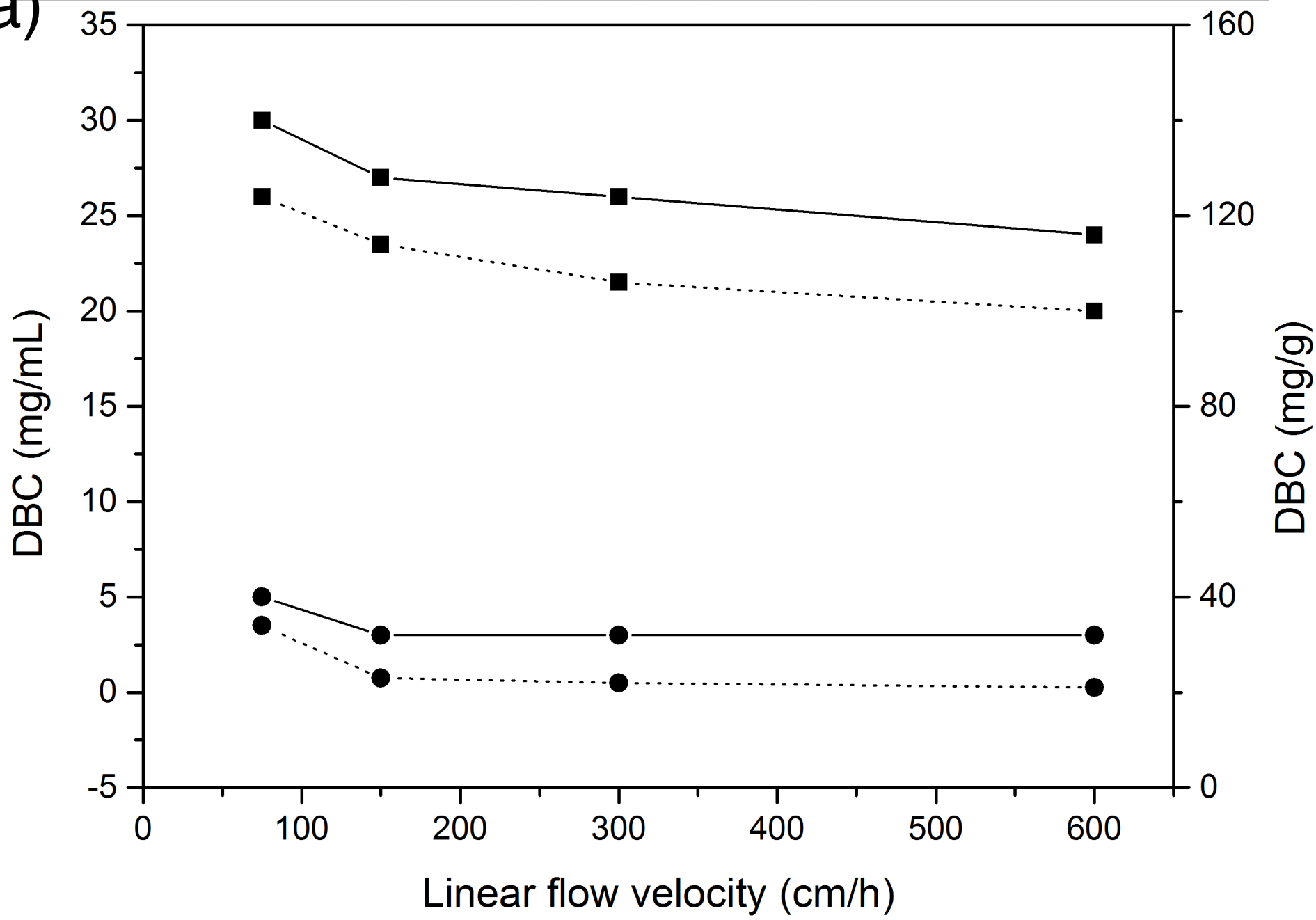

f)
15kV X650 20µm SEM Jacobs

Figure 3


0.24
0.20
0.16
0.12
0.08
0.04
0.00
HETP (cm)
0
100
200
300
400
500
600
Linear flow velocity (cm/h)



a)



b)