Gözde Gözke¹ Frank Kirschhöfer²Carolin Precht³Gerald Brenner-Weiss²Nikolay Venelinov Krumov⁴Ursula Obst²Clemens Posten⁵

Research Article

Electrofiltration improves dead-end filtration of hyaluronic acid and presents an alternative downstream processing step that overcomes technological challenges of conventional methods

¹Department of Chemical and Process Engineering, Faculty of Engineering, Yalova University, Yalova, Turkey

²Institute of Functional Interfaces, Karlsruhe Institute of Technology, Leopoldshafen, Germany

³Vetter-Pharma GmbH & Co. KG, Ravensburg, Germany

⁴Clinical Development and Licensing, Lonza AG, Visp, Switzerland

⁵Institute of Process Engineering in Life Sciences, Bioprocess Engineering, Karlsruhe Institute of Technology, Karlsruhe, Germany

Hyaluronic acid (HA) dispersion obtained from the bacteria *Streptococcus equi* was concentrated by electrofiltration. In the conventional downstream processing of HA, extraction and precipitation lead to increase in environmental issues, structural changes, and time and energy related costs. Using electrofiltration as an alternative technology delivers solutions to these limitations. Experiments were conducted in order to test the applicability of electrofiltration to downstream processing of the negatively charged HA. The structural changes and molecular weight distributions, often a consequence of the employed separation method, were tested by analysis of the initial dispersions and final products. In comparison to the conventional filtration, concentration factors were increased up to almost four times without any detectable structural change in the final product.

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1 Introduction

Hyaluronic acid (HA) is an anionic linear polysaccharide composed of alternating glucuronic acid and *N*-acetylglucosamine units with a molecular weight in the range of 10^4 – 10^7 Da depending on its origin and production technology [1–3]. HA is present in the extracellular matrix of vertebrate epithelial, neural, and connective tissues. Physicochemical properties such as biodegradability, hydrophilicity, and biocompatibility define HA as an important biopolymer with a variety of biomedical, pharmaceutical, and cosmetic applications [4–6]. Depending on applications, the value of HA products varies from US\$ 2000 to 60,000 per kilogram [7].

Commercially, HA is either extracted from rooster combs or produced by fermentation of certain strains of *Streptococcus*, which synthesize the biopolymer as part of their outer capsule [8–10]. Extraction of HA from rooster combs has some dis-

advantages such as probability of inflammatory reactions. Additionally, HA obtained from animal sources forms complexes with proteoglycans, which complicates the purification process and increases production costs [11]. Besides these disadvantages, optimization potential of product yield and quality through control of cultural conditions and genetic engineering justify the recent increased interest in the microbial production of HA [7].

Common separation and purification of HA mainly comprises precipitation with organic solvents such as ethanol, acetone, isopropanol, etc. Nevertheless, the use of organic solvents is a time-consuming process, not environmentally friendly and also can lead to change in the quality of HA [12, 13]. Molecular weight distribution being a key parameter for the quality of the product is highly affected by the applied purification strategy [14]. In biotechnology industry, the current downstream processing trends are directed toward integrated, faster, and more effective processes, incorporating mild operational conditions. Alternative approaches including membrane filtration can be introduced to solve the technological challenges. However, pressure-driven membrane processes lead to membrane fouling and subsequent permeate flux decline [15].

On the other hand, most biopolymers are charged and application of an electrical field leads to the migration of molecules

Correspondence: Dr. Gözde Gözke (gozde.gozke@yalova.edu.tr), Department of Chemical and Process Engineering, Faculty of Engineering, Yalova University, Kazım Karabekir Mah. Rahmi Üstel Cad. No. 1, Yalova 77100, Turkey

Abbreviations: HA, hyaluronic acid; RI, refractive index

Figure 1. Lab-scale electrofiltration cell. Product chamber (in the middle) and flushing chambers (on the left and right sides).

in electric field [16]. The electric charge of HA enables its purification by electrofiltration, which is a separation technique combining membrane filtration and electrophoresis in a dead-end process. The aim of this work was to investigate the applicability and potential of electrofiltration as an alternative downstream strategy in biotechnological production of HA. By application of both pressure and electric field, an improvement of the filtration kinetics was expected. Structural changes in HA and molecular weight determinations are the critical factors affecting the applicability.

2 Materials and methods

2.1 Materials and experimental conditions

HA was purchased from Sigma-Aldrich (Seelze, Germany) with product number 53747, which was produced by *Streptococcus equi*. Investigations were performed in a lab-scale electrofiltration cell designed with flushing electrodes (Fig. 1) and the detailed experimental approach was previously described [17]. Online control of filtrate mass, pH, and conductivity was performed in order to ensure the proper process realization. The use of flushing chamber system enables pH and temperature control, prevents the contact of the biopolymer with electrodes, and rinses out the electrolysis products formed at the electrodes. The various buffer solutions, which were prepared according to the conductivity and pH of the initial HA dispersions, contained potassium dihydrogen phosphate–dipotassium hydrogen phosphate (buffer I), potassium hydrogen phthalate–NaOH (buffer II), and acetic acid–sodium acetate (buffer III). HA was dispersed in demineralized water with a concentration of 2 g/L and pH of 5.6. In order to obtain reproducible results, all experiments were repeated several times and new membranes with a surface area of 0.001 m² (polyethersulfone, 0.1 μm; Pall, USA) were used for each experiment.

2.2 Characterization methods

2.2.1 Zeta potential measurements

Zeta potential is an important characteristic defining the stability and electrophoretic mobility of the products. In order to evaluate

the electrofiltration capacity, zeta potential measurements of HA dispersions were performed using Malvern Zetasizer 5000 with PCS software V1.52, Rev1 (Malvern Ltd, UK). Each sample was measured five times and a purchased standard solution (Malvern Ltd.) was used for calibration.

2.2.2 Molecular weight distribution

As a key parameter for the quality of the product, the molecular weight of HA was determined by using SEC combined with multiangle laser light scattering (DAWN DSP; Wyatt Technology Corporation, Santa Barbara, CA, USA) and refractive index (RI) detection. The system was composed of an Agilent 1100 Series Chromatography system with an RI detector using a TSKgel G6000PWxl column (Tosoh Bioscience GmbH, Stuttgart, Germany) of 7.8 mm inner diameter and 30 cm length with a guard column. The multiangle laser light scattering detector was calibrated with Toluene (VWR, Germany) and normalized with BSA (5 mg/mL) in 30 mM ammonium acetate buffer. RI calibration constants were generated with anhydrous sodium chloride. Analyses were performed at room temperature using ammonium acetate buffer as the mobile phase at a flow rate of 0.8 mL/min. A sample volume of 100 μL was injected and the average of three replicates was used for evaluation.

2.2.3 FT-Raman spectroscopy

The influence of the purification technology upon the structure of the product was estimated by applying FT-Raman spectroscopy. The used FT-spectrometer Vertex 80 with attached Raman module RAM II (Bruker Optik GmbH, Germany) employed a 1064 nm NdYAG excitation laser, an interferometer, and a high sensitivity near infrared Ge-detector. For Raman analysis, an adequate amount of each sample was lyophilized for at least 20 h. The applied laser power was maintained at 400 mW in order to improve data comparison. The received spectra were evaluated by OPUS 6.5 software (Bruker Optik GmbH).

3 Results and discussion

3.1 Filtration kinetics is influenced by process parameters

The effects of applied electric field strength and pressure were investigated by varying both parameters. The t_f/V_f (filtration time/filtrate volume) versus V_f (filtrate volume) graphs interpreted the filtration characteristics based on the slope alteration of the curves, describing the specific filter cake resistances. The standard progress of electrofiltration experiments has three distinct sections divided according to the changes in specific filter cake resistances [18]. In the section I due to the higher effect of hydrodynamic resistance force in comparison to the electrophoretic force, the specific filter cake resistance is high. After the equilibrium of the forces in section II, no further filter cake is formed on the cathode side for a negatively charged product. In the section III, as a result of the constant accumulation of product on the anode side membrane, the formed filter cake

Figure 2. t_f/V_f versus V_f diagrams of HA dispersions under conditions of 2 V/mm and 1 bar with various buffer solutions (pH 5.6, temperature: 15°C) used. Buffer I: dihydrogen phosphate–dipotassium hydrogen phosphate, Buffer II: potassium hydrogen phthalate–NaOH, and Buffer III: acetic acid–sodium acetate.

reaches the cathode side filter film, increasing the specific filter cake resistance.

In order to determine the influence of specific buffer solutions on the filtration kinetics of HA dispersions, several experiments with various buffer compositions were conducted. The t_f/V_f versus V_f graph of the applied buffer solutions is shown in Fig. 2.

All curves in Fig. 2 representing specific filter cake resistances exhibit almost the same tendency in the first two sections of the curves. However, the experiments using buffer II and buffer III delivered, in the third section, a higher specific filter cake resistance in comparison to the experiment with the buffer I. The result could be explained by the conductivity of the membranes, which depends on the variety of ions. Concerning the lower specific filter cake resistance, the use of buffer I led to higher permeate mass for the same experimental time and therefore was used for further experiments. The experiments demonstrated that different buffer solutions have different influences on filtration kinetics and could be further investigated for a detailed consideration.

The effect of various process parameters upon the efficiency of electrofiltration of HA dispersions was investigated by conducting different experiments with 1, 2, and 4 bars overpressure with and without applied electric field. The effect of applied pressure on filtration kinetics of HA dispersions is demonstrated in Fig. 3 based on the gradient of filtrate mass per membrane surface area.

According to Darcy's law, application of higher pressure results in more mass of filtrate. However, compressibility of the biopolymers hinders the proportional increase in filtrate mass with pressure. According to the initial HA concentration (2 g/L), concentration factors were 2.8, 4.5, and 5 for 1, 2, and 4 bars conditions, respectively, and the obtained corresponding average filtrate fluxes were determined to be 15, 23, and 25 L/m²h. The differences between the permeate masses obtained with 2 and 4 bars are insignificant within the experimental frame. Fig-

Figure 3. Filtrate mass/membrane area versus filtration time diagrams of HA dispersions at 0 V/mm with different applied overpressures (pH 5.6, temperature: 15°C). Gradient of filtrate mass by the increase in pressure presents the compressibility of the product.

Figure 4. t_f/V_f versus V_f diagrams of HA dispersions at 4 bars with different applied electric field strengths (pH 5.6, temperature: 15°C). The slope of the curves is characteristic for the specific filter cake resistances.

ure 3 demonstrates that application of higher pressure compresses the HA filter cakes, increasing filter cake resistances. Without application of electric field, the hydrodynamic resistance force is the only force that has effect on the filter chamber and the filter cakes are formed on anode and cathode side membranes. The compression results in the blocking of the membranes on both sides, preventing the filtration capacity of HA dispersions.

The effect of applied electric field is demonstrated in Fig. 4 in terms of t_f/V_f versus V_f and compared with conventional filtration. HA contains carboxylic groups designating its negative charge [19]. The zeta potential of HA dispersion was determined to be −37 mV at pH 6. Based on the high migration capability

Figure 5. Filter cake composition of HA at 4 bars and 4 V/mm applied. The product has high viscosity with a transparent appearance.

of the molecules, experiments revealed promising results considering the filtration technology of HA dispersions. The charge facilitates the separation of the biopolymer by means of electric field. The application of electric field leads to a decrease in specific filter cake resistances as demonstrated by the slopes of the curves. The experiment without electric field is characterized by a steeper curve compared to the ones derived after application of electric field of 2 and 4 V/mm. The results present the high positive effect of electric field on the filtration kinetics of HA. This result is based on the electrophoretic migration of negatively charged HA molecules toward anode side membrane. Cathode side membrane remains almost free of filter cake allowing an undisturbed perfusion of the filtrate. The experiments under electric field follow the already described theoretical division of the electrofiltration process into sections. However, only the experiment with 4 V/mm reached the third section where the specific filter cake increases significantly. The result was caused by the rapid filtration of the dispersion with higher applied electric field strength. The concentration factors at 4 bars were 5, 15, and 18 for 0, 2, and 4 V/mm conditions, respectively, and the corresponding average filtrate fluxes were determined to be 22, 60, and 80 L/m²h for the elapsed time. In comparison to conventional filtration, the concentration factor based on the permeate mass of samples was increased up to almost four times for the same experimental time for HA dispersions, when 4 V/mm electric field strength under 4 bars was applied. For a defined filtrate mass (49 g), the filtration duration was 3 h without application of electric field, whereas for the same amount of filtrate mass the duration was only 14 min representing a time saving of approximately 92%. The composition of filter cake after application of 4 bars and 4 V/mm is presented in Fig. 5. Furthermore, the energy need was 8100 J/100 g filtrate, whereas energy requirement for a conventional method such as extraction is higher [20]. In case of higher salt contents as may be present in real fermentation broths, electric current increases with a negative impact on energy balance [21]. Ongoing research is targeted to overcome the limitations by in situ desalination during the filtration process.

Figure 6. Raman spectra of HA samples before and after electrofiltration at a pressure of 4 bars and applied electric field strengths of 0, 2, and 4 V/mm. No structural deformation was detected by the increase in electric field strength.

3.2 Product characteristics are not significantly influenced by electrofiltration parameters

The alteration of the chemical structure of HA is another criterion to evaluate the applicability of electrofiltration. In order to detect possible changes in the structure due to the applied electric field, samples which were taken before and after electrofiltration were analyzed using FT-Raman spectroscopy. The spectra of the HA samples derived from experiments under maximum applied pressure (4 bars) with 0, 2, 4 V/mm are presented in Fig. 6 together with the sample of unprocessed HA dispersion.

The main bands are composed of N-H stretching (1), C-H stretching (2), Amid I and C=C stretching (3), C-N stretching and C-H deformation (4), C-H bend (5), Amid III (6), and C-C and C-O stretchings (7) [22]. The results reveal no significant difference between the spectra before and after electrofiltration and, respectively, the different experimental conditions. Therefore, the application of electrofiltration expresses no negative effect on the structure of HA molecules. Slight differences between the intensity of the HA spectra were caused by diminutive differences in molecule size. However, in general there was no detectable oxidative or reductive change in any functional group induced by electrofiltration.

Another important product characteristic of HA influencing its properties is the molecular weight. With (4 V/mm) and without applied electric field (0 V/mm), the average molecular weight remained in the range of 451–462 kDa (Table 1). Together with the Raman spectra, no structural change is revealed with respect to molecular weight. The evaluation of possible structural changes and characteristics arising from the purification method demonstrated that electrofiltration is a relevant and promising technique in downstream processing of HA delivering required unchanged specifics.

4 Concluding remarks

The present work suggests electrofiltration as a novel downstream processing method in the production of HA. In the state

Table 1. The weight average molecular weights (M_w) and the number average molecular weights (M_n) of samples obtained after application of 0, 2, and 4 V/mm

Electric field strength (V/mm)	M_w in Da $\pm$ SD	M_n in Da $\pm$ SD	Polydispersity
0	4.51 E5 $\pm$ 1.3%	6.29 E5 $\pm$ 1.3%	1.4
2	4.51 E5 $\pm$ 3.1%	6.44 E5 $\pm$ 6.8%	1.4
4	4.62 E5 $\pm$ 0.7%	6.37 E5 $\pm$ 1.0%	1.4

of the art of HA production processes, filtration is not possible, as presented in the experiments without electrical field. Increasing HA concentration in technical scale is only possible by precipitation with organic solvents. The inherent disadvantage of using organic solvents can be overcome by electrofiltration making HA production potentially more sustainable. The results confirmed that the application of electric field in the filtration technology of HA dispersions reduces processing time and increases concentration factors compared with no electric field applied.

It has been demonstrated that the average molecular weight and structural composition of HA remained the same after introduction of electric field during processing. In addition, the influence of different buffer solutions upon filtration kinetics was explored. The obtained results established electrofiltration as an alternative technology to common downstream processes. Depending on production procedure, salt content of fermentation broth varies, and consequently influences filtration behavior. As a result of increased ionic strength, electric current increases leading to an increase in energy consumption. In order to reduce overall energy requirement, integration of an in situ desalination system into the electrofiltration set up can be a valuable contribution. For the optimization of the purification process and its successful implementation in industrial-scale technologies, further experiments including separation of HA after biotechnological production are required.

Practical application

Hyaluronic acid is a high molecular weight polysaccharide present in the extracellular matrix of mammalian connective tissues as well as in some bacteria. Separation of hyaluronic acid is usually performed by application of organic solvents, which leads to high costs and change in product quality. Owing to surface charge of hyaluronic acid caused by carboxylic groups, electrofiltration can be applied as an alternative technology. Instead of conventional methods in the downstream processing of the biopolymer, application of electrofiltration results in a significant increase in concentration and the lack of unwanted structural changes in HA molecules. Therefore, electrofiltration opens new possibilities for HA production meeting the current downstream processing trends.

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