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Optimization of a chromatographic stationary phase based on gellan gum using central composite design

A.I.C. Gonçalves [a], L.A. Rocha [a], J.M.L. Dias [b], L.A. Passarinha [a,*], A. Sousa [a]

[a] CICS-UBI – Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, 6201-506 Covilhã, Portugal
[b] University of Cambridge, Cambridge System Biology Centre, Sanger Building, 80 Tennis Court Road, Cambridge CB2 1GA, England, United Kingdom

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ABSTRACT

To develop a new stationary phase of easy production, low cost, biocompatible, biodegradable and low unspecific adsorption, a three-dimensional network was prepared by combining the natural polysaccharide of gellan with divalent cations. The stability of this cation exchange chromatographic matrix was optimized by using an experimental design tool. The optimal conditions proposed for the gellan gel formulation were 48 mM $ZnSO_4$, 0% DMF, 25 °C, 0.75% gellan and 0.5 h. The applicability of gellan matrix was tested by chromatographic assays with three model proteins (bovine serum albumin (BSA), α-chymotripsin and lysozyme). The results showed that the retention occurred in function of the net charge of each protein in MES buffer pH 6.2 and the elution was performed by increase of ionic strength to 750 mM NaCl in MES buffer pH 6.2. Lysozyme was the more retained protein due to its positive charge more effective than α-chymotripsin, while BSA did not interact with the matrix due to its negative charge at these conditions. Dynamic binding capacity assays were accomplished to characterize this matrix and to compare with commercial resins. The values of dynamic binding capacity from gellan gel were 3.9 mg/mL and 17.4 mg/mL, at 10% and 50% of breakthrough, respectively. In this way, gellan gel might be a promising chromatographic matrix to explore ionic interactions and to be applied in different purification strategies, getting the best benefit from its use at low cost.

1. Introduction

Chromatography is one of the most studied methods, due to its simplicity, versatility and high reproducibility, to separate and purify biomolecules that can have therapeutic interest. Depending on the physicochemical characteristics of target biomolecules and matrices, several chromatographic techniques can be used, by exploring different interactions between the support and these molecules. Basically, there are two mechanisms used for chromatographic separation of proteins: adsorption (ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography and affinity chromatography) and molecular filter chromatography (size exclusion chromatography) [1]. Thus, the main goal of chromatography is to separate the target biomolecule from the contaminants [2,3]. Ideally, a chromatographic stationary phase should be selective, macroporous, with low unspecific adsorption and high binding capacity, present an adequate mass transfer, incompressible, reusable and mechanical and chemically stable [4–6].

Ion exchange chromatography is amply utilized in separation and purification of proteins and biopharmaceuticals due to its simple methodology, which preserves the biological activity of the target biomolecule [7,8]. Additionally, this chromatographic technique offers a high resolution, and it can be performed in the presence of non-charged detergents and high urea concentrations [5]. The interaction between the matrix and target molecules is based on their opposed net charges, being this chromatographic principle divided in two subtypes [8]. If the matrix is negatively charged it is applied anion exchange chromatography, whereas, when the matrix has a positive charge it corresponds to cation exchange chromatography [9].

Gellan gum has some properties that are important in a chromatographic matrix, including porosity, hydrophilicity, high binding capacity and negative charge to establish ionic interactions with positively charged biomolecules. In addition, this polymer in the presence of metallic ions forms clear gels, resistant to temperature and extreme acidic conditions [10–13].

* Corresponding author at: Health Sciences Research Centre (CICS-UBI), Faculty of Health Sciences, University of Beira Interior, Av. Infante D. Henrique, 6200-506 Covilha, Portugal. Tel.: +351 275 329 069; fax: +351 275 329 099.
E-mail addresses: lpassarinha@fcsaude.ubi.pt, lapp@ubi.pt, lapp@bi.pt (L.A. Passarinha).

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Gellan gum is an anionic high molecular weight polymer with a linear monomer consisting of β-D-glucose–glucuronic acid–glucose–α-L-rhamnose residues [14,15]. This polymer can be produced in an extracellular mode by microbial fermentation of *Sphingomonas elodea* and it is commonly used in the food processing industry as a thickener and stabilizer, nonetheless, it has been recently investigated in other areas [16–19].

Gellan gum gelation can occur by combining the conditions of low temperature and pH with the presence of cations, because this phenomenon involves the formation of double helical junction zones followed by aggregation of the double helical segments to form a three-dimensional network by complexation with cations and hydrogen bonds with water [11,20]. Divalent cations are more effective than monovalent cations, because they are necessary in less quantity and the direct "cross-link" occurs decreasing the electrostatic repulsion between helices [10]. The opposite happens with monovalent cations, which form indirect "cross-linking" of the gellan double helix. Therefore, the presence and progressive addition of divalent cations form ordered structures, becoming the gel extremely thermostable [21–23].

In the present work, taking into account that gellan gum has negative charge, the preparation of a gellan chromatographic matrix and its applicability in cation exchange chromatography will be explored with three model proteins.

2. Experimental

2.1. Materials

All the solutions were prepared using deionized water with ultra-pure grade, purified with a Milli-Q system from Millipore/Waters (Billerica, MA, USA). Gellan gum (Gelzan™ CM), 4-morpholineethanesulfonic acid (MES), zinc sulfate ($ZnSO_4$), *N,N*-dimethylformamide (DMF), lysozyme, bovine serum albumin (BSA), α-chymotrypsin and sodium chloride (NaCl) were acquired from Sigma–Aldrich (St. Louis, MO). Acrylamide 30%/Bis-acrylamide solution was obtained from BioRad (Hercules, CA). Tris (hydroxymethyl)aminomethane was bought from Fisher Scientific (Epson, United Kingdom). Sodium dodecyl sulfate (SDS) was acquired from Himedia (Mumbai, India). Ammonium persulfate (PSA) was obtained from Eurobio (Courtaboeuf, France). N,N,N′,N′-tetramethylethylenediamine (TEMED) was purchased from Merck (Darmstadt, Germany). NZYTech color protein marker II was bought from NZYTech (Lisbon, Portugal). The employed buffers were filtered through a 0.20 μm pore size membrane (Schleicher Schuell, Dassel, Germany) and ultrasonically degassed.

2.2. Gellan gels stability assays

To evaluate the stability of gellan gels, several experimental conditions were tested. Accordingly, for the gel formulation, the following factors were tested: gellan concentration (%, w/v), counter ion concentration, reaction time and temperature. Finally, the contribution of presence or absence of DMF (%, v/v) to increase the gels stability was also studied. The counter ion studied was zinc. Gellan gels were prepared taking into account changes in concentration of gellan gum (0.75–2%), zinc (30–120 mM), reaction time (0.5 h to overnight), temperature (temperature ambient – 110 °C) and amount of DMF (0–30%). The gel formulation consisted in dissolving the counter ion in water, with constant magnetic stirring at room temperature. Then, DMF was added and the solution was progressively heated. Afterwards, gellan addition was performed with the mixture being kept with a constant magnetic stirring of 300 rpm for a determined reaction time. Finally, the obtained suspension was left at room temperature in order to cool it down. After this process, 15 mL of gellan gel (12 mm diameter × 130 mm long) was packed in an Econo-Pac column (BioRad, Hercules, CA) and equilibrated with 10 mM MES buffer pH 6.2. The column also contains a surface area water content of 1.13 mm^2 and a void volume of 0.22 mL (12 mm diameter × 2 mm long). Stability assays were then processed, which consist on registering the time that 15 mL of 10 mM MES buffer pH 6.2 took to pass through each gel formulation before losing its integrity. Consequently, this strategy allowed assessing the number of column volumes (CVs) that each gel formulation remained without being affected by the loss of stability.

2.3. Experimental design

After testing several gel formulations, a central composite design (CCD) was applied to define ideal experimental conditions that allowed a more stable gel.

2.3.1. Artificial neural network

A feed-forward artificial neural network was applied to predict the number of column volumes as function of the concentration of gellan gum, $ZnSO_4$, DMF, time and temperature. The ANN models were implemented in MATLAB™ using the Neural Network Toolbox. The ANN structure included an input layer with five neurons (one for each input variables), an output layer with one neuron (number of column volumes) and one hidden layer with four neurons (5/1/4), resulting in 29 model parameters. The transfer function of the input and output layers was linear function 'purelin' and of the hidden layer was log-sigmoid function 'logsig'. The output function can be represented according to the following expression:

$$f(x, w) = w_{output} \cdot s(w_{hidden} \cdot x + bias_{hidden}) + bias_{output}$$

with w_{hidden}, w_{output}, b_{hidden} and b_{output} the parameter matrices associated with connections between the nodes of the network, w is a vectored form of the previous parameters and s is the activation function:

$$s(t) = \frac{1}{1 + e^{-(t)}}$$

with t representing:

$$t = w_{hidden} \cdot x + bias_{hidden}$$

The ANN structure was built using the "newff" function. ANN was trained with the Levenberg–Marquardt back-propagation function, up to 1000 epochs, using the "train" function. The learning rate and the momentum constant were set at 0.01 and 0.9. The remaining training parameters were set at the default values defined by MATLAB™. The input and output data were scaled according to the 'mapminmax' function. The well-known early stopping method was used, i.e. the mean squared error between measured and predicted number of column volumes of the validation dataset was monitored during training. The training was stopped when the validation increases over a certain threshold. The ANN was trained until either the maximum number of epochs or the performance goal was reached.

2.3.2. Sensitivity analysis

A sensitivity analysis was performed to elucidate the relative importance of the inputs variables on the prediction of number of column volumes. This analysis was implemented according to the following equations:

$$S = \frac{df(x, w)}{dx} \cdot \frac{x}{f(x, w)}$$