Journal of Chromatography A, xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Designing flexible low-viscous sieving media for capillary electrophoresis analysis of ribonucleic acids

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ARTICLE INFO

Article history:
Received 20 February 2018
Received in revised form 9 May 2018
Accepted 24 May 2018
Available online xxx

Keywords:
Capillary gel electrophoresis
Design of experiments
Sieving media
Polyvinylpyrrolidone (PVP)
Multi-objective optimization
RNA

ABSTRACT

Modified messenger RNA (mRNA) has recently become a new prospective class of drug product. Consequently, stability indicating separation methods are needed to progress pharmaceutical development of mRNA. A promising separation technique for the analysis of mRNA is capillary gel electrophoresis (CGE). We designed a flexible, low-viscous sieving medium for CGE, based on high mass linear polyvinylpyrrolidone (PVP) and glycerol. A Central Composite Face-centered design resulted in a strong model that allowed us to predict suitable sieving media compositions by using multi-objective optimization. The way of working proposed in this paper gives analysts the freedom to design a suitable sieving medium for their response(s) of interest, for purity and stability analysis of polynucleotides with a size around 100–1000 bases. Depending on the criteria for the analysis there will be a trade-off between different suitable conditions. By using this method, we created a sieving medium that was able to improve resolution, peak height and analysis time of an RNA ladder compared to the current commercially available separation gels.

1. Introduction

Polynucleotides such as modified messenger RNA (mRNA) are a new type of active pharmaceutical ingredient (API) [1–3]. The improvement in intracellular stability and translational efficiency of modified mRNA into proteins have led to the development of drug products comprising mRNA that are tested in clinical studies. These modified mRNA are typically single-stranded polynucleotides of about 1000 bases. The increased focus towards this new type of API has created a need for effective and reliable characterization methods to be used during pharmaceutical development of the mRNA drug products. It is essential to develop robust and efficient methods for purity and stability analysis of these large polynucleotides, as this is crucial for quality control of APIś and drug products.

A promising separation technique for analysis of mRNA is capillary gel electrophoresis (CGE). Evolving from conventional slab gel electrophoresis, the utility of CGE for analysis of large nucleic acids has been demonstrated since the early 90′s by Swerdlow et al. [4]. In the early years of CGE the most used sieving matrices were highly concentrated cross-linked gels such as polyacrylamide, closely mimicking the gels used in slab-gel electrophoresis. These 'permanent gels' [5] generate enough resolution for being used in DNA sequencing but have several challenges when it comes to incorporating the gel in the capillary. In addition, long separation times, air bubble formation and degradation of the gel during analysis make them impractical. Another group of gels are the reversible gels, e.g. agarose. Although the name suggests something else, these gels behave as permanent gels during analysis, with the same advantages and drawbacks, but can be released from the capillary after analysis by heat treatment. For automation, a simpler and more robust separation medium is needed. For example, low-viscous polymer solutions can be used as the sieving medium for separation of polynucleotides. Low-viscous sieving media deliver faster, more robust separations, allow more flexibility and pressure injection [5,6].

Moreover, low-viscous sieving media made of hydrophilic polymers such as polyvinylpyrrolidone (PVP) have the advantage of also being a capillary coating agent, eliminating electroosmotic flow (EOF). PVP can be used for dynamic coating and removes time consuming pre-coating procedures [7,8].

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https://doi.org/10.1016/j.chroma.2018.05.048
0021-9673/

Separation of smaller oligonucleotides is more effective in higher concentrations of low molar mass linear polymers while the large polynucleotides are best separated in dilute solutions of a high molar mass linear polymer [9]. Furthermore, polyhydroxy compounds such as glycerol can be used as additives in low-viscosity polymer solutions to enhance the separation [10]. Such media can also be used with microchip electrophoresis for analyses of nucleic acids [11]. However, designing and testing various sieving media is easier with a CE instrument. Notably, the majority of reports show separation of DNA and small RNA, whereas application on RNA with a size range above 100 bases has only been sparsely described [12–28].

The aim of this study is to design a flexible, multi-objective CGE-UV method for analysis of modified mRNA by focusing on the components of the low-viscous polymer matrix. To facilitate purity and stability analysis of polynucleotides with a size-range around 100–1000 bases, PVP 1.3 MDa was used as high mass linear polymer. Design of experiments (DoE) was used to gather sufficient data for multi-objective optimization.

2. Experimental

2.1. Reagents and chemicals

Polyvinylpyrrolidone (PVP) average $M_r \sim 1{,}300{,}000$ Da by light scattering, Tris-Borate-EDTA buffer (TBE) 10 x concentrate suitable for electrophoresis, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) 1 M BioPerformance Certified, Glycerol for molecular biology (purity ≥99%) and Sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (St. Louis, USA). RNase-free water was prepared by filtering purified water from a MilliQ water system through a Biopak filter from Polisher (Sydney, Australia). Methanol was used as an anti-RNase cleaning agent for glassware such as flasks and the capillary. Commercial gel (DNF-265) was used as received from Advanced Analytical (Ankeny, USA). The RNA ladder was acquired from Thermo Fischer Scientific (Vilnius, Lithuania) at 1 μg/μL in 0.1 mM EDTA. It consisted of 7 single stranded RNA transcripts (100, 200, 300, 400, 500, 750 and 1000 bases). The single stranded model mRNA compound coding for erythropoietin (EPO) (used in pre-tests), constituting 858 bases, was anti-reverse cap analog (ARCA) capped and fully substituted with 5-Methyl-C and Pseudo-U. It was purchased from TriLink Biotechnologies (San Diego CA, USA) and delivered as 1.0 mg/mL in RNase-free water.

2.2. Buffer preparation

The 10X TBE buffer (890 mM Tris-Borate, 20 mM EDTA) was added to the polymer solution as is. The pH of the HEPES buffer (750 mM) was adjusted to 7.5 with 1 M NaOH.

2.3. Sieving media preparation

Depending on the concentration of PVP and glycerol in the polymer solution, specific amounts of each component were added. The amount of buffer was kept constant at 15%(m/m). The amount of PVP was weighed and slowly added to an Erlenmeyer flask containing RNase-free water, glycerol and buffer under magnetic stirring. This solution was left to stir for 2 h and then transferred to polypropylene (PP) vials for CE.

2.4. Coating solution preparation

1%(m/m) PVP was added to RNase-free water and stirred for 1 h.

2.5. Sample preparation

The RNA ladder was stored at −80 °C and the EPO mRNA sample was stored at −20 °C. Before analysis the samples were first thawed and then heated at 80 °C for 2 min, cooled down in an ice-bath for 2 min and transferred to a PP vial with insert. Samples not subject to analysis were kept in a freezer at −20 °C and carefully mixed by pipetting upon thawing.

2.6. Instrumentation and material

All CGE experiments were performed on an Agilent CE7100 system with bare fused silica capillaries purchased from Agilent (Santa Clara CA, USA). The capillary had an inner diameter (I.D.) of 50 μm, an outer diameter (O.D.) of 363 μm, a total length of 80.5 cm, an effective length of 72.0 cm and an extended light path. The CE auto sampler tray was thermostatted to 16 °C with a LAUDA ecoLine RE106 thermostat. Before injection of the sample the capillary was first coated with the PVP coating solution and afterwards filled with the sieving medium, this was automated. Separations were carried out at a voltage of −25 kV and the capillary temperature was maintained at 25 °C. Samples were injected by 80 mBar pressure with an injection time of 30 s. Each sample was injected after maximum 130 min of preparation. After each sequence, the capillary was flushed for 20 min with RNase-free water, methanol and nitrogen by pump. The instrument and data collection were controlled by OpenLAB CDS ChemStation Edition software (Agilent Technologies, Santa Clara CA, USA). Absorption was monitored at 260 nm, and a UV filter 260 nm detector Filter Assembly, CE7100 G7100-62700 was used.

2.7. Viscosity measurements

The viscosities of selected polymer solutions were measured with a Modular Compact Rheometer MCR 302 (Anton Paar, Graz, Austria) equipped with a Viscotherm VT2 temperature regulating accessory with following Geometry: Double Gap rotational measuring system with 26.7 mm Bob inner diameter. The measurement temperature was 25 °C and the viscosity was measured over a shear ramp of 1–100 1/S.

2.8. Determination of the separation performance

The model compound consisted of an RNA ladder starting at 100 bases with increment of 100 bases, except for the last two fragments (6 and 7). It is expected that the resolution will decrease with larger fragments. It was found that the half peak width varies differently for the individual peaks in the different media. The majority of peaks became partly resolved into two peaks depending on the media. Therefore, the resolution per base unit (R), of fragment 6 to fragment 7 was calculated as the time difference (Δt) divided by the size difference factor (ΔF). ΔF = size difference in bases/100

$$R = \frac{\Delta t}{\Delta F} \quad (1)$$

For the resolution of the other fragments (1–5) the following formula was used [29]:

$$R = \sqrt{2\ln(2)}\frac{t2 - t1}{w1 + w2} \quad (2)$$

Where t_2 and t_1 are the migration times between the subsequent fragments and w_1, w_2 are the half-height peak widths of the subsequent fragments.

Please cite this article in press as: L. De Scheerder, et al., Designing flexible low-viscous sieving media for capillary electrophoresis analysis of ribonucleic acids, J. Chromatogr. A (2018), https://doi.org/10.1016/j.chroma.2018.05.048