Talanta 188 (2018) 516–521

Contents lists available at ScienceDirect

Talanta

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

Transverse diffusion of laminar flow profiles as a generic capillary electrophoresis method for in-line nanoreactor mixing: Application to the investigation of antithrombotic activity

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ARTICLE INFO

Keywords:
Nanoreactor
Capillary electrophoresis
TDLFP
Activity assay
Thrombin

ABSTRACT

Capillary electrophoresis (CE) instrument was used for the generation of a robust and reliable nanoreactor for enzymatic assays in the context of antithrombotic drug screening. The activity of the screened molecules was monitored in a quick and fully automated fashion using only few nanoliters of reactants. To achieve this goal, the targeted enzyme (thrombin) and the chromogenic substrate with or without the screened inhibitor were injected as separate plugs. The mixing of the reactants was then realized using electrophoretically mediated microanalysis (EMMA) or fast transverse diffusion of laminar flow profiles (TDLFP) procedure. The latest provided better mixing performance and was chosen to investigate the inhibitory potency of a fragment library. This very straightforward and fast CE activity assay showed results in good accordance with a previously developed CE affinity assay that confirms the potential of CE at the early stages of drug discovery, providing not only an efficient nanoscale bioreactor but also a selective and integrated separation device.

1. Introduction

Evaluating the bioactivity of chemical entities is a crucial step in medicinal chemistry, particularly in the hit discovery phase. Nowadays, two approaches are mainly used to discover hits against a defined target. In the first one, a high throughput screening (HTS) campaign is undertaken with up to several hundred thousands of compounds tested. These bioassays are usually based on cellular or enzymatic assays in a multiwell plate format. Unfortunately due to several artifacts, HTS can lead to 'nuisance' compounds [1]. Counter-screens and careful analysis of the identified hits are thus always needed to confirm their interest. The other approach is based on fragment based drug discovery (FBDD). Schematically, the screening is done on molecules, also named fragments, that typically contain less than 20 heavy atoms [2]. This ensures that a large chemical space can be explored with only a few thousands of fragments. For the screening, the used methodologies should be able to evaluate weak ligands with affinity or inhibition constant in the range of micromolar to milimolar. With such low affinities, the data measurement is often made near the detection limit and it is now admitted that orthogonal screening approaches should be employed to confirm the fragment hits.

For enzyme target whatever for HTS or FBDD, enzymatic assays, in which the reaction velocities are followed in the presence of the tested molecules, play a major role in the screening process. In this context, capillary electrophoresis is an attractive technique that can combine in the same device incubation in a nanoreactor, separation of the reaction products and their detection by various kinds of detectors for a wide range of applications [3–19].

The use of such in-line nanoreactors could be particularly interesting for the screening of libraries at the early stages of drug discovery, since only few microliters of target and reagents are needed for the realization of hundreds of analyses. However, the generic mixing

Abbreviations: μ_{ep}, electrophoretic mobility; ACE, affinity capillary electrophoresis; Akt, protein kinase B; BGE, background electrolyte; BZM, benzamidine hydrochloride; CE, capillary electrophoresis; DMSO, dimethyl sulfoxide; EMMA, electrophoretically mediated microanalysis; FBDD, fragment based drug discovery; HEPES, 4-(2-hydroxyethyl) – 1-piperazine ethanesulfonic acid; HTS, high throughput screening; IS_{CA}, Internal standard corrected area; KB, kinetic buffer; K_i, inhibitory constant; mTOR, the mammalian target of rapamycin; NAFA, nafamostat mesylate; NAPAP, Pefabloc TH®; NF, nitrofurantoin; PABZM, 4-aminobenzamidine dihydrochloride; PEG 6000, polyethylene glycol 6000; PEO, poly(ethylene oxide); PI3k, phosphatidylinositol-3-kinase; PNA, p-nitroaniline; PNA_{CA_I}, PNA normalized peak area obtained in the presence of the screened inhibitor; PNA_{CA_I0}, PNA normalized peak area obtained in the absence of inhibitor; PNA_{CA}, PNA corrected area; QC, quality control; RSD, relative standard deviation; SDS, sodium dodecylsulfate; TDLFP, transverse diffusion of laminar flow profiles; Thr, thrombin; Tris HCl, tris(hydroxymethyl)aminomethane hydrochloride

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https://doi.org/10.1016/j.talanta.2018.06.014
Received 28 April 2018; Received in revised form 3 June 2018; Accepted 4 June 2018
Available online 07 June 2018
0039-9140/

procedure of reactants remains a bottleneck of integrated micro-analyses, mainly because of the nonturbulent flow inside the capillary [5]. Up to now, three approaches have been suggested for in-capillary mixing: mixing by electromigration, mixing by longitudinal diffusion and, more recently, mixing by transverse diffusion of laminar flow profiles (TDLFP) [5]. The mixing by electromigration, also called electrophoretically mediated microanalysis (EMMA), is only possible if reactants have different velocities. This method consists of injecting the reactant with the lowest electrophoretic mobility (μ_{ep}) followed by the reactant with higher μ_{ep}. Then a voltage or a voltage switch is applied and the mixing is realized due to the migration of the fast migrating reactant into the plug of the slow migrating reactant [20,21]. However, this method is not generic since it is impossible to mix molecules that have similar electrophoretic mobilities or if both have no mobilities [5]. Moreover, sophisticated optimization has to be undertaken particularly if more than 2 molecules have to be mixed or if the molecules are dissolved in different buffers [5,22,23]. To overcome some of the EMMA drawbacks, mixing by longitudinal diffusion has been proposed [24]. Since longitudinal diffusion is not governed by the velocities of molecules, this approach could be used for molecules with similar μ_{ep}. However this kind of mixing is time-consuming (diffusion velocity depends on molecule MW and medium viscosity) and could be used in practice mainly for the mixing of two short plugs of small size molecules. If one of the reactant is a high MW molecule or if more than 2 plugs have to be mixed, the longitudinal diffusion becomes unpractical since it would require prolonged analysis time [5].

More recently, Krylov et al. proposed a generic method for mixing two or more reactants inside the capillary [3]. This kind of mixing is based on the assumption that the pressure used for the plug injection generates a parabolic profile due to the laminar flow created inside the capillary. As a result, each injected plug deeply penetrates the plug previously injected [3]. The same team proposed two strategies to improve the TDLFP mixing. The first strategy consists of injecting a long plug of solvent after the introduction of reagents. Recently Morin et al. successfully used this approach in the context of Human neutrophil elastase inhibition study by CE-LIF [25] in which they injected several reagent plugs of 3 nl and a longer buffer plug of 22 nl. With the same idea, Bénédetti et al. evaluate inhibitors of the PI3k/Akt/mTOR signalling pathway [15]. A second Krylov's strategy was a “shaking” method, in which a series of negative and positive pressure pulses were applied to the capillary inlet. The “shaking” sequence was found to improve the reproducibility of mixing without decreasing the method sensitivity [5].

Reminek et al. proposed another improvement of TDLFP mixing, by modifying the injection protocol [18]. In their assay the solutions of an enzyme and its substrates were injected by hydrodynamic pressure as a series of repeated consecutive plugs. Using this protocol, the kinetic and inhibition studies of cytochrome P450 isoform 2C9 were performed using diclofenac as probe substrate and sulfaphenazole as probe inhibitor and the kinetic values obtained were in good agreement with the literature results. More recently, the same group used the previously described TDLFP optimized procedure to study β-secretase activity [19]. The results obtained were compared to the ones obtained using the EMMA methodology. The optimized methods were fully validated and used for the determination of the enzyme kinetic parameters and to study its inhibition by two potent probe inhibitors.

Previously, we have developed an EMMA assay to evaluate the inhibitory potency of argatroban, a potent reversible inhibitor (inhibitory constant, K_i 19 nM, [26]) used in clinical practice toward thrombin [13]. In the present study, we further developed this assay to allow its use to fragment library screening. First, we considered the importance of the mixing procedure using EMMA and TDLFP approaches with known inhibitors displaying various inhibitory potencies (K_i from 0.02 µM to 220 µM). In the second part, the ability of our optimized assay in a screening process was assessed using a library of compounds. The results obtained were compared to our orthogonal CE-affinity method.

2. Materials and methods

2.1. Chemicals and reagents

Benzamidine hydrochloride (BZM), 4-aminobenzamidine dihydrochloride (PABZM), N-(pyridin-4-ylmethyl) guanidine, 4-aminomethyl benzamidine dihydrochloride, 6-(trifluoromethyl)pyridine-3-amidine hydrochloride, 3-aminomethyl benzamidine dihydrochloride, 3-chloro-benzamidine hydrochloride, 3,5-difluoro-benzamidine hydrochloride, nafamostat mesylate (NAFA), 4-methoxybenzamidine, 4′-acetamidophenyl 4-guanidinobenzoate hydrochloride, argatroban, Pefabloc TH® (NAPAP), N-(1-benzyl-4-piperidinyl)guanidine hydrochloride, tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl), nitrofurantoin (NF) and poly(ethylene oxide) (PEO) (MW 200,000 g/mol) were purchased from Sigma Aldrich (Saint-Louis, MO, USA).

Fragments Z2238944429, Z234894831, Z1211228967, Z1263820361, Z1473553993, Z234895267, Z839575938, Z234894451, Z2238944431, Z234895181, Z228583172 were purchased from Enamine (New Jersey, USA). The RF2 was synthetized in Namedic (UNamur) and histamine dihydrochloride was purchased from Fagron (Nazareth, Belgium).

Human alpha thrombin (Thr) was acquired from Haematologic Technologies (Vermont, USA), while S-2366 was obtained from Chromogenix (Milano, Italy). 4-(2-hydroxyethyl) − 1-piperazine ethanesulfonic acid (HEPES), NaCl, triethanolamine, sodium dodecylsulfate (SDS) and dimethyl sulfoxide (DMSO) were purchased from Acros Organics (NJ, USA). NaOH and polyethylene glycol 6000 (PEG 6000) were purchased from VWR (Leuven, Belgium). All chemicals and reagents were of analytical grade. Ultrapure water was supplied by a Milli-Q equipment (Millipore, Bedford, MA, USA). All solutions were filtered through a cellulose based membrane (0.20 µm) with Chromafils syringe filters from Macherey-Nagel (Düren, Germany).

2.2. Preparation of buffer and stock solutions

The kinetic buffer contained 0.01 M Tris–HCl, 0.01 M HEPES, 0.1 M NaCl, 0.1% PEG 6000 and was adjusted to pH 7.5 with triethanolamine. In order to perform the separation of the reaction products, the background electrolyte (BGE) was prepared by adding 15 mM SDS to the kinetic buffer.

A stock solution of Thr 5 µM was prepared in the kinetic buffer. This stock solution was stored at − 20 °C in low adhesion Eppendorf tubes. S-2366 stock solution was also prepared in kinetic buffer at a concentration of 5 mM. NF 0.5 mM stock solution (internal standard, IS) was prepared in a DMSO and kinetic buffer mixture (10/90; v/v).

The fragments stock solution was prepared at a concentration of 20 mM in MeOH, unless otherwise stated. The 20 mM stock solution of argatroban and NAFA were prepared in DMSO. The stock solutions were kept at − 80 °C, protected from light. Before analysis, the stock solutions of the screened molecules were diluted to reach the appropriate final concentrations (10 µM unless specified otherwise).

All these solutions were stored in the fridge protected from light and extemporaneously diluted with the kinetic buffer to reach the appropriate final concentrations.

2.3. Electrophoretic conditions

The experiments were performed on a qualified $HP^{3D}CE$ system (Agilent Technologies, Waldbronn, Germany) supplied with an autosampler, an on-column DAD and a temperature control system (15–60 °C ± 0.1 °C). Chemstation (Hewlett-Packard, Palo Alto, CA, USA) was used for instrument control, data acquisition and data analysis.

The analyses were realized using uncoated fused silica capillaries (ThermoSeparation Products, San Jose, CA, USA) having 50 µm i.d. and 48.5 cm total length (8.5 cm effective length). The tray and the capillary