Talanta 161 (2016) 489–496

Contents lists available at ScienceDirect

Talanta

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

Novel bead-based platform for direct detection of unlabelled nucleic acids through Single Nucleobase Labelling

Seshasailam Venkateswaran [a,1], Maria Angélica Luque-González [b,c,1], Mavys Tabraue-Chávez [d], Mario Antonio Fara [d,e], Barbara López-Longarela [d], Victoria Cano-Cortes [b,c], Francisco Javier López-Delgado [b,c], Rosario María Sánchez-Martín [b,c], Hugh Ilyine [e], Mark Bradley [a], Salvatore Pernagallo [d,e,*], Juan José Díaz-Mochón [b,c,e,**]

[a] School of Chemistry, EaStCHEM, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3FJ, United Kingdom
[b] Pfizer-Universidad de Granada-Junta de Andalucía Centre for Genomics and Oncological Research (GENYO), Parque Tecnológico de Ciencias de la Salud (PTS), Avenida de la Ilustración 114, Granada 18016, Spain
[c] Faculty of Pharmacy, University of Granada, Campus Cartuja s/n, Granada 18071, Spain
[d] DestiNA Genomica S.L. Parque Tecnológico Ciencias de la Salud (PTS), Avenida de la Innovación 1, Edificio BIC, Armilla, Granada 18016, Spain
[e] DestiNA Genomics Ltd., 7-11 Melville St, Edinburgh, EH3 7PE United Kingdom

ARTICLE INFO

Article history:
Received 15 June 2016
Received in revised form
19 August 2016
Accepted 28 August 2016
Available online 31 August 2016

Keywords:
Dynamic chemistry
Peptide nucleic acid (PNA)
Magnetic microspheres
Nucleic acid
MicroRNA (miRNA)
Nucleic acid test (NAT)
Single Nucleobase Labelling (SNL)

ABSTRACT

Over the last decade, circulating microRNAs have received attention as diagnostic and prognostic biomarkers. In particular, microRNA122 has been demonstrated to be an early and more sensitive indicator of drug-induced liver injury than the widely used biomarkers such as alanine aminotransferase and aspartate aminotransferase. Recently, microRNA122 has been used in vitro to assess the cellular toxicity of new drugs and as a biomarker for the development of a rapid test for drug overdose/liver damage. In this proof-of-concept study, we report a PCR-free and label-free detection method that has a limit of detection (3 standard deviations) of 15 fmoles of microRNA122, by integrating a dynamic chemical approach for "Single Nucleobase Labelling" with a bead-based platform (Luminex®) thereby, in principle, demonstrating the exciting prospect of rapid and accurate profiling of any microRNAs related to diseases and toxicology.

1. Introduction

MicroRNAs (miRNAs) are small non-coding RNAs of 19–24 nucleotides in length that regulate gene expression by base pairing with the 3′-untranslated region of a target gene's messenger RNA (mRNA), leading to degradation and/or translational repression of that gene [1–3]. miRNAs are implicated in many biological events and their deregulation is associated with many serious disease states [4,5].

miRNAs while present in biological fluids in a stable and reproducible manner are differentially expressed under pathological conditions, such that the expression patterns of circulating miRNAs can thus be used as fingerprints for various diseases [6,7]. As such miRNAs possess ideal characteristics of a biomarker – i.e., they can be specific to the disease or pathology of interest, reliably indicate disease before clinical symptoms appear, be sensitive to changes in the pathology (disease progression or therapeutic response), and also allow detection from samples of biological fluids (e.g. blood, urine) [8–14]. Additionally, circulating miRNAs are remarkably stable in body fluids, resisting ribonucleases and variations in physicochemical conditions such as pH [15].

Recently, microRNA122 (miRNA122) has been found to be substantially elevated in the plasma of patients with drug-induced liver injury (DILI), and can be detected much earlier than current clinical biomarkers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [16,17]. ALT and AST levels increase 12–16 h post overdose of drugs like acetaminophen, while miRNA122 can detect liver injury within 4 h [18]. Moreover,

* Corresponding author at: DestiNA Genomica S.L. Parque Tecnológico Ciencias de la Salud (PTS), Avenida de la Innovación 1, Edificio BIC, Armilla, 18016 Granada, Spain.
** Corresponding author at: Pfizer-Universidad de Granada-Junta de Andalucía Centre for Genomics and Oncological Research (GENYO), Parque Tecnológico de Ciencias de la Salud (PTS), Avenida de la Ilustración 114, 18016 Granada, Spain.
E-mail addresses: salvatore@destinagenomics.com (S. Pernagallo), juanjose.diaz@genyo.es (J.J. Díaz-Mochón).
[1] These authors contributed equally and should be considered co-first authors.

http://dx.doi.org/10.1016/j.talanta.2016.08.072
0039-9140/

miRNA122 has been demonstrated as an in vitro marker of drug-induced cellular toxicity for acetaminophen and diclofenac, with sensitivity similar to conventional assays that measure lactate dehydrogenase activity and intracellular adenosine triphosphate. Thus, miRNA122, as a biomarker, also has the potential to be used during early phases of the drug development processes [19].

Therefore, the development of a rapid test for miRNA122 would be useful in healthcare and drug development [18,20,21]. However, technical difficulties to perform robust and comparable profiling of circulating miRNAs have impeded progress to develop an approved clinical diagnostic assay [22,23]. A reliable detection platform which removes laborious sample-preparation steps (e.g., enzymatic steps for PCR-based amplifications) would represent a step forward, and allow miRNAs to be part of the approved clinical diagnostic test arsenal.

To date, most of the detection methods developed for direct detection of unlabelled nucleic acids are based just on hybridisation events using tagged probes. There are sandwich hybridisation approaches with two probes [24], which can also be assisted by ligases to elongate short sequences of miRNAs [25,26], methods based on triple-stem DNA probes [27] and methods which use a single probe to create specific duplexes which can then be identified either by modified surfaces, [28,29] or p19 protein that specifically recognises nucleic acid duplexes [30,31]. These molecular assays based on hybridisation without further molecular recognition are then integrated within different detection systems such as electrochemical sensors [27,29,31–33] and fluorescence-based platforms [25] with variable limit of detections. In particular, there are three reports which claim the direct detection of miRNA122 using electrochemical sensors with limit of detections of 2.7 pmoles [32], 1 pmole [33] and sub-attomole [27] respectively.

This work describes the potential for direct quantification of miRNAs by combining a PCR-free approach that enables detection of nucleic acids using dynamic chemistry [34–36], combined with a bead-based platform (Luminex®) already in wide clinical use [37–40]. Nucleic acid analysis by dynamic chemistry has already been successfully used for genotyping Single Nucleotide Polymorphisms (SNPs) [35] and here we describe the application of this technology to detect circulating miRNAs. Its high specificity together with its advantage of PCR- and label-free testing of nucleic acids is applied here to detect unlabelled miRNA sequences through what we describe as 'Single Nucleobase Labelling' (SNL) defined as "a non-enzymatic labelling of nucleic acids using dynamic chemistry" and which is ideal for short nucleic acid strands.

Nucleic acid analysis by dynamic chemistry harnesses Watson–Crick base pairing to template a dynamic reaction on a strand of an Abasic peptide nucleic acid [PNA; a DNA mimic in which the sugar-phosphate backbone is replaced with N-(2-aminoethyl) glycine]. This is achieved by hybridising an Abasic PNA probe to the target nucleic acid strand such that a nucleobase-free position on the PNA (a so-called 'blank' position) lies opposite to a nucleotide on the nucleic acid strand. The reversible reaction, between an aldehyde-modified nucleobase (SMART Nucleobases) and a free secondary amine on the PNA probe, generates an iminium intermediate which can be reduced to a stable tertiary amine, (reaction known as reductive amination). Four iminium species (one for each base) will be thus generated, but the one with the correct hydrogen bonding motif (obeying Watson–Crick base-pairing) will be the most thermodynamically stable product [34] (Fig. 1). Moreover, complementary nucleic acid strands act also as catalysts as accelerate the rate of the reductive amination. When there is not complementary nucleic strands, reductive aminations do not happen within the assay timeframe. In summary, this technology requires two specific molecular events to create a signal, (i) perfect hybridisation between nucleic acid strands and Abasic PNA and (ii) specific molecular recognition, through Watson–Crick base-pairing rules, by the SMART Nucleobase (Fig. 1).

With these features in mind, we aimed to use this technology for the direct detection and quantification of miRNAs. In this case, native miRNAs act as template molecules which drive the specific incorporation of a labelled SMART Nucleobase onto a specific Abasic PNA. In cases where there are other nucleic acids which are not fully complementary to the Abasic PNA, reaction does not happen. Quantification is also possible as the yield of the reaction depends on the amount of templating miRNA.

To achieve the merging of this technology with Luminex® bead-based detection platform, Abasic PNA probes complementary to target miRNAs were covalently bound to Luminex® microspheres (Fig. 2). Labelling was achieved *via* an aldehyde-modified nucleobase tagged with biotin (Fig. 2, Step 1) and Streptavidin-R-Phycoerythrin Conjugate (SAPE) (Fig. 2, Step 2) to allow microspheres to be read using a Luminex® MAGPIX® platform (Fig. 2, Step 3). Validation of the assays developed was further confirmed using two alternative technologies, i.e., flow cytometry and confocal microscopy.

2. Experimental section

2.1. General

Carboxylated magnetic microspheres (MagPlex, MC10012) and

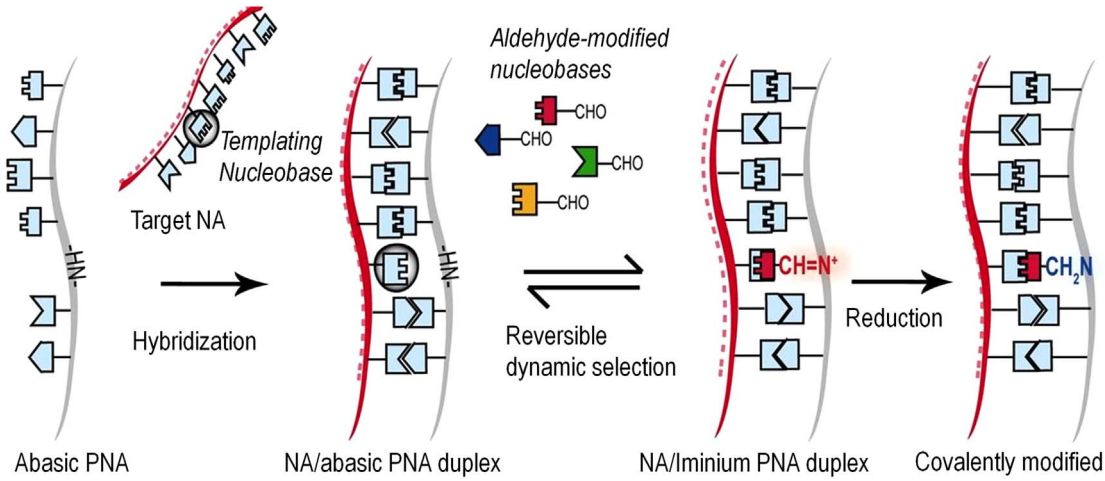

Fig. 1. Schematic representation of dynamic chemistry for nucleic acids analysis. (From [34]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA, Reproduced with permission).