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Citation for published version:
Venkateswaran, S, Luque-González, MA, Tabraue-Chávez, M, Fara, MA, López-Longarela, B, Cano-Cortes, V, López-Delgado, FJ, Sánchez-Martín, RM, Ilyine, H, Bradley, M, Pernagallo, S & Díaz-Mochón, JJ 2016, 'Novel bead-based platform for direct detection of unlabelled nucleic acids through Single Nucleobase Labelling', *Talanta*, vol. 161, pp. 489-496. https://doi.org/10.1016/j.talanta.2016.08.072

Digital Object Identifier (DOI):
10.1016/j.talanta.2016.08.072

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

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Key words

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, 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 [20-22]. However, technical difficulties to perform robust and comparable profiling of circulating miRNAs have impeded progress to develop an approved clinical diagnostic assay [23-24]. 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 [25], which can also be assisted by ligases to elongate short sequences of miRNAs [26, 27], methods based on triple-stem DNA probes [28] and methods which use a single probe to create specific duplexes which can then be identified either by modified surfaces, [29, 30] or p19 protein that specifically recognises nucleic acid duplexes [31, 32]. These molecular assays based on hybridisation without further molecular recognition are then integrated within different detection systems such as electrochemical sensors [28, 30, 32-34] and fluorescence-based platforms [26] 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 [33], 1 pmole [34] and sub-attomole [28] 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 [35-37], combined with a bead-based platform (Luminex®) already in wide clinical use [38-41]. Nucleic acid analysis by dynamic chemistry has already been successfully used for genotyping Single Nucleotide Polymorphisms (SNPs) [36] 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 hybridizing 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 [35] (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).

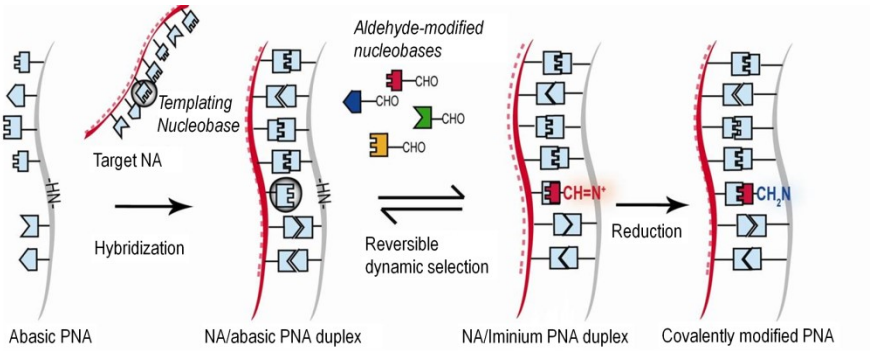

Fig. 1. Schematic representation of dynamic chemistry for nucleic acids analysis. (From F.R. Bowler, J. J. Diaz-Mochon, M. D. Swift, M. Bradley, DNA Analysis by Dynamic Chemistry, Angew. Chem., Int. Ed., 49 (2010), 1809–1812. Copyright Wiley-VCH Verlag GmbH & Co. KGaA, Reproduced with permission).

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 labeled 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.

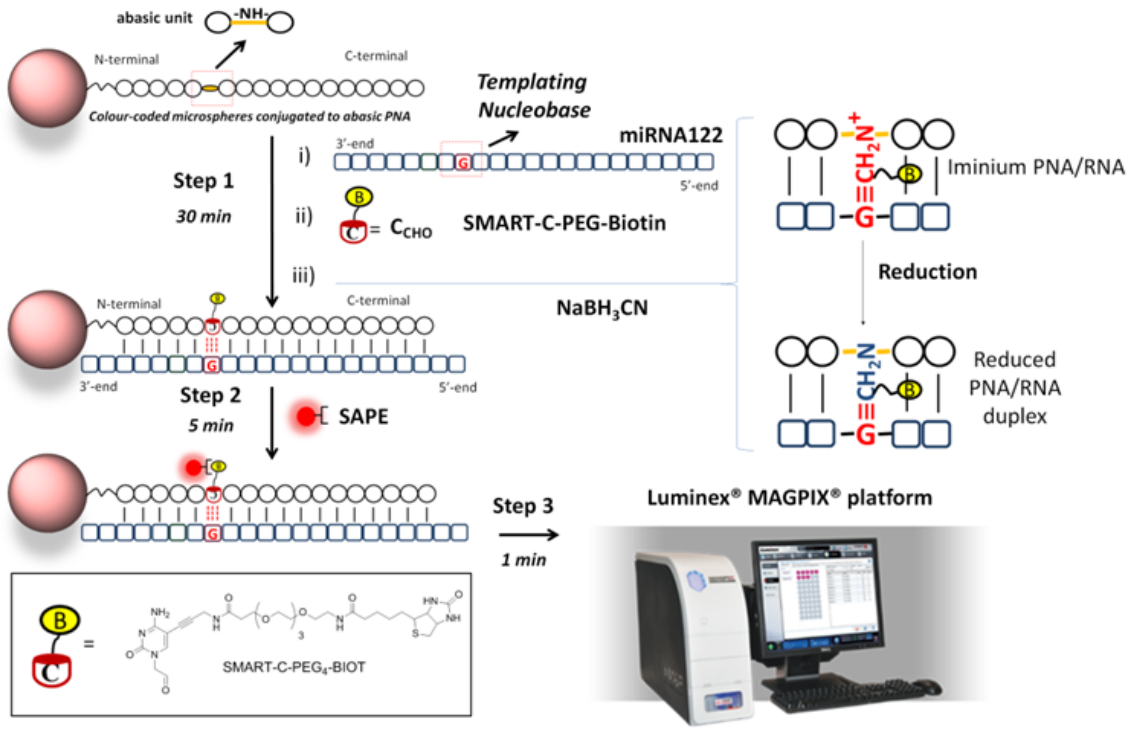

Fig. 2. Merging of dynamic chemistry with Luminex® MAGPIX® platform for 'Single Nucleobase Labelling' (SNL) of miRNAs. Step 1: Modification of the Abasic PNA, which is conjugated to color-encoded microspheres, with the biotinylated aldehyde-modified cytosine (SMART-C-PEG-Biotin). This process requires three steps: i) perfect hybridisation between the Abasic PNA and miRNA122; ii) generation of a reversible iminium specie between the secondary amine of the "Abasic" unit and the aldehyde group of the SMART-C-PEG-Biotin nucleobase driven by the templating nucleobase. In this case, the templating

guanine can just template the incorporation of an aldehyde-modified cytosine as otherwise the iminium specie is not stable enough to be reduced and iii) reduction of the iminium specie by sodium cyanoborohydride to yield to a non-reversible tertiary amine within the PNA conjugated to the microspheres [35]. Step 2: Microsphere labelling with Streptavidin-R-Phycoerythrin Conjugate (SAPE). Step 3 Fluorescent detection using a Luminex® MAGPIX® platform (45 min total time, including washings).

2. Experimental section

2.1. General

Carboxylated magnetic microspheres (MagPlex, MC10012) and MagPlex-TAG microspheres with surface-bound oligonucleotide (MTAG-A012) were obtained from Luminex® Corporation (Netherlands) and stored at 4°C in the dark. All chemicals were obtained from Sigma Aldrich and used as received. SCD buffer was prepared from 2× saline sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) with the pH adjusted to 6.0 using HCl. All synthetic DNA oligomers (desalted) were purchased from Microsynth AG (Balgach, Switzerland). Streptavidin-R-Phycoerythrin Conjugate (SAPE, 1 mg/mL) was purchased from Thermo Fisher Scientific.

2.2. Instrumentation

Abasic PNA probe concentrations were determined using a Thermo Fisher NanoDrop 1000 spectrophotometer. Hybridisation and Single Nucleobase Labelling were conducted in a Techne Thermal cycler (TC-5000). Flow cytometry was conducted using a BD FACSCanto II λex 488 nm and emission collected with a 585/42 band pass filter. Confocal imaging was performed with a Zeiss LSM 710 confocal laser scanning microscope using the Zeiss ZEN 2010 software. A Petroff-Hausser Counting Chamber (Hausser Scientific) was used to count the microspheres.

2.3. Probes and SMART-C-PEG-Biotin

Two peptide nucleic acids probes containing an Abasic position (DGL-122-U and DGL-122-C) and terminated with an amino-pegylated group were designed and synthesised by standard solid phase chemistry on an INTAVIS MultiPep Synthesiser (Intavis AG GmH, Germany) (Table 1). The 18-mer sequences were designed to allow anti-parallel hybridisation with

miRNA122 strands (Table 2). The Abasic site was positioned at +13 from their C-terminal end so that post-hybridisation, the mature miRNA122 strands, present a guanine at that position (+15 from the 5′-terminus) thereby allowing incorporation of a cytosine into the Abasic pocket (Table 2, nucleobases shown in red). Aldehyde-modified cytosines, tagged with a biotin by either a tetraethylenglycol or a dodecylethylenglycol spacer (SMART-C-PEG4-Biot or SMART-C-PEG12-Biot), (ESI, Fig. S1 and S2, were prepared following the synthetic route described elsewhere [42].

2.4. Functionalization of Luminex® microspheres with probes FB-1 and FB-2.

DGL-122-U and DGL-122-C were coupled to MagPlex carboxylated microspheres (MC10012) to give the functionalised beads, FB-1 and FB-2 respectively. Briefly, one million microspheres were suspended in MES Buffer (20 µL, pH ~ 4.5) in an eppendorf tube and freshly prepared solutions of EDC (10 mg/mL in de-ionised water, 10 µL) and DGL probe (0.1 mM, 5 µL) were added and vortexed followed by incubation (30 min, shaking in the dark). The microspheres were washed (×2 0.02% Tween- 20, 200 µL and ×2, 0.1% SDS, 200 µL), re-suspended in water (~1 million microspheres per 100 µL) and diluted in SCD buffer (100 microspheres per µL).

Table 1. DGL Probes

Reference	Oligomer sequence (N'-- C')	N'-modification
DGL-122-U	xx AAC AC _A TTG TCA CAC TC	Amino
DGL-122-C	xx AAC* AC*_A* TTG* TCA* CAC* TC	Amino

'xx': PEG linker (Fig. S3); '*': PNA nucleobase containing a “propanoic acid side chain” (negative charge); '_': abase monomer (blank) (see Fig. 3 and Fig. S3).

Table 2. Single-stranded DNA oligonucleotides

Reference	Oligonucleotides sequences (5'-3')	5'-modifications
LUMA012-biot	CAT AAT CAA TTT CAA CTT TCT ACT	Biotin
miRNA122-biot	TGG AGT GTG ACA ATG GTG TTT G	Biotin
miRNA122	TGG AGT GTG ACA ATG GTG TTT G	-
miRNA122-A	TGG AGT GTG ACA ATA GTG TTT G	-
miRNA21-biot	TAG CTT ATC AGA CTG ATG TTG A	Biotin
miRNA21	TAG CTT ATC AGA CTG ATG TTG A	-

'In red': The nucleobase at +15 from the 5′-terminus of miRNA122 under interrogation *via* dynamic chemistry with biotinylated SMART nucleobases

2.5. Hybridisation assessment of FB-1 and FB-2 microspheres.

The performance of FB-1 and FB-2 were confirmed by hybridising a complementary oligonucleotide labelled at their amino-5′-terminus with biotin (miRNA122-biot) (Table 2), followed by labelling with SAPE and analysing on a MAGPIX®. Briefly, 12.5 µL of either FB-1 or FB-2 (100 microspheres per µL), 30 µL of SCD buffer and 7.5 µL of 100 nM miRNA122-biot, were mixed in a 200 µL eppendorf tube. Hybridisation was conducted at 41° C for 20 min, followed by addition of 10 µL of SAPE (20 µg / mL), vortexed for 1 sec and incubated at 41°C for 5 min in a thermal cycler prior to analysis. A non-complementary biotinylated synthetic oligonucleotide (miRNA21-biot) was used as a negative control (Table 2).

2.6. Single Nucleobase Labelling (SNL) by dynamic chemistry.

23.5 µL of SCD buffer, 12.5 µL of the functionalised microspheres FB-2 (dispersed in SCD buffer, containing 100 microspheres per µL), 4 µL of SMART-C-PEG4-Biotin (500 µM), 7.5 µL of either miRNA122 or controls miRNA21 and miRNA122-A (to give final concentrations of 1 µM, 100 nM, 10 nM or 1 nM) and 2.5 µL of reducing agent, sodium cyanoborohydride (20 mM) were added in a 200 µL eppendorf, vortexed and incubated (41° C for 30 min, thermal cycler). The microspheres were then washed twice with SCD buffer, re-suspended (in 50 µL of SCD buffer), followed by addition of 10 µL of SAPE (20 µg/mL), vortexing and incubation in a thermal cycler (41° C for 5 min) before analysing with the MAGPIX® instrument or preparation for flow cytometry or confocal microscopy.

2.7. Luminex® MAGPIX®

The MAGPIX® instrument was calibrated using the verification and calibration kit and standard instructions of the manufacturer, with detection of phycoerythrin (PE) using a LED (λex 511 nm ± 27 nm) and a CCD camera. Microspheres post hybridisation or Single Nucleobase Labelling were vortexed (1 sec) and analysed in the MAGPIX® System (injection volume 20 µL) with a minimum microsphere count of 100.

2.8. Flow cytometry

Following Single Nucleobase Labelling with 7500, 750, 75 and 7.5 fmoles of miRNA-122 or miRNA-21 as a negative control and SAPE incubation, the microspheres were washed twice with SCD buffer, resuspended in 150 µL of SCD. The results of flow cytometry were analysed with Flowjo (version 7.2.4). The conclusions section should come in this section at the end of the article, before the acknowledgements.

3. Results and discussion

3.1. Polyanionic Abasic PNA probes improve hybridisation efficiency.

It is well known that standard PNA molecules tend to adopt collapsed conformations, due to their neutral backbone and hydrophobic interactions between nucleobases [43-45]. In the case of Abasic PNA probes bound to polystyrene microspheres, these conformations were anticipated to be further enhanced due to hydrophobic interactions between polymers and PNA oligomers. In order to resolve this, we hypothesised that adding anionic groups across the probe backbone would reduce this tendency, also reducing self-aggregation. Polyanionic Abasic PNA probes bound to microspheres would thus be more readily available to hybridise complementary nucleic acid strands and, hence, allow more efficient dynamic incorporation of the SMART Nucleobases. To achieve this, PNA building blocks containing propanoic acid chains at gamma positions with S-configuration were chosen (Fig. 3) [46-48].

Two DGL probes, both containing the same sequence of nucleobases to allow hybridisation to the mature miRNA122 strand were prepared, with one containing unmodified PNA monomers (DGL-122-C) and the other carrying six PNA monomers containing the propanoic acid modifications at the gamma positions (DGL-122-C) (Table 1) were conjugated to MagPlex microspheres (MC10012) to give FB-1 and FB-2 respectively (Fig. 3).

Hybridisation performance of microspheres FB-1 and FB-2 were assessed by hybridisation with miRNA122-biot or the non-complimentary control, miRNA21-biot, (750 fmoles in a reaction volume of 50 µL, i.e., 15 nM) followed by SAPE labelling. Microspheres FB-2 (functionalised with DGL-122-C) showed better hybridisation efficiency than FB-1 (functionalised with DGL-122-U) and hence were chosen for further experiments (Fig. 4). Propanoic acid groups improve the hybridisation feature of DGL-122 probes due to (i) the chiral configuration which creates a right-handed duplex and (ii) the electrostatic repulsion provided by the negative charges distributed across its backbone.

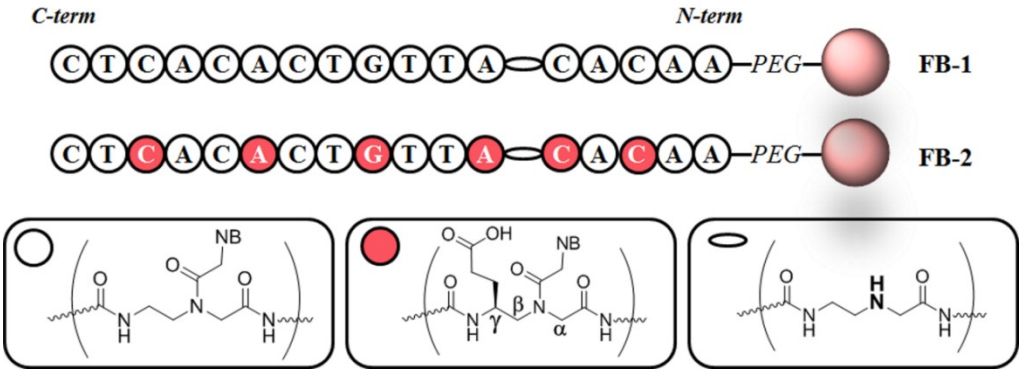

Fig. 3. Two DGL probes (Table 1) with the same sequence of nucleobases complementary to the mature miRNA122 strand were conjugated to MagPlex microspheres (FB-1 & FB-2). FB-1 contained unmodified PNA monomers (white circles) and FB-2 carried six PNA monomers containing chiral modifications at gamma positions based with propanoic acid (red circles). The yellow ellipse shows, the Abasic unit (blank).

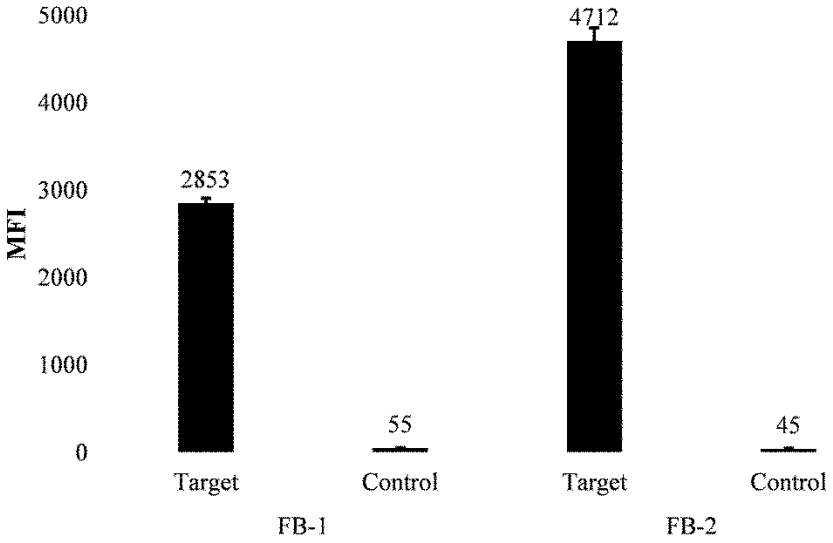

Fig. 4. Median Fluorescence Intensities (MFI) obtained from MAGPIX® after hybridisation and SAPE treatment using FB-1 and FB-2. Target: miRNA122-biot; control: miRNA21-biot. n = 3.

3.2. Detection of miRNA122 using Single Nucleobase Labelling.

Once hybridisation performance of DGL probes on the magnetic microspheres were established, Single Nucleobase Labelling was conducted using FB-2 microspheres and the SMART-C-PEG4-Biot base in varying quantities (7500, 750, 75 and 7.5 fmoles in a total reaction volume of 50 µL, i.e., 150, 15, 1.5 and 0.15 nM) of unlabelled miRNA122 (Fig. 5A), which fully matches Abasic PNA sequences on FB-2, and miRNA21, negative control (Fig. 5B). After SAPE labelling, microsphere samples were analysed using MAGPIX® (Fig. 6). The plot of MFI versus miRNAs concentrations showed a linear correlation for the target (Fig. 6), confirming the suitability of Single Nucleobase Labelling to detect unlabelled nucleic acids. The experiments were repeated five times presenting coefficient of variant for each condition, ranging from 10% to 15% (see ESI, Table S1).The average signal to background ratios obtained were 14.2 ± 1.5, 9.7 ± 0.7, 4.2 ± 0.6 and 1.6 ± 0.3, for the four miRNAs concentrations (signal = MFI of target and background = MFI of negative control) with a detection limit of 15 fmoles (0.3 nM). In order to know if whether this is adequate to analyse relevant clinical samples, we have reviewed the latest publications regarding miRNA122. Dear et al, the leading group proposing the use of miRNA122 as biomarker of drug toxicity, has very recently reported a mean value of 71.3 million copies of miRNA122 per mL of serum (95% confidence interval [CI] 29.3–113.2 million) in a group of 18 healthy volunteers 71 million miRNA122 copies per mL of non-intoxicated patients is equivalent to around 0.15 fmoles [49]. As James Dear previously reported [16, 20, 21], the increases in miRNA122 level of patients with liver injury from acetaminophen toxicity are from 100- to 10000-fold higher when compared with basal levels. Under the methodology presented, it would then require 3 mL of serum, obtained from 7.2 mL of blood to be within its limit of quantification.

The chain length of the PEG spacer in the SMART nucleobase did not have an influence on the detection limit when two biotinylated SMART Nucleobases, SMART-C-PEG4-Biot and SMART-C-PEG12-Biot, were compared (ESI, Section S1, Fig. S4).

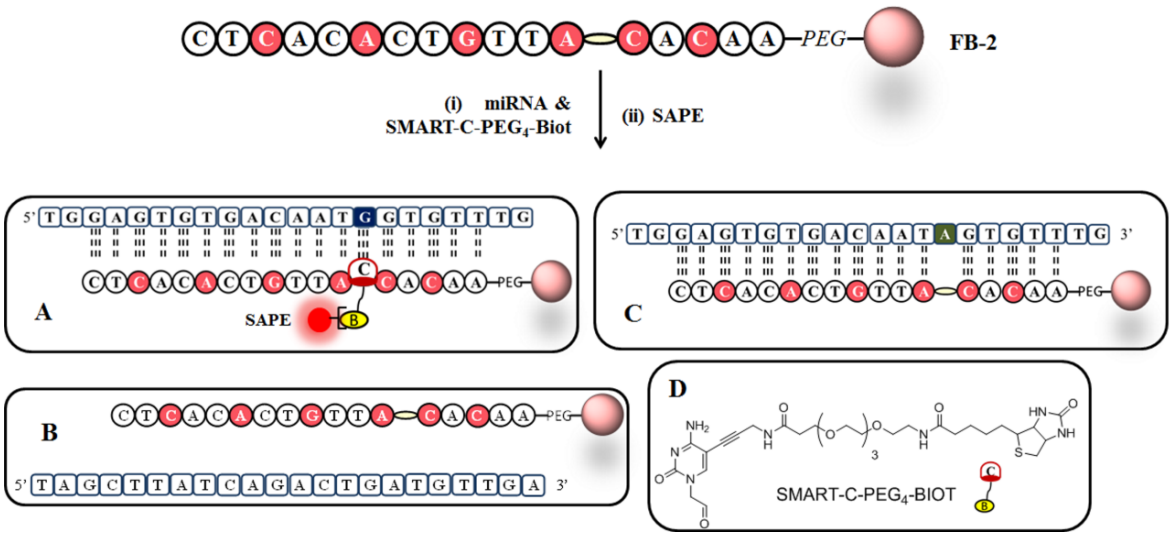

Fig. 5 Representation of Single Nucleobase Labelling (SNL) using SMART-C-PEG4-Biot and MagPlex microspheres. (A) FB-2 MagPlex carboxylated microspheres, which are conjugated to DGL-122-C, with its unlabelled complementary nucleic acid sequence miRNA122, which template, through the guanine nucleobase, the incorporation of the SMART-C-PEG4-Biot nucleobase into the Abasic position. After SAPE labelling, microspheres thus become fluorescent and capable of being detected by MAGPIX. (B) FB-2 microspheres with its non-complementary sequence miRNA21. The lack of duplex formation avoids the dynamic reaction between the Abasic position and SMART-C-PEG4-Biot. (C) FB-2 microspheres hybridise with its unlabelled complementary nucleic acid sequence, which in this case, would template the incorporation of a SMART-thymine nucleobase but not the SMART-C-PEG4-Biot. Therefore, after SAPE labelling, microspheres are not labelled by SAPE. (D) Structure of biotinylated SMART-Cytosine Nucleobase (SMART-C-PEG4-Biot).

3.3. Specificity assessment of Single Nucleobase Labelling

The specificity was investigated using a fully complementary target sequence (miRNA122) (Fig. 5A) and a target containing a single nucleotide mismatch (miRNA122-A) (Fig. 5C) which presents an adenosine in front of the Abasic PNA position instead of a guanine. When the perfectly complementary target miRNA122 was used the signal showed a linear correlation with miRNA122 concentration while with miRNA122-A the signals were unaffected and comparable to that of the non-complementary strand. These results show the

high discrimination capability offered by this approach, which can be exploited to analyse iso-miRNAs (Fig. 7).

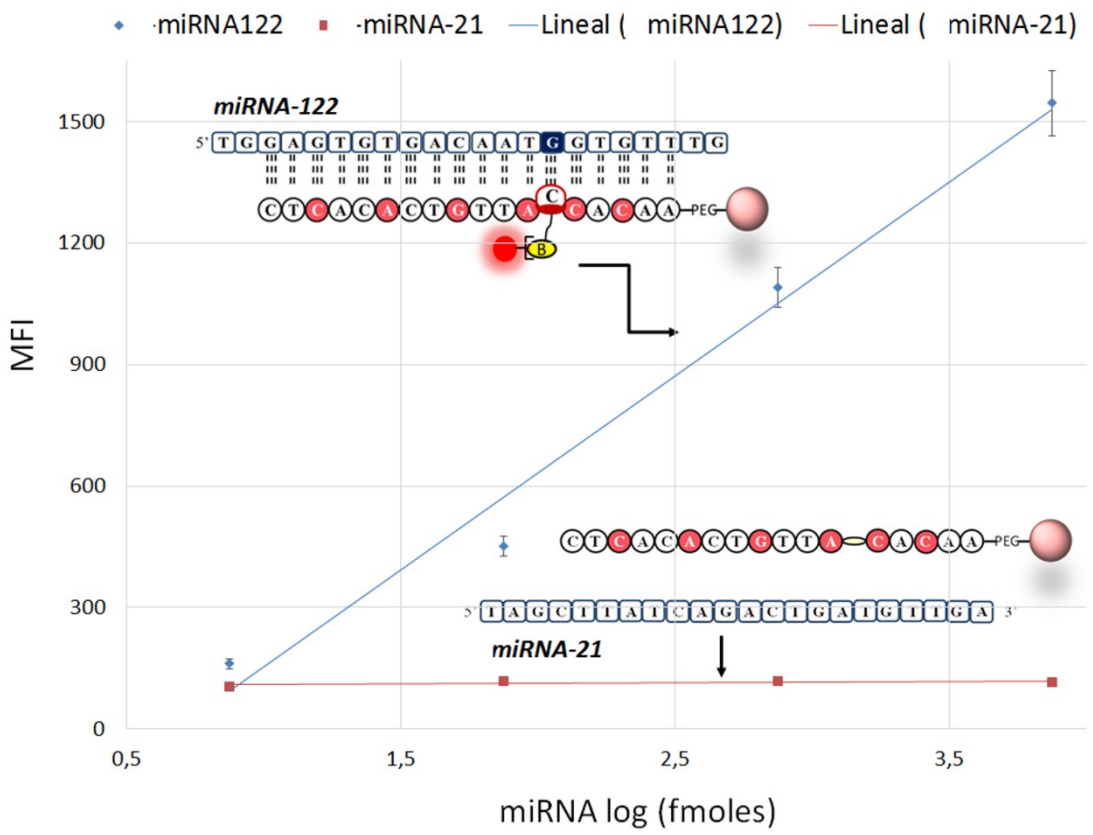

Fig. 6. Direct detection of nucleic acids by dynamic chemistry. Median fluorescence intensities (MFI) obtained using MAGPIX® after Single Nucleobase Labelling (SNL) and SAPE treatment, reveal selective incorporation of the SMART base only for the target. FB-2, SMART-C-PEG4-Biot, and four quantities (7500, 750, 75 and 7.5 fmoles) of unlabelled target (miRNA-122) and control (miRNA-21) were used. n =5.

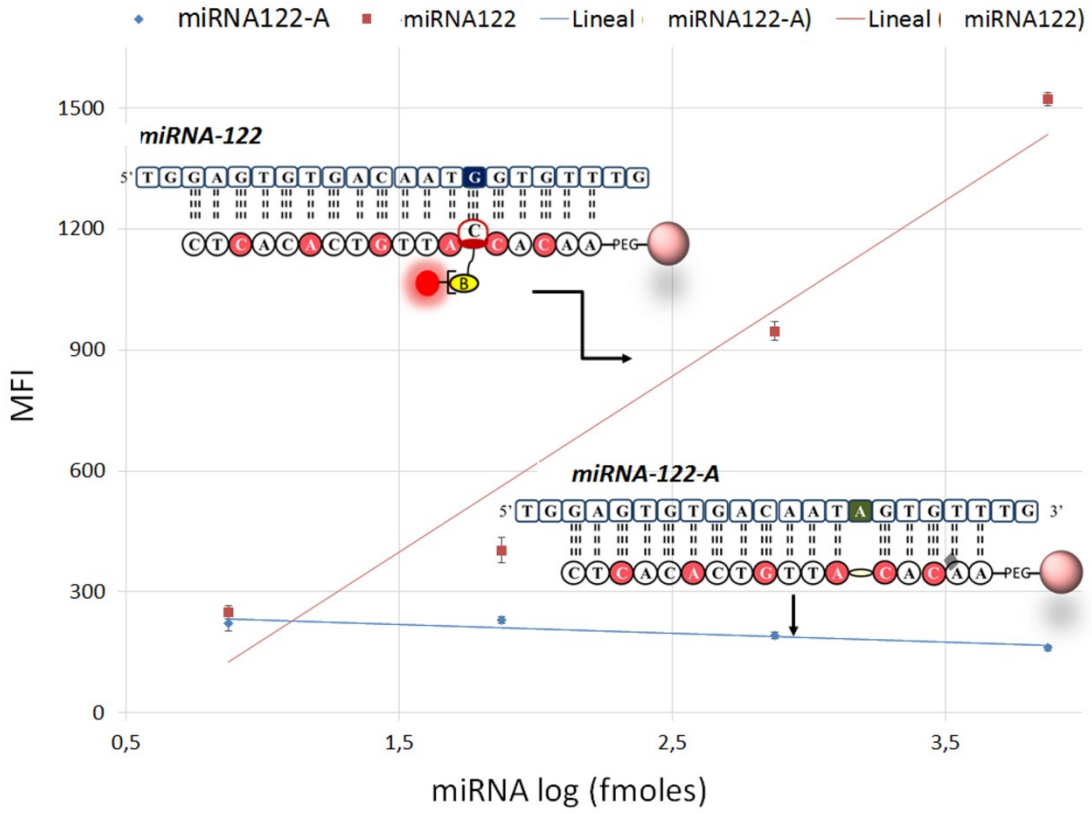

Fig. 7. Direct detection of nucleic acids by dynamic chemistry on MAGPIX® - specificity assessment. Median fluorescence intensities (MFI) obtained from MAGPIX® after Single Nucleobase Labelling and SAPE treatment using FB-2, SMART-C-PEG4-Biot, and four different quantities (7500, 750, 75 and 7.5 fmoles) of unlabelled miRNAs (miRNA-122 and miRNA-122-A). n =5.

3.4. Validation of Single Nucleobase Labelling through flow cytometry

Flow cytometry was used to validate the results from MAGPIX®. The validation experiments also served to determine whether better discrimination could be achieved with FLEXMAP 3D®, a more advanced multiplexing platform that uses flow cytometry based technology [50]. Single Nucleobase Labelling was conducted with varying quantities of miRNA122 (7500, 750, 75 and 7.5 fmoles) and miRNA21 as negative control. After SAPE incubation, samples were analysed in a BD FACSCanto and dot-plots obtained through Flowjo (Fig. S5). Population shifts on the PE channel were clearly seen even with the lowest amounts of miRNA122 used (7.5 fmoles). In this case, better signal to background ratio at the lowest

concentration was achieved when compared with the data obtained using MAGPIX® indicating that it might be possible to achieve better detect limits miRNA with the more advanced platform. Furthermore, to provide a visual confirmation of selective dynamic incorporation of SMART-C-PEG4-Biot, Single Nucleobase Labelling was conducted with 7500 fmoles of miRNA122 and miRNA21 (control) and, after SAPE incubation, microspheres were analysed by fluorescence microscopy (ESI, Section S2 and Fig. S6). As expected, FB-2 microspheres which reacted with complementary miRNA122 and SMART-C-PEG4-Biotin were fluorescently labelled while those microspheres treated with non-complementary miRNA21 where non-fluorescent.

4. Conclusions

A "Proof of Concept" study for the integration of Single Nucleobase Labelling with Luminex xMAP® technology for the detection of miRNA122 is presented. Effective conjugation of the DGL probes to MagPlex microspheres allowed detection of miRNA122 via dynamic chemistry demonstrating the potential for rapid and accurate profiling of miRNA122 in particular and miRNAs in general. A detection limit of 15 fmoles (0.3 nM) together with high specificity and the capacity to differentiate sequences containing a single nucleobase mismatch opens the possibly for direct detection of miRNAs from biological fluids with neither pre-amplification nor pre-labelling of target nucleic acids.

While the length of the PEG spacer on the SMART base did not improve detection, the introduction of anionic groups at the backbone of the Abasic PNA probes improved hybridisation efficiencies when coupled to microspheres. Our results show that Abasic PNAs containing monomers modified at the gamma-position by a propanoic acid residue have superior sequence selectivity when compared to unmodified PNAs, matching previous studies [43-45].

The synergistic combination of these novel technologies provides an accurate tool to profile miRNA122, allowing direct detection of miRNAs from biological fluids in a single device. Multiplexing can be achieved by coupling various target-specific probes onto microspheres with different spectral signatures. Hence the novel platform promises to provide a consistent, rapid, cost-effective and straight-forward method for clinical diagnostics and drug

development by screening of miRNAs, not only associated with liver disease but also with genetic diseases, cancers, drug toxicology and heart diseases.

Acknowledgements

This research was supported by a Scottish Enterprise SMART Grant number SMART/13/070/13/9259. JJDM thanks Spanish Ministerio de Economía y Competitividad for a Ramon y Cajal Fellowship. This research was also partially supported by: a) Marie Curie Career Integration Grants within the 7th European Community Framework Programme (FP7-PEOPLE-2011-CIG-Project Number 294142) to RMSM; b) Marie Curie Career Integration Grants within the 7th European Community Framework Programme (FP7-PEOPLE-2012-CIG-Project Number 322276) to JJDM; c) EASTBIO (the East of Scotland BioScience Doctoral Training Partnership funded by the BBSRC). d) Spanish Ministerio de Economía y Competitividad within a FPI Grant BES-2013- 063020 to ALG. These studies were approved and supported by Luminex Corporation and DestiNA Genomics Ltd.

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