A simple and sensitive enzyme-free approach for the detection of multiple microRNAs using a single sequence-independent nanostructured enhancer of surface plasmon resonance imaging

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ABSTRACT

Here we describe a simple approach for the simultaneous detection of multiple microRNAs (miRNAs) using a single nanostructured reagent as surface plasmon resonance imaging (SPRi) enhancer. The strategy involves the preparation and optimization of neutravidin-coated gold nanospheres (nGNS) functionalized with a previously biotinylated antibody (Ab) against DNA/RNA hybrids. The Ab guarantees the recognition of any miRNA sequence adsorbed on a surface properly functionalized with different DNA probes; at the same time, gold nanoparticles permit to detect this interaction thus producing enough SPRi signal even at a low ligand concentration. After a careful optimization of the nanoenhancer and after its characterization using transmission electron microscopy, ultra-violet and radio-labelling methods, the final assay allowed the simultaneous detection of multiple miRNAs with a limit of detection (LOD) of up to 0.55 pM (equal to 275 attomoles in 500 μ L) by performing a single enhancing injection. The proposed strategy shows good signal specificity and permits to discriminate wild type, single- and triple-mutated sequences much better than non-enhanced SPRi. Finally, the method properly works in complex samples (total RNA extracted from blood) as demonstrated by the detection of four miRNAs potentially related to multiple sclerosis used as case study. This proof-of-concept study confirms that our approach provides the possibility to detect a theoretically unlimited number of miRNAs using a simple protocol and an easily prepared enhancing reagent, and may further facilitate the development of affordable multiplexing miRNA screening for clinical purposes.

KEYWORDS

SPR; nanoparticles; nanobiosensor; miRNA; enhancement; multiplexing

INTRODUCTION

The small size, low concentrations, various abundance and sequence similarity of microRNAs (miRNAs) make them challenging to detect and means that, unlike the quantification of mRNA (i.e. gene expression measurements) for which existing analytical approaches satisfy most standard analytical and practical requirements, the methods of profiling and quantifying miRNAs are still to be improved ¹. The currently used platforms, most of which are based on quantitative polymerase chain reaction (PCR), deep sequencing and microarrays, do not always combine good analytical performance (sensitivity, specificity and multiplexing capacity) with sufficient usability (high-throughput power, simplified workflows and cost-effectiveness) ^{2,3}. This is one reason why, despite the potential use of miRNAs as circulating stable diagnostic markers or predictive markers of disease progression, they have not yet been screened in large patient cohorts thus probably delaying their introduction into clinical practice. There is therefore a need to develop new and technologically improved analytical approaches.

Much has recently been done in this direction and, among the promising new approaches based on optical, electrochemical and nanotechnological strategies (reviewed in ³⁻⁵), surface plasmon resonance (SPR) has made a large contribution to the field of miRNA detection ⁶⁻⁸. The relevance of SPR to DNA and RNA detection is mainly based on the direct capture of label-free signals arising from the simple adsorption of target molecules onto complementary probes, but the low molecular weight of miRNAs and their very small concentration in clinical samples require SPR enhancements. The pioneering SPR-based method of miRNA detection using a signal enhancer was described by Corn *et al.*, who demonstrated the detection of miRNA after coupling gold nanoparticles (NPs) and enzymatic reactions ⁹. Some of the other enhancement strategies developed with the aim of improving and simplifying miRNA detection are based on sequence-

sandwich (or super-sandwich) approaches to coupling miRNA targets with enhancers including Au and Ag nanoparticles ¹⁰⁻¹², streptavidin ^{13,14}, and graphene oxide-Au NPs ¹⁵. Most of these methods offer very good analytical performances and high sensitivity. Besides, some currently proposed sequence-sandwich strategies may imply the partial hybridisation of the miRNA target with a first semi-probe immobilised on the SPR chip, thus potentially reducing hybridisation efficiency and specificity given the shortness of miRNA sequences; others require the use of as many sequence-specific enhancers as the number of miRNAs to be detected, thus reducing multiplexing capability, or the use of multiple reagents and/or multiple enhancing steps.

An alternative solution is to use "universal" enhancers able to recognise the presence of miRNA targets onto an array in a sequence-independent and sequence-sandwich-free manner, i.e. by avoiding the partial hybridization of two (or more) semi-probes to the target sequence. For example, anti-DNA/RNA hybrid antibodies ¹⁶ and protein 19 ¹⁷ have been used as SPR signal enhancers reporting sensitivity above pM. More sensitive universal sequence-free strategies have been reported but these are based on specific enzymatic reactions (able to discriminate the presence of miRNA molecules, such as ligation ¹⁸, polyadenylation ⁹ and nucleases reactions ¹⁹) that may limit the protocol simplicity due to specific enzymatic reaction conditions, long reaction time, reproducibility biases and elevated costs.

The aim of this study was to develop a single, universal SPR enhancer capable of simultaneously detecting multiple miRNAs with sensitivity below pM and without the use of enzymatic reactions, partial sequences hybridization steps, multiple reagents or multiple enhancing steps. To this end, we just combined 1) the universal sequence-independent recognition of miRNA thanks to the use of an antibody against all DNA/RNA hybrids and 2) the SPR-enhancing properties of gold NPs properly functionalized with the mentioned antibody (See the graphical abstract "TOC"). This

commercially available antibody recognises DNA/RNA hybrids without sequence specificity, and is similar to the S9.6 antibody previously used for microarray-based ²⁰, SPR-based ¹⁶ and graphene/composite electrode-based methods of miRNA detection ²¹, but never conjugated with GNS for enhanced SPRi strategies. Passing through careful protocol optimization and deep characterization steps anti DNA/RNA Abs were conjugated with gold nanospheres (GNS) using simple biotin/avidin strategy and the new nano-enhancer was tested by means of commercial, high-throughput and fully automated SPR imaging (SPRi).

Multiple sclerosis (MS), a highly heterogeneous, chronic and immune-mediated demyelinating disease ²², was used for a case study. On the basis of the most recent guidelines, a clinical evaluation and magnetic resonance imaging (MRI) are still the only available means of staging and monitoring MS ²³. Some blood MS biomarkers have been proposed, but none of them has been clinically implemented for diagnostic, sub-typing or staging purposes ²⁴. Some miRNAs have been also reported as promising MS markers according to what reported by several studies ^{25–29} and by relevant reviews ^{30,31}, but these still need to be validated in large patient cohorts, and further MS-related miRNAs may be discovered by means of multiple miRNAs studies. MS is therefore a paradigmatic example of diseases for which the development of efficient and versatile multiplexing miRNA detection methods may help patient care. On the basis of these premises, we tested our enzyme-free universal SPRi enhancer to detect multiple MS-related miRNAs on total RNA extracted from blood, which is the standard sample used for miRNA detection.

RESULTS AND DISCUSSION

Spotting optimisation. Contact spotting procedures for depositing thiolated oligonucleotides on a chip's surface are practical and cost-effective, but the spotting needs to be suitably optimised. Before starting the enhancement experiments, a number of tests were carried out in order to select the spotting buffer that guaranteed the strongest SPRi signal and the greatest reproducibility.

We first studied the effects of various surfactants typically used for printing and spotting, including some reported in literature ³²: Tween-20, sucrose monolaurate, Triton X-100, pentaethylene glycol monododecyl ether (C12E5) and 0.05% glycerol were tested in PBS pH 7.4, and compared with pure water and PBS without any surfactant. As can be seen in Fig. 1A, the choice of surfactant affected both the shape of the spots and the final SPRi signal, whereas pure water and PBS alone did not produce good spots and led to very poor signals. Glycerol was completely inefficient; C12E5 led to relatively good signals but irregular spots with coffee-ring features and poor reproducibility; and only Triton X-100, sucrose monolaurate and Tween20 all produced spots with regular boundaries and good homogeneity. Of the last three surfactants, Tween20 guaranteed the strongest signal Triton X-100 produced over-large spots and, when the distance between the spots was small, sometimes caused spots to merge (data not shown).

Subsequently, various buffer solutions selected from the literature ^{33,34}, as being among the most used for microarray spotting (i.e. PBS, pH 7.4; phosphate buffer 0.2 M, pH 3.8; acetate buffer 0.1 M, pH 6; and saline-sodium citrate 3x (SSC3x), pH 7.4) were mixed with Tween-20 0.05% in order to establish the best combination of buffer and surfactant, which proved to be PBS pH 7.4 + Tween-20 0.05% (Fig. 1B).

Figure 1. Spotting optimisation. Panels A and B both show the SPRi signal related to the injection of miR-223 (500 nM, signal at equilibrium) flowed onto a chip spotted with the same complementary thiolated DNA probe previously dissolved in different solutions. (A) SPRi signals detected in correspondence with the thiolated DNA probes dissolved in solutions containing different surfactants. The CCD differential image (reflectivity variation) acquired during the measurement (association phase at equilibrium) is shown beside the bar plot. (B) SPRi signals detected in correspondence with the thiolated DNA probes dissolved in different buffers, all mixed with Tween-20 0.05% as the best surfactant. Each bar corresponds to the average signal obtained from three parallel spots and the subsequent average of two consecutive and identically administered injections (500 μ L, 50 μ L min⁻¹).

Universal nano-enhancer production: neutravidin-GNS (nGNS) synthesis and functionalisation with anti-DNA/RNA hybrid Ab. The proposed SPRi enhancing strategy is based on the use of nGNS functionalised using anti-DNA/RNA hybrid Ab. GNS with a diameter of around 14 nm were prepared using citrate as the reducing agent, and stabilised using a carboxylated self-assembled monolayer (SAM). The coated nanoparticles showed excellent stability and did not show any sign of aggregation for a few months. In order to define the best approach to guarantee a good neutravidin conjugation but also to prevent a loss in the stability of the nGNS leading to the higher unspecific adsorption of the surface of the SPRi chip, we optimised the protocol in order to define the best amount of neutravidin required to functionalise all the GNS. Neutravidin was conjugated onto the SAM layer using EDC/sulfo-NHS coupling and the neutravidin conjugation was followed by gel electrophoresis, UV-vis spectroscopy and TEM measurements (Fig. S1).

Coupling data acquired by means of ICP-AES and by radio-labelling protein determination using iodine-125 radionuclide made possible to quantify the number of neutravidin molecules attached to each nanoparticles. Radio-labelling allows the precise quantification of very low protein levels, and is one of the few methods that can be used to measure the amount of protein linked and not linked to the nanoparticles at the same time. The results showed that a mean 2.58 ± 0.19 neutravidin molecules were conjugated per GNS in the case of EDC/NHS covalent coupling, and 1.6 ± 0.25 in case of non-covalent adsorption (see Table S1 for details) thus demonstrating that neutravidin was covalently conjugated on the surface of the nanoparticles and not simply adsorbed.

Considering the importance of using a robust and controlled procedure for the production of the nanoenhancer³⁵, before functionalising the nGNS with the biotinylated anti-DNA/RNA Abs, we

tested various Ab:biotin molar ratios (1:5, 1:25, 1:50 and 1:75) considering a biotinylation reaction yield of around 10% according to protocol suggested by manufacturer. This test was done in order to compare the activity of the original Ab and with that of the differently biotinylated Abs injected immediately after the injection of a high concentration of miRNA (500nM) and determine signals at equilibrium binding conditions (Fig. 2A-C). As expected, increasing the biotinylation molar ratio led to a progressive reduction in Ab activity due to the chemical modification of the antigen-binding site (Fig. 2A). On the other hand Fig. 2B-D and Fig. S2 show the results when the different Abs were mixed with nGNSs and injected after the injection of a low concentration of miRNA (1 nM) with the aim of selecting the optimal Ab:biotin molar ratio and verifying the best conditions for SPRi enhancement. The biotinylated Abs mixed with nGNS greatly enhanced SPRi, thus confirming nGNS functionalisation and Ab coupling. The SPRi signal progressively increased when moving from the non-biotinylated Ab (Ab:biotin molar ratio 1:0) to a ratio of 1:25 biotinylated antibodies, which led to approximately 900-fold enhancement (compared to non-enhanced SPRi miRNA detection), and began to decrease when the higher ratios of 1:50 and 1:75 were used. Taken together, these findings show that the 1:25 ratio offered the best compromise between Ab activity and the number of Abs linked to the nGNS. As shown in Fig. 2D and Fig. S2, the nGNS alone did not show any significant signal arising from aspecific interactions between the nGNSs and the SPRi chip surface, and the original non-biotinylated Abs used alone showed similar enhancement to that observed when the nGNSs were mixed with non-biotinylated Abs (19-fold and 17-fold). This suggests that the non-biotinylated Abs do not spontaneously bind nGNS and that their combination does not lead to any significant additional signal due to aspecific interactions. It is finally worth noting that in these experiments and all of the further experiments

involving model or real samples, the mixture of nGNS and the Ab used as the only enhancing reagent was stable for days without any further purification if stored at 4°C.

Radio-labelling experiments were also used to determine the number of Abs effectively linked to the nGNS and clarify the importance of Ab biotinylation, (Fig. 2E). Using the theoretical molecular weight of the nGNS ($1.67 \cdot 10^7 \text{ g} \cdot \text{mol}^{-1}$), the molecular weight of the antibodies (about 150 kDa), and the gamma emissions produced by functionalised nGNSs after the reaction, the number of linked can be easily calculated (see more details in "Materials and methods" and Table S2). The result confirmed that, under our experimental conditions, the number of biotinylated Abs linked to each nGNS are between 0.7 to 4.1 and this is compatible with the number of biotin-binding sites provided by the number of neutravidin molecules immobilised on the nanoparticles (i.e. about 2.58 neutravidin molecules x a maximum of four binding sites = a maximum of ~10 Abs/nGNS). More in details, nGNS conjugated with 1:5 biotinylated antibodies were linked with less than one Ab (0.73); the most active nGNS (i.e. those conjugated with an Ab:biotin ratio of 1:25) were linked with an average of 1.9 Abs, whereas the less active nGNS conjugated with more biotinylated Abs (i.e. at molar ratios of 1:50 and 1:75) were linked with respectively 2.4 and 4.1 Abs. These numbers are also close to the number of biotins supposed to be covalently bound to each antibody considering the biotinylation reaction yield of around 10% (i.e. 0.5, 2.5, 5, 7.5 biotin/Ab for 1:5, 1:25, 1:50 and 1:75 molar ratio respectively). As the enhancement factor did not linearly correlate with Ab activity or the number of immobilised Abs (see Fig. 2B), different biotinylation ratios may not only affect Ab activity and functionalisation efficiency but also the orientation of the immobilised Abs. All these data confirmed that the nGNS linked with more Abs were less active due to Ab damage, wrong Ab orientation or steric hindrance, and that the preparation of active nanoparticles-Ab conjugates require proper screening and optimization steps.

Figure 2. nGNS functionalisation tests. (A) Ab activity tests. The sensograms relates to the activity of the original Ab and the differently biotinylated antibodies (Ab:biotin molar ratios 1:5, 1:25, 1:50, and 1:75) injected (5 nM, 500 μ L, 10 μ L ml⁻¹) after the injection of of miR-223 (500 nM, 500 μ L, 10 μ L min⁻¹) (not shown). (B) Enhancement test (see Fig. S2 for larger graphs). The sensogram relates the injection of nGNS (0.25 O.D. at Abs. λ 524 nm) mixed with 5 nM of the original Ab and the differently biotinylated antibodies (Ab:biotin molar ratios 1:5, 1:25, 1:50, 1:75) (800 μ L, 10 μ L min⁻¹) after the injection of 1 nM of miRNA 223 "(1)" (500 μ L, 50 μ L/min). Each Ab+GNS injection, was followed by a dissociation phase (running buffer "(2)") and chip

regeneration phase (double injection of NaOH 50 mM "(3)"). The signal in A and B are the average of three spots after the subtraction of the signals from the negative references (PolyA sequences) spotted in parallel on the same chip. Both sensograms also show the signal related to miR-159 (not injected). (C) Activity of Abs functionalised with increasing amounts of biotin; the SPRi signals shown in panel "A" were normalised using a non-biotinylated Ab as the reference (100%). (D) SPRi enhancement induced by the original Ab, the nGNS, and the nGNS conjugated with differently biotinylated Abs; enhancement was calculated by dividing the SPRi signals (see panel "B") produced by the Ab+nGNS by those produced by 1 nM of miR-223 alone injected before the Ab+nGNS. (*) See the sensograms in Fig. S2 for the enhancement produced by the nGNS and the original Ab alone. E) Results of the radio-labelling experiments; the values refer to the average number of Abs linked to each nGNS (see Table S2 for details).

Analytical performances. The specificity of the signal produced by the Ab+nGNS can be seen in the sensogram (Fig. 2B) and the chip CCD differential image (Fig. 3A), neither of which shows any significant signal related to the chip surface or negative control spots. The interaction between the Ab+nGNS and the SPRi chip was investigated by SEM at the end of an enhancement experiment that was without the regeneration steps normally used to remove interacting/adsorbed molecules. As shown in Fig. 3B and 3C, the positive spots are covered by nanoparticles of the expected size and probably clustered in correspondence with the presence of SH-DNA/miRNAs hybrids; the negative spots do not show any significant interactions.

Figure 3. SPRi chip images. (A) CCD differential image (reflectivity variation) of the SPRi chip acquired during Ab+nGNS-enhanced detection of four different miRNAs [1 nM]; the PolyA spots were used as references for each miRNA family, miR-159 was used as negative control. The two capital letters indicate the chip positions visualised by means of SEM and reported in B and C. (B) SEM image of the positive spot showing nGNS adsorbed on miR-223:DNA probe hybrids. (C) SEM image of one negative spot relating to miR-159, which was not injected in this experiment. Scale bar = 400 nm (200000 X). The two regions indicated by the dashed lines are magnified in the boxes enclosed by the solid lines (scale bar = 50 nm (450000 X)).

In order to evaluate the enhancing properties and dynamic range of the proposed method, two synthetic miRNAs (miR-422 and miR-223) injected in a wide range of concentrations were detected without enhancement; using only the Ab; and using the Ab+nGNS. Fig. 4A shows all of the calibration curves, which demonstrate the improvement in detection when using the two enhancing strategies. The dynamic ranges of the three approaches were from about 1 nM to >500 nM for direct detection, from about 50 pM to 100 nM (20x enhancement) for antibody-enhanced detection, and from about 0.5-1.0 pM to 1 nM (1000-2000x enhancement) for Ab+nGNS-enhanced detection. The sensitivity and the multiplexing capacities of the proposed method were tested by the simultaneous detection of four miRNAs that have been suggested as potential MS biomarkers (miR-422, miR-223, miR-126, and miR-23a)^{25,26,28,36}. These miRNAs were mixed, serially diluted in running buffer from 0.16 pM to 100 pM, injected in the SPRi instrument, and then detected using Ab+nGNSs as a single enhancing reagent. As shown in Fig. S3, the SPRi chip surface was completely regenerated between injections. Most of the binding curves (Figs. 4B and S4) were clearly separated from noise and their maximum values were plotted using a log-log scale for each miRNA (Fig. 4C). The data can be interpolated linearly through most of the tested concentrations, and the linear equation was used to calculate the limit of detection (LOD: the average of three replicated blank injections + 3 SD) for each miRNA: 0.55 pM for miR-422, 0.88 pM for miR-223, 1.19 pM for miR-126, and 1.79 pM for miR 23a. The amounts of detectable miRNA given the 500 μ L of injected volume are therefore between 275 and 890 attomoles. These data, show that our universal enhancement strategy does not seem to be affected by different sequences as the four calibration curves reside in similar SPRi signals intensity ranges. This is in line with the findings of a previous fluorescence microarray-based study that used an anti-DNA/RNA antibody to analyse small RNA expression²⁰.

In parallel, the sequence selectivity, necessary in case of mutated miRNA or miRNA isomers detection, was tested by comparing the SPRi signal obtained after injecting wild-type miR-23a and miR-23a carrying one or three mutations (Fig. 4D, Table S3) in three different experiments comparing the selectivity of non-enhanced SPRi (miRNA injected at 100 nM), Ab-enhanced SPRi (miRNA injected at 1 nM) and Ab+nGNS-enhanced SPRi (miRNA injected at 100 pM). These experiments were conducted using different analytes concentration according to the dynamic ranges of the three different signal detection approaches. As shown in Fig. 4D, the use of both Ab- and Ab+nGNS-induced enhancement increased the selectivity of the SPRi biosensor. In the case of the miR-23a carrying a single mutation, SPRi alone was unable to distinguish it (i.e. 96% of response signal in comparison with the wild-type sequence), Ab still produced 77% of the signal but the use of Ab+nGNS reduced the sensor response to 52%. This demonstrates that Ab, but especially Ab+nGNS enhancement, easily allow to recognise the mutated sequence probably because of the three-dimensional changes arising from the base-base mismatch. Ab and Ab+nGNS enhancement similarly improved sequence specificity in the case of the sequence with three mutations.

Figure 4. Sensitivity and sequence selectivity. (A) The calibration curves of the detection of miR-422 and miR-223 by means of standard SPRi (blue and red), Ab-enhanced SPRi (jade and orange) and Ab+nGNS-enhanced SPRi (grey and violet). (B) SPRi sensograms showing the Ab+nGNS-enhanced detection of miR-223 within the low concentration range of 0-100 pM that was used to plot the calibration curves in A and C (see Fig. S4 for the sensograms of all of the miRNAs). (C) Calibration curves of all of the studied miRNA families plotted using a log-log scale and fitted linearly (see equations in Table S4). The LOD values shown in B and C were calculated as the blank (0 pM) signal + 3 SDs). (D) Sequence selectivity. The bars represent the SPRi signals

corresponding to the detection of non-mutated (WT) (100%) miR-23a, and miR23a carrying one and three mutations. The three variants were detected by means of non-enhanced SPRi (after injecting miR-23a 100 nM), Ab-enhanced SPRi (after injecting miR-23a 1 nM) and Ab+nGNS-enhanced SPRi (after injecting miR-23a 100 pM) in separate experiments.

Test on complex samples. In order to test our approach in a clinical relevant environment, we used total RNA extracted from serum samples taken from three healthy control subjects, which were processed as is usually done in pre-clinical research laboratories before RT-PCR quantification^{28,29}. Total RNA samples were isolated from serum using a commercial extraction kit, diluted in 500 μ L of running buffer, and directly injected into the instrument, followed by the Ab+nGNS. All three samples were automatically and consecutively injected onto the SPRi chip, which had been previously used to define the calibration curves. The four studied miRNAs (miR-422, miR-223, miR-126, and miR-23a), which were selected because they have previously associated with MS^{25,26,28,36}, were then detected and quantified (Fig. 5A). These data show the varied contents of miRNAs in different samples as expected and reported in literature and show that, at least for these four selected miRNAs, the proposed approach is adequate for the realistic dynamic range for clinical purposes. A parallel quantification by RT-PCR was carried out thus demonstrating a good agreement between the data obtained by the proposed SPRi approach and demonstrating that the total RNA complex sample does not interfere the SPRi measurement (Fig. 5B). Worthy of note, also RT-PCR was not able to detect miR-422 in Sample 1, probably because at a very low concentration in the real sample. It is obviously beyond the scope of this study to provide clinical evidence concerning significant differences in miRNA expression between subjects. On the other hand, our experiments aim to confirm that multiple miRNA can be detected

starting from complex samples and passing through standard sample preparation followed by a very simple detection approach.

Figure 5. Tests on complex samples. (A) Total RNA extracted from serum samples taken from three healthy control subjects were analysed by SPi. The concentrations of four different miRNAs (miR-223, miR-126, miR-23a, and miR-422) were calculated on the basis of the calibration curves shown in Fig. 4 and were corrected (1:0.687 dilution factor) considering that the RNA extracted from 800 μL of serum was then suspended in a final volume of 550 μL ; 500 μL of those were injected ($10 \mu\text{L min}^{-1}$) followed by 800 μL Ab+nGNS ($10 \mu\text{L min}^{-1}$). (B) Comparison between the new proposed method and miRNA quantification by RT-PCR. The same sample of total RNA extracted from serum (Sample 1) was used as starting material for both quantifications. The relative signal intensity was based on the expression ratio between the four miRNA and miR-223. For SPi results were used the quantities and the standard deviation reported in Fig. 5A. For the RT-PCR results relative expression fold between each miRNA and miR-223 were used.

CONCLUSION

This paper describes a new and practical approach to the sub-picomolar simultaneous detection of multiple miRNAs without the use of enzymes or multi-step enhancing protocols. The major advantage is that just one, stable and easy-to-prepare reagent consisting of gold nanoparticles functionalised using a commercially available antibody is sufficient for the detection of a theoretically unlimited number of miRNAs. This is possible because of the coupling of an antibody able to recognize RNA/DNA regardless of sequence with the very good enhancing properties of gold nanoparticles. Our protocol also allows specific interactions (without GNS adsorption on the chip surface or on negative spots) and better sequence selectivity than the intrinsically low sequence selectivity obtained by means oligonucleotide hybridisation on a bare SPRi chip. Most of these benefits are due to the optimisation of GNS synthesis and functionalisation: the nano-structured enhancer was thoroughly characterised, including the absolute radio-labeling quantification of the average number of neutravidin molecules and antibodies immobilised on each single nanoparticle. As proof-of-concept, we tested our method by simultaneously detecting four miRNAs related to MS in real samples.

Further studies are planned in order to carefully validate the method with standard methods and to increase the number of real samples, and the number of miRNAs that can be detected on the same chip, which is only limited by the number of spots that can be deposited on the chip surface. This should allow the development of more feasible and affordable methods of screening and validation of miRNAs related to the diagnosis, subtyping and progression of MS. At the same time, this approach can obviously also be used to detect miRNAs related to any other disease or process of clinical or research interest.

METHODS

Chemicals and solutions. The reagents used in this study were purchased from Merck KGaA, Darmstadt, Germany, unless otherwise specified. Diethyl pyrocarbonate(DEPC)-treated Rnase- and Dnase-free water was used to prepare the thiol-modified DNA and RNA solutions. The oligonucleotide sequences (Eurofins Genomics, Germany) were purified by means of high-performance liquid chromatography (HPLC) by the manufacturer and the thiol-modified sequences were reduced with tris(2-carboxyethyl) phosphine (TCEP) before use. All of the oligonucleotide sequences are shown in [Table S3](#). The iodine-125 radionuclide was purchased from PerkinElmer, Spain.

GNS synthesis and conjugation with neutravidin. Gold nanospheres (GNS) of 14 nm with maximum optical density (OD) at 520 nm were synthesised using the citrate-based approach as previously described excepting for the order of the addition of reagents which is here inverted ³⁷ (Fig. S1). Three hundred millilitres of ultrapure water were brought to the boil, after which 12 mL of sodium citrate 50 mM were added and stirred for 15 minutes; subsequently, 2.5 mL of tetrachloroauric acid ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) 20 mM were added and the solution was stirred under boiling conditions for ~1h in order to obtain monodispersed GNS characterized by an intense red color. Finally, the solution was slowly cooled to room temperature under stirring for two hours. Bare GNS were coated with alpha-(11-Mercapto-undecanoylamido)-omega-carboxy dodeca (ethylene glycol) (HS-FA-PEG-COOH) (818 Da) using a protocol previously reported ³⁸. Briefly, 10 nM (see “*nGNS characterisation* “below for GNS quantification) of GNS were mixed with 0.028 % sodium dodecyl sulfate and 2.0 mM HS-FA-PEG-COOH. NaOH was further added to a final concentration of 25 mM and the mixture was incubated in agitation for 16 hr at room temperature. The excess PEG chains were removed by centrifugation at 14,000 x g for 30 min at

4°C and the supernatant was discarded. This washing process was repeated three times and the pellet with GNSs was resuspended in ultrapure water and stored at 4°C. The bare GNS were then stored at 4°C, a temperature at which they remain stable for several months if protected from direct light. Before beginning the conjugation with neutravidin, the GNS were concentrated by centrifuging 1 mL of GNS (0.5 OD) at 14,000 x g for 20 minutes, and resuspended in 250 µL of water. A mixture of 60 µL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC 10 mM), 12 µL sulfo-*N*-hydroxysuccinimide (sulfo-NHS 10 mM) and 48 µL of water was added to the vial containing the 250 µL of GNS immediately after its preparation, and the suspension was gently stirred for 15 minutes. The GNS were then centrifuged at 14,000 x g for 20 minutes, and resuspended in 880 µL of phosphate buffer, pH 5.2 (1 mM). Thereafter different amount (0, 2, 4, 6, 10, 15, 20 µL) of neutravidin (Thermo Fisher Scientific, Waltham, MA, USA, product Nr. 31000) solution 280 µg mL⁻¹ were added to different GNS solutions (reaching final neutravidin concentration from 0 to 104 nM) in order to screen the best amount of neutravidin, finally defined as 6 µL (32 nM) (see the table in Fig. S1A). Each suspension was then gently stirred for 20 minutes. Subsequently, 160 µL of taurine 100 mM in sodium borate buffer (100 mM, pH 9) were added, and the suspension was gently stirred for 10 minutes. The neutravidin-GNS (nGNS) were centrifuged at 12,000 x g for 20 minutes, and resuspended in 10mM phosphate buffer saline (PBS) 10 mM pH 7.4. The conjugation of the GNS and neutravidin was followed by gel electrophoresis and also verified by means of ultraviolet-visible (UV-vis) spectroscopy. For gel electrophoresis experiments nGNS were resuspended in 20 µL of sucrose 20 mg mL⁻¹, instead of buffer, loaded onto 0.6% agarose gel, and the electrophoretic run was held for 30 minutes at 225V in TBE 0.5X, pH 8.

nGNS characterisation. The transmission electron microscopy (TEM) images for nGNS characterisation were collected using an FEI Tecnai T20 (FEI Europe, Eindhoven, The Netherlands) working at 200 kV. Image J software was used to measure the mean diameter of the nGNSs. For elemental analysis, the samples were evaluated by means of inductively coupled plasma atomic emission spectroscopy (ICP-AES) using an Optima 8300 (Perkin Elmer, MA, USA). By correlating the absorbance at 450 nm revealed by means of UV-vis spectroscopy with the Au concentration measured by means of the ICP-AES analysis, it was possible to obtain a conversion factor (ϵ) of 12.6 mL mg ml⁻¹ cm⁻¹ for GNSs at 450 nm. Assuming that the GNS were spherical and using the diameter measured by means of TEM, the density of Au fcc (19.3 g cm³), the Avogadro number, and the theoretical molecular weight (MW) of GNSs was calculated (1.67*10⁷ g mol⁻¹). These data were used to calculate the protein:GNS molecular ratio and for radiolabeling experiments.

nGNS functionalisation with anti-DNA/RNA hybrid antibody. The monoclonal anti-DNA/RNA hybrid antibody (Ab; Covalab, mab0105-P, clone D5H6) was dialysed in PBS for using a Slide-A-Lyzed mini-dialysis unit with a 3.5 kDa MW cut-off limit (Thermo Fisher Scientific, Waltham, MA, USA, product nr. 69550) in order to remove the Tris, sucrose and glycine used as storage additives. The Ab was then diluted in PBS (0.5 mg ml⁻¹, 3.33 μ M), and mixed with freshly prepared NHS-PEG4-biotin (EZ-Link™, Thermo Fisher Sci., 21330). Different amounts of the NHS-PEG4-biotin stock solution (1 mM) were added to the Ab in order to obtain solutions with 5, 25, 50 and 75 biotin:Ab molar ratios. After one hour of incubation at room temperature, the mixture was dialysed twice in PBS pH 7.4 in order to remove the unreacted biotinylation reagent, and the biotinylated Ab was stored at 4°C until use. The neutravidin-coated GNS (nGNS) were functionalised with the biotinylated antibodies before each enhancement experiment by

diluting them in PBS Tween 0.01% (0.25 OD measured at 524 nm) and mixing them with the biotinylated Ab in order to obtain a final antibody concentration of 5 nM. The mixture was incubated by means of gentle shaking for at least 30 minutes before use.

Radio-labelling protein quantification. A previously described radio-labelling protein quantification method ³⁹ was used with minor modifications. See details in *Supplementary Information*.

SPRi chip preparation. Bare gold SPRi biochips (HORIBA Scientific SAS, Palaiseau, France) were cleaned with Piranha solution for 20 minutes at room temperature and washed with ultra-pure water before use. They were then microspotted by means of contact printing (SPRi-Arrayer, HORIBA Scientific SAS, Palaiseau, France) using Xtend metal-ceramic capillary pins with a 700 µm spot diameter. Spotting was optimised by dissolving the thiol-modified (5' thiohexyl (C6)) DNA oligonucleotides in different spotting solutions (PBS, pH 7.4; KH₂PO₄, pH 3.8: acetate 0.1 M, pH 6; or saline-sodium citrate 3x) containing different surfactants (Tween-20 0.05%, sucrose monolaurate 0.05%, Triton X-100 0.05%, pentaethylene glycol monododecyl ether (C12E5) 0.05% or glycerol 0.05%) in order to obtain a final concentration of 30 µM. The optimal spotting solution was PBS, pH 7.4, and Tween-20 0.05%. At least three spots were made for each probe, and negative control (reference) probes (PolyA DNAC 5-S) were spotted in parallel in order to minimise spikes related to differences in the position of the response and reference spots. The spotting procedure was performed at about 70% relative humidity and 20°C. Before the spotting of each probe, at least three pre-spots were made on a separate chip in order to prepare the pins for definitive spotting. The printing pins were automatically washed with EtOH 10% and dried by air at least four times between the spotting of different samples. The spotted SPRi biochip was then incubated overnight at 20°C with a relative humidity of 75% controlled using a saturated salt

solution of sodium chloride, after which it was briefly washed with ultra-pure water and then blocked using PolyA DNA 5-S (10 μ M) in water for four hours at room temperature with gentle shaking. Finally, the chip was washed in water for 12 hours, dried, and immediately used or stored at 4°C.

SPRi experiments. The SPRi experiments were carried out using XelPleX (HORIBA Scientific SAS, Palaiseau, France), a fully automated high-throughput SPRi system based on acquisitions at incident angles selected during the preparation phase on the basis of the best resonance condition for each spotted probe (a maximum of ten angles simultaneously). No more than three incident angles were simultaneously monitored. After each experiment, the system and the SPRi chip were conditioned with the running buffer (PBS and Tween 0.01%) for more than 20 minutes and by means of two injections of NaOH 50 mM (100 μ L) with a flow of 100 μ L min⁻¹ in order to stabilise the chip surface. The CCD response of each spot was then calibrated and normalised by injecting 200 μ L of sucrose 3 mg ml⁻¹. The flow rates used to inject the miRNAs, antibodies and nGNS were 10 or 50 μ L min⁻¹ depending on the type of experiments. The surface of the SPRi chip was regenerated by injecting one or twice 100 μ L of NaOH 50 mM.

The calibration curve was calculated by performing a linear fitting of data after plotting them using a log-log scale. For each miRNA the LOD signal was calculated as the average of three SPRi enhanced signals obtained after the injection of running buffer summed to 3 times the corresponding standard deviation (i.e. average of background signal + 3 SD). The miRNA concentration corresponding to each LOD signal was calculated using the fitting equations reported in Table S4.

Scanning electron microscopy (SEM). SEM images of the SPR chips were acquired using a field emission SEM Inspect F50 with an EDX INCA PentaFET×3 system (FEI Company, Eindhoven, The Netherlands) in an energy range of between 0 and 30 keV.

Tests on complex samples. The tests on complex samples were performed using blood from three healthy control subject enrolled at IRCCS Fondazione Don Carlo Gnocchi. The subjects included in this study gave written informed consent in accordance with the protocols approved by the ethics committee of the same institution and according to the principles of the Declaration of Helsinki. Peripheral blood was collected in BD Vacutainer® SST™ II Advance Tubes (Becton Dickinson, Franklin Lakes, NJ, USA). At the end of clotting time (60 minutes), serum was obtained by centrifugation at 1800 g for 10 min at room temperature. The clear supernatant was aliquoted into RNase/DNase-free tubes and stored at -80°C until use. Serum was then thawed on ice, centrifuged at $16,000 \times g$ for 5 min in a 4°C , and then total RNA was extracted from 200 μL of serum using spin column chromatography (miRNeasy Serum/Plasma, Qiagen GmbH, Hilden, Germany) according to protocol suggested by manufacturer. RNA was eluted by adding 15 μL of RNase-free water for each column. A total of 60 μL of RNA obtained from 800 μL of serum was then resuspended in 550 μL and analysed by SPRI.

ASSOCIATED CONTENT

Supporting Information. The following files are available free of charge.

“Supplementary Information” (PDF)

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Funding Sources

Research funding was provided by the Italian Ministry of Health within the framework of the European EuroNanoMedII Project (Call 2015) entitled "NanoPlasmiRNA"; DGA-FSE (Diputación General de Aragón–Fondo Social Europeo); Ministerio de Educación, Cultura y Deportes of Spanish Government, FPU grant (FPU014/06249).

ACKNOWLEDGMENT

The authors thank Dr. Domenico Caputo for patients' recruitment and for his support for clinical aspects. They also thank Nicholas Corneli for his support for spotting and buffer optimization. The authors gratefully acknowledge 'The Advanced Microscopy Laboratory' (INA-Universidad de Zaragoza) for access to their instrumentation and expertise. They also thank J.C. Raposo of

the Servicio Central de Análisis de Bizkaia from SGIker of Universidad del País Vasco (EHU) for the ICP technical support and J. Puertas of the Radioisotope Service of Universidad de Zaragoza for the radiolabeling technical support.

ABBREVIATIONS

Ab, antibody; C12E5, pentaethylene glycol monododecyl ether; CCD, charge-coupled device; DEPC, diethyl pyrocarbonate; EDC, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; GNS, gold nanospheres; ICP-AES, Inductively coupled plasma atomic emission spectroscopy; LOD, limit of detection; miRNA, microRNA; MS, multiple sclerosis; nGNS, neutravidin-coated gold nanospheres; NHS, N-Hydroxysuccinimide; NP, nanoparticle; OD, optical density; RT-PCR, real time PCR; SAM, self-assembled monolayer; SPR, surface plasmon resonance; SPRi, surface plasmon resonance imaging; SSC3, saline-sodium citrate; TCEP, tris(2-carboxyethyl) phosphine.

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