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One-Step Detection of miRNA-21 Cancer Marker in Serum using Molecularly Imprinted Nanoparticle Recognition and Surface Plasmon Resonance

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Abstract

Background

The rapid one step detection of cancer markers at early stages of cancer is critical to increase the efficiency of the treatments and survival. miRNA-21 is the most commonly upregulated microRNA in solid tumours and haematological malignancies and is associated with poor prognosis and survival rate. Therefore, its detection at the early stage is vital, ideally using a rapid, simple method with a low detection limit. Current methods often require sophisticated method and labelling to detect miRNAs.

Results

Here, we show that nanosized molecularly imprinted polymers (nanoMIPs), can selectively and sensitively detect miRNA-21 without labelling. NanoMIPs were synthesized via solid-phase polymerization. The template molecule of 5' phosphor-capped miRNA-21 was attached to a functionalised glass bead via phosphorylimidazolid chemistry. Selected functional monomers/cross-linkers were used to generate selective nanoMIPs with recognition based on electrostatic and ionic interactions. NanoMIPs were characterized to investigate their physicochemical properties and interaction with target molecule. These nanoMIPs were then used for miRNA-21 detection studies exploring affinity and selectivity to the target with Surface Plasmon Resonance in both buffer and serum. High affinity materials (equilibrium dissociation constants – K_D 's in the nM/pM range) were demonstrated with high selectivity and when linked to the SPR sensor, a theoretical lower detection limit (LOD) of miRNA-21 was calculated as 0.49 pM in serum.

Significance:

This work provides new perspectives in developing miRNA-21 specific synthetic molecular recognition materials using nanoMIPs. The nanoMIPs has potential to be suitable sensor platform, that could be promising tools for early cancer diagnostics in complex matrices.

Keywords: miRNA-21, molecularly imprinted polymers, early cancer detection, surface plasmon resonance

Highlights:

- A highly sensitive nanoMIPs sensor was developed for selective miRNA-21 detection.
- 5' phosphorylated miRNA-21 as an imprinting target was used for molecular imprinting.
- SPR was used as an analytical method for rapid, one step detection.

- Circular Dichroism (CD) and other techniques were used to investigate nanoMIP-miRNA-21 interactions.

1. Introduction

MicroRNAs (miRNAs) are non-coding ribonucleic acids (RNAs) that are evolutionarily conserved and length 10-20 nucleotides. miRNAs negatively regulate gene expression at the post transcriptional level by promoting transcript degradation or translational inhibition of messenger RNA [1,2]. miRNAs can regulate a range of physiological functions, such as development, cell differentiation, cell proliferation and cell apoptosis, and are found in a variety of tissue samples and body fluids [3-5].

Recent studies have shown that miRNAs could prevent cell death in certain tumours across a range of cancers [6,7]. The upregulation degree of miRNA can also be correlated with survival rate in a various cancer type including lung, breast, gastric cancers [3,8,9]. However, abnormal expression of miRNAs is also related to the inhibition and progression of disease in various cancer types, neurological and autoimmune diseases, and myocardial infarction [6,10-13]. miRNAs possess two different characteristic features as being tumour suppressors or oncogenes; the expression of miRNAs such as miRNA-34, miRNA-let7, miRNA-143 can act as tumour suppressors down regulating cancer cells, while oncogenic miRNAs such as miRNA-21, miRNA-373, miRNA-15a, miRNA-16-1 can be upregulated due to downregulation of tumour suppressor genes [4,14,15]. Therefore, the degree of miRNA biomarkers present will be related to cancer levels [5,14].

Studies have shown that miRNA-21 is the most common upregulated miRNA in solid tumours and haematological malignancies with poor prognosis and survival, and it also has a role in tumorigenesis regulation of the tumour suppressor genes; Tropomyosin 1 (TPM1), Phosphatase and TENsin homolog (PTEN), and Programme Cell Death 4 (PDCD₄) [16-18]. As such, miRNA-21 has been associated a variety of activities including cell invasion, proliferation, migration, apoptosis and metastasis, and chemoresistance [7,19-22].

Accurate and sensitive detection of miRNAs can be useful for diagnostic and prognosis to determine the cancer type depending on the expressed tissue [23,24]. However, the detection of miRNA-21 is currently challenging, due to the low levels present in the cell and small size of the molecules being tested, while using low sensitivity techniques [25].

Various methodologies have been implemented for sensitive, selective, and high throughput detection of miRNAs. These methods are based on electrochemical methods, optical methods, reverse transcription quantitative polymerase chain reaction (qRT-PCR), digital PCR, Northern Blot and high throughput sequencing [4,26-28]. In example, Huang and colleagues used enzyme-free DNA sensor and achieved highly sensitive and selection detection, yet their detection probe was based on DNA. Although their method was promising to detect miRNA-21, their system relied on natural DNA, and the hairpin probes were only stable for 8 hours [23]. Similarly, Chen and colleagues reported another single strand-double strand DNA dependent probe and used Ti_3C_2 nanosheets for the fluorescent resonance energy transfer assay [29]. Li and colleagues also reported the two hairpin structures, H1 and H2 and nanoparticles (AuNPs) hybrid probe for mRNA-21 detection [30]. Although these methods have showed the limits of detection from fM to pM sensitivity, the recognition probes require a large amount of purified sample and long incubation times, marker labelling, time consuming preparation and analysis [23,25,29-35]. Hunt developed an electrochemical biosensor for the detection of miRNA-21 using ssDNA-21 probe and gold inkjet printing onto photopaper and gold nanoparticle electrodeposition. They also used 1000-fold dilution to avoid matrix effect and obtain low detection. Although the detection limit for this work was in the femtomolar range, the stability of the biosensor shelf life was limited to one month [36]. As highlighted in these works, stability is a major issue. These limitations of current detection techniques require new approach to detect miRNA-21 in real-world samples. Sustainable synthetic recognition materials could overcome these disadvantages of current miRNA systems.

Molecularly imprinted polymers (MIPs) are an attractive alternative for the miRNA detection, due to their proved high stability, cost effectiveness, 3R's profile and ability to be tailored for a range of specific analytes [37-40]. MIPs can produce binding sites which are complimentary to the size, shape and attached functional groups using a template molecule. This process involves the use of a cross linker combined with functional monomers which complex (usually non-covalently via hydrogen bonding, Van der Waals and dipole-dipole interactions) around a template molecule. These undergo polymerization to form a matrix in which the template recognition complex is entrapped. The template is then removed keeping the molecularly imprinted polymer matrix and binding pocket intact due to the crosslinked structure of the particles [41].

MIPs are currently used in a range of biological applications including enzyme linked assays, sensors, *in vivo* applications, drug delivery and cancer diagnostics for numerous biological templates such as drugs, proteins, peptides, lipids, amino acids, viruses and cells. However, example nucleic acid recognition has not been extensively explored [38,42-52], with miRNA-

21 a rare target for this work, probably due to the difficulty of targeting a single strand nucleic acid sequence [41]. Kranz and colleagues developed an electrochemical sensor using peptide nucleic acid polymer hybrids (PNA) which, while offering the required sensitivity (pM level detection), utilised a complex multi-stage and expensive synthesis [53]. Nouraei and colleagues developed MIP-coated silica beads for sample separation, but their detection required qRT-PCR for measurement, as well [54].

Development of MIPs of MiRNA-21 could offer cost effective, and reliable rapid methods of detecting disease early therefore of great interest, as this can lead to earlier treatment and potentially increased survival.

Surface plasmon resonance (SPR) is a real time, label-free optical biosensing method that is effective in monitoring biomolecular interactions including antigen-antibody, protein-protein and small-molecule interactions. SPR provides high sensitivity even with compounds with low molecular weights, and it requires minimal sample preparation [25,42]. Due to this, it is considered the industry standard for ligand-target analysis. Previously confined to laboratories, portable SPR systems have entered the market in the past ten years making this a potentially powerful tool for clinical applications. Dubois and colleagues used SPR sensor method for immunoglobulin A detection in plasma and reported the limit of detection as 400 ng mL⁻¹ in diluted plasma [55]. Yu and their team developed portable SPR sensor to detect human IgG at nanomolar concentration. Their approach highlighted a promising method for cost effective, high performance and scalable detection [56]. With this respect, SPR has been also an attractive alternative for one-step, sensitive, rapid, real-time, label-free biomarker detection [25,39,57,58]. Jebelli and colleagues investigated the metal nanoparticle label-based detection and label free detection for biosensing using SPR, ELISA and electrochemical sensors. According to their comparative analysis, label-free SPR allowed real time kinetic, high sensitivity, quantitative, kinetic/affinity extractions; strong analytical control, whereas ELISA detection require labelling and multistep workflow, and electrochemical detection often require labelling and creates calibration challenges; matrix effect [25]. Li and their colleagues discussed miRNA detection based on DNA probes using laminar flow SPR and reported high sensitivity and selectivity without fluorescent labels [58]. Corn's group reported two different studies for miRNA detection with a nanoparticles and DNA probe with the effective and ultrasensitive SPR measurements [59]. Aoki and colleagues also used DNA propene for microRNA detection based on SPR imaging for enzymatic signalling [60]. To date, no one has used SPR combined with MIPs for microRNA-21 detection.

In this study, we investigated the miRNA-21 detection using miRNA-21 MIPs to develop one-step, rapid, cost-effective detection method for the early detection of this critical cancer marker, enabling direct measurements in serum with no sample preparation. We present a straightforward simple synthesis and characterization of miRNA-21 imprinted nanoscale MIPs via a solid-phase methodology [39]. The physicochemical properties of nanoMIPs were determined using Dynamic Light Scattering (DLS); the interactions between miRNA-21 and nanoMIPs were investigated using surface zeta potential (ζ) measurements, and circular dichroism (CD). The binding affinity and selectivity studies were carried out with SPR. These miRNA-21 imprinted nanoMIPs attached to an SPR enabled not only direct, sensitive, label-free qualitative analysis with low theoretical LOD (0.49 pM) but also provide rapid (1h) detection in foetal bovine serum (used as a model representative matrix without dilution). This method represents a significant step towards early-stage cancer detection using MIP technology and optical biosensor device.

2. Materials and Methods

2.1 Materials

Sodium hydroxide (NaOH), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were obtained from Thermo Scientific (UK). Ethanol, acetone, anhydrous toluene Aminopropyltrimethoxysilane (APTMS), Ethylenediaminetetraacetic acid (EDTA) Hydrochloric acid (HCl), were purchased from Sigma-Aldrich (UK). Imidazole was obtained from Fluorochem Ltd. (UK).

For polymerization, N-(3-Aminopropyl)methacrylamide hydrochloride (NAMPA) and N-Isopropylacrylamide (NIPAm), and acrylic acid (AA) were obtained from Acros Organics (UK); Tert-butylacrylamide (TBAm) was obtained from Alfa Aesar (UK); N,N'-Methylene-bis-acrylamide (BIS), Ammonium persulfate (APS), Tetramethylethylenediamine (TEMED), Phosphate buffered saline (PBS) tablets, Potassium chloride, Sodium chloride, methanol, Tween® 20 and glycerine were obtained from Fisher Scientific (UK). For SPR studies, sodium acetate anhydrous was obtained from Fisher Scientific (UK); N-hydroxysuccinimide (NHS) was obtained from Alfa Aesar (UK); Ethanolamine (EA) was obtained from Acros Organics (UK). Glass beads (90 μ m) were purchased from Acros Organics (Belgium). Gold Sensor Slides (5000000 Da carboxymethyl Dextran) were purchased from Reichert Inc (USA). Foetal Bovine Serum Premium Plus (FBS) was purchased from Gibco (Ref A56694-01, USA). The miRNA-21, random RNA and ssDNA sequences were obtained from Kaneka Eurogentec S.A. (Belgium).

2.2 Synthesis of miRNA-21 imprinted nanoMIPs

2.2.1 Preparation of Template derivatized glass beads

The template derivatized glass beads were prepared using well-established protocol described previously [39]. Briefly, 10 mL sodium hydroxide (4 M) was added to the 10 g glass beads and glass beads were boiled in this solution for 15 minutes and then washed with double distilled water (8 x 125 mL) to ensure a pH of 7 after washing. The beads were then washed with acetone (2 x 125 mL) and dried in an oven at 80 °C for 3 hours. Into a round bottomed flask, a solution of APTMS (180 µL, 3 % v/v) in anhydrous toluene (6 mL) was added to the glass beads (10 g), then degassed using bubbled nitrogen for 15 minutes at 20°C. The flask was quickly sealed before being placed in an oven at 60 °C for overnight incubation. After incubation, the glass beads were washed using acetone (4 x 250 mL) followed by methanol (3 x 125 mL), then dried under vacuum. The beads were dried in an oven at 60°C for 30 minutes.

An imidazole buffer (4 mL, 100 mM, pH 6.0) was added to the glass beads in a round bottomed flask. EDC (0.4 mg) was added to deionised H₂O (1 mL), and the EDC solution (20 µL) was added to PBS (180 µL) which contained EDTA (10 mM, pH 7.2), and the miRNA-21 template (1 mg) at 20°C, to obtain a template solution. Instantly the template solution (100 µL) was added to the glass beads and placed in the oven at 50 °C for 2 hours, before being left to incubate overnight at room temperature. After incubation with the RNA, the glass beads (10 g) were washed using deionised water (8 x 125 mL) and dried under vacuum, before being added to a round bottomed flask containing PBS (5 mL).

2.2.2 Synthesis of miRNA-21 nanoMIPs via solid phase polymerization

A monomer mixture for the nanoMIPs preparation comprised: TBAm (33.28 mg dissolved in 0.5 mL ethanol, NAMPA (5.83 mg), BIS (2.02 mg) and NIPAm (39.66 mg), AAc (22 µL of 100µL/mL aqueous solution). The components were dissolved in 50 mL PBS, sonicated for 5 min. 5 mL of this monomer mixture was exposed to the glass beads. The beads and monomer mixture were degassed under nitrogen for 20 minutes. The polymerization was initiated by the addition of solution (0.5 mL) of APS (31.07 mg) containing TEMED (25 µL) at 20°C. This solution was briefly vortexed, and quickly 100 µL of this solution was added to the glass beads. The flask was left to incubate at ambient temperature (20 °C) for 3 h. The beads were transferred into an SPE cartridge (60 mL) filled with a 20 µm porosity PE frit (Sigma Aldrich, UK), unreacted monomers and other low affinity materials were removed by washing with double-distilled water (6 x 125 mL) at ambient temperature. Next, the high affinity nanoMIPs

were collected by washing with filtered and pre-warmed water at 60 °C (5 x 20 mL). The nanoMIPs were dried with a freeze-drier and stored at -18 °C.

2.3 Physicochemical Characterization of the nanoMIPs

2.3.1 Dynamic light scattering (DLS) analysis

DLS analysis was performed using a Malvern Instruments Zetasizer Nano ZS instrument fitted with a 4mW He-Ne laser ($\lambda=633$ nm). An avalanche photodiode detector collected scattered light at an angle of 173°. The size, size distribution and zeta potential for the miRNA-21 nanoMIPs, miRNA-21, and a combination of both were measured. All measurements were recorded in triplicate, using ten sub-runs per run at 20°C. The hydrodynamic diameter was calculated using the Stokes-Einstein equation, with the assumption of perfectly monodisperse, non-interacting spheres.

2.3.2 Circular Dichroism (CD) Spectroscopy

The conformation changes to the structure of miRNA-21 once combined with the nanoMIPs was studied by CD spectroscopy (Chirascan V100, Applied Photophysics, Leatherhead, UK) in the UV region (200 – 300 nm). All measurements except temperature ramping were performed in triplicate at 20°C. The normalized unfolding trend of miRNA-21 alone and miRNA-21 and miRNA-21 imprinted MIPs were calculated using Global 3 software allowing the degree of unfolding to be compared.

2.3.3 Immobilisation of nanoMIPs onto the Surface Plasmon Resonance (SPR) sensor surface

A gold chip coated with carboxymethyl dextran hydrogel layer (#13206066, Reichert, Buffalo, USA) was conditioned with 10 mM PBS including 0.01% Tween 20 buffer (PBST) at 10 $\mu\text{L min}^{-1}$ within the SPR. 1 mL PBS solution containing EDC (40 mg) and NHS (10 mg) was then passed over the gold chip (6 min at 10 $\mu\text{L min}^{-1}$). 1 mg of nanoMIPs in 1 mL PBS solution containing sodium acetate (0.82 mg, 10 mM) was injected over the working channel of the chip for 1 min. Ethanolamine (1 mM) was injected over the working and reference channels for 8 min to quench the surface of unreacted $-\text{NH}_2$ groups. This was followed by a flow of PBS solution at 10 $\mu\text{L min}^{-1}$. All experiments were carried out at 20°C

2.4. Binding affinity and selectivity studies of nanoMIPs

SPR was studied using a Reichert 2 SPR system (Reichert Technologies, Buffalo, USA) with attached autosampler and degasser. The nanoMIPs immobilized gold sensor slides were used for the binding affinity and selectivity studies. The rebinding of analyte (target miRNA-21 sequence and random RNA and ssDNA sequences) to the nanoMIPs was performed in a cycle using 5 $\mu\text{L min}^{-1}$ infuse flow rate of 2 min association (2, 4, 8, 16, 32 and 64 nM analyte concentrations in PBST or FBS), 5 min dissociations with only PBST, and a regeneration cycle (10 mM Glycine-HCl, pH 2 for 1 min), followed by a final stabilisation cycle (PBST for 1 min), all at 20°C. The first run consisted of an initial injection of PBST as the blank, with increasing analyte concentrations for subsequent runs. The reference channel signals were subtracted from the working channel, and all rebinding were studied in triplicate. The K_D values were calculated from 2, 4, 8, 16, 32 and 64 nM analyte concentrations using TraceDrawer software.

3. Results and Discussion

The miRNA-21 imprinted nanoMIPs were synthesised using solid phase polymerisation using the well-established method (Figure 1) initially developed by Piletsky and built upon by others [37,39,61]. Briefly, glass beads were activated in 4M NaOH solution. The activated glass beads were washed through DI water and applied further washing step with acetone and dried at 80 °C for 3 hours. After then, glass beads were incubated APTMS to obtain NH_2 groups on the surface of the glass beads. These NH_2 activation allowed to bind the 5' phosphorylated miRNA-21 (5'phos-UAG-CUU-AUC-AGA-CUG-AUG-UUG-A-3') template sequence onto the glass beads.

The monomer mixture was introduced to form interactions around the target sequence. Monomers were selected to enable a range of interactions with the sequence [62,63]. The concentration of cross linker is known to have a direct effect on the binding affinity, density of binding sites and selectivity of the nanoMIPs due to the flexibility or rigidity of the polymers [64,65]. Cross linking agent N'N-methylene bisacrylamide (BIS) was used in a low ratio to the monomers, to ensure that the polymer chains were linked, but to allow for flexibility of the nanoMIPs. This not only allows for the nanoMIPs to attach to the target miRNA-21 molecule, but also to detach easily from the template during synthesis elution and collection. NIPAm was selected to further enable this particle release from the solid phase. The polymer and template interactions are easy to break as it is assumed that multiple weak bonds such as hydrogen and dipole-dipole interactions bond the template to the polymer [63]. The template bound nanoMIPs were initially washed with room temperature water, to remove not only the unreacted monomers, but also the low affinity nanoMIPs. After the cold wash, the high affinity miRNA-21 nanoMIPs were collected with hot water wash for further studies.

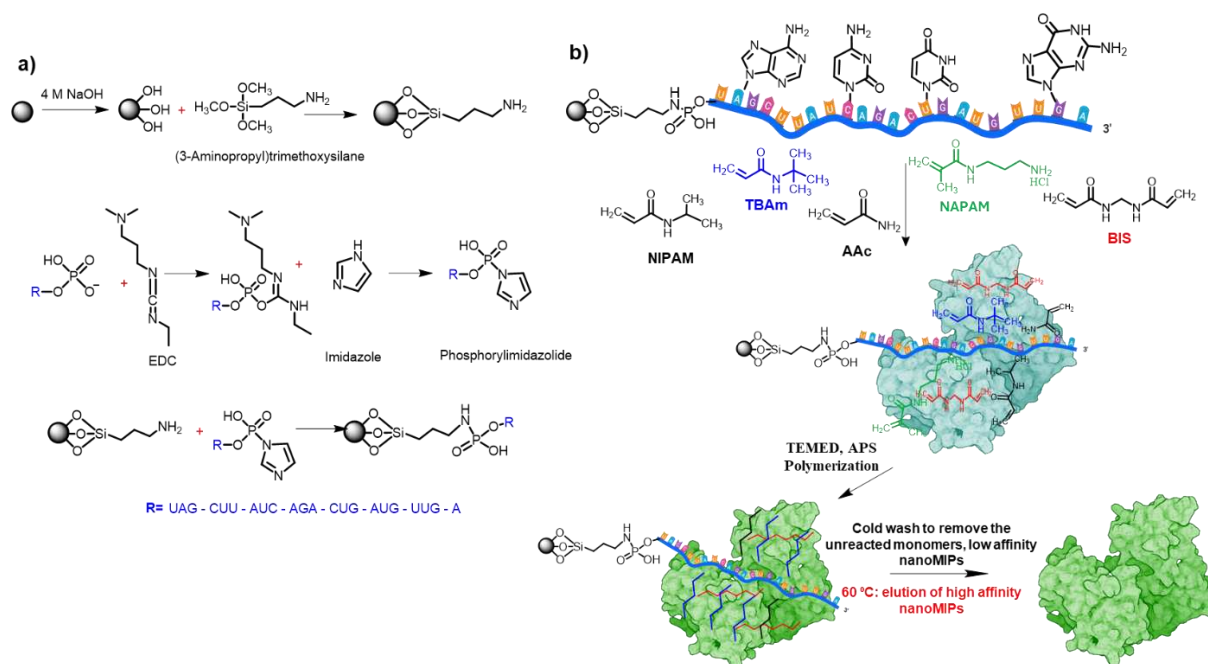


Figure 1. a) Preparation of miRNA-21 bonded glass beads, b) miRNA-21 imprinted nanoMIPs via solid-phase polymerization.

The size and size distribution studies were recorded using DLS. These studies confirmed that size of nanoMIPs stayed stable in aqueous media and PBS due to cross-linked nature of nanoMIPs (Fig.2a) while polydispersity slightly increased with PBS effect. When mRNA-21 sequence gave interaction with miRNA-21 imprinted nanoMIPs, the size of miRNA-21 imprinted nanoMIPs slightly increased and the recorded peak at 2.7 nm disappeared because of the interaction between miRNA-21 and nanoMIPs. (Fig. 2b).

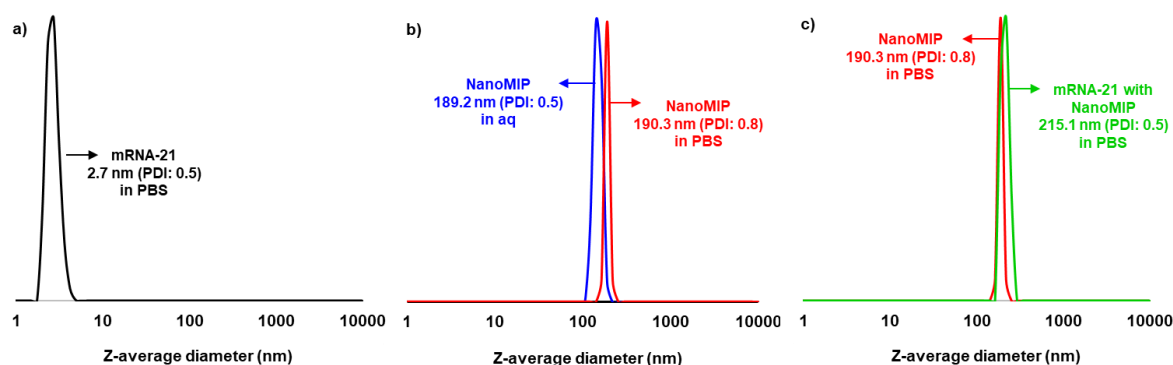


Figure 2. The average particle diameter (nm) of (a) miRNA-21 in PBS, and (b) comparison the miRNA-21 imprinted nanoMIPs in aqueous media and in PBS (c) comparison of the size miRNA-21 imprinted nanoMIPs, and the size once combined (in PBS). Note the loss of the RNA only peak highlighting binding.

Aqueous electrophoresis studies were performed on miRNA-21 imprinted nanoMIPs (Fig. 3a). These nanoMIPs exhibited zeta potential between +2.5 mV to -25.5 mV from pH 2 to 12, which is match with ionic nature of nanoMIPs due to used monomer combination. The similar study was conducted for miRNA-21 (Fig. 3b). The zeta potential of miRNA-21 sequence was recorded highly negative (approximately -35 mV) across the whole pH range. When these miRNA-21 and miRNA-21 imprinted nanoMIPs was merged, the zeta potential was recorded between these two trends (approximately -30 mV at or above pH 3). This new trend change indicated miRNA-21 sequence sticks on the surface of miRNA-21 imprinted nanoMIPs as expected and the zeta potential shifted to high negative range.

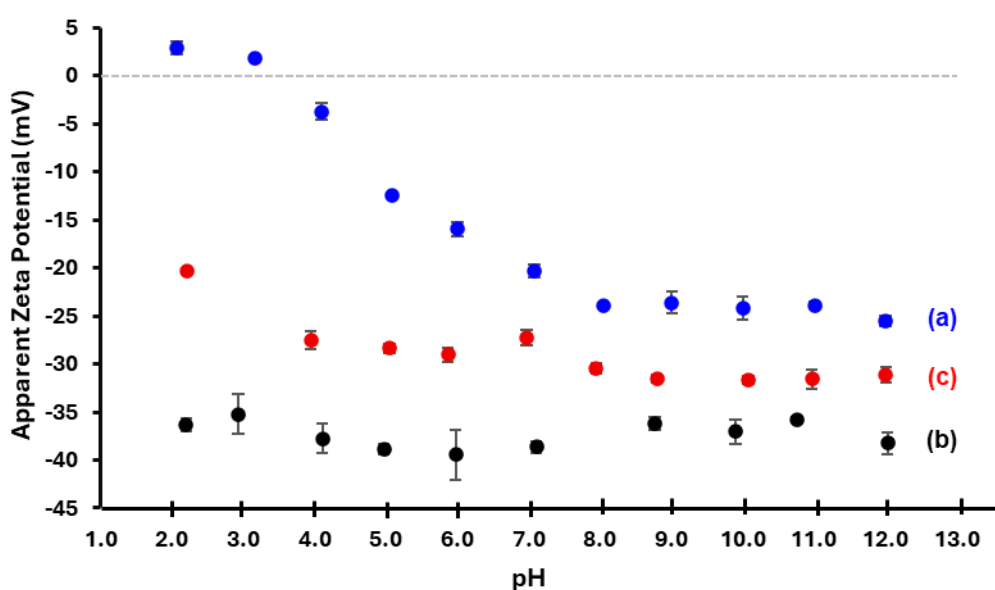


Figure 3. Zeta Potential at varying pH for (a) miRNA-21 imprinted nanoMIPs, (b) miRNA-21, and (c) a combination of both miRNA-21 and the nanoMIPs.

The interactions between miRNA-21 and miRNA-21 imprinted nanoMIPs was also confirmed with circular dichroism (CD) (Fig. 4a). CD is an extremely sensitive, inexpensive fast, and simple spectroscopic technique for conformational isomerization of DNA and base-base interactions of the double-stranded helix [66]. Specially, CD can give clear information about gradual changes within a single DNA confirmation and cooperative isomerization between discrete structural states [67]. This sensitive analysis method is able to rapidly evaluate macromolecules providing understanding of their 3D structure, and subsequently any structural changes caused by heat, pH and additives [68]. Thus, CD spectroscopy offers reliable investigation of miRNA-21 interactions with DNA probe [69].

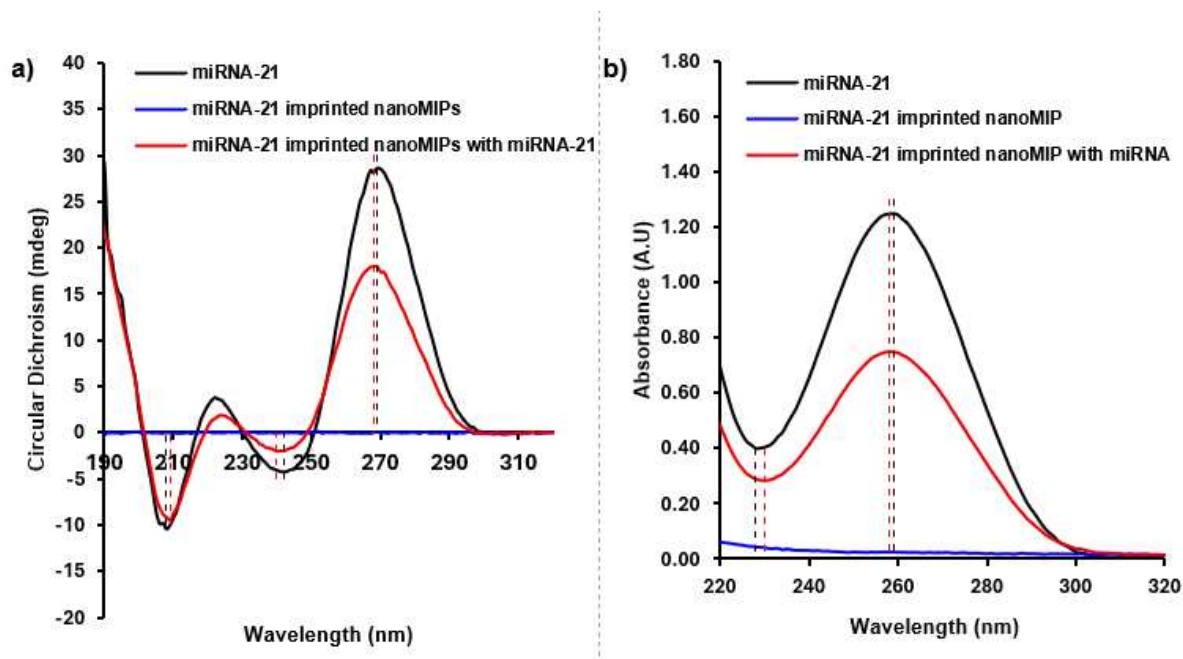


Figure 4. (a) Circular Dichroism of miRNA-21 and miRNA-21 imprinted nanoMIPs combined with miRNA, and (b) UV-Vis absorbance spectrum of miRNA-21 and miRNA-21 imprinted nanoMIPs combined with miRNA.

According to CD data, miRNA-21 displayed a strong positive band at around 270 nm and two negative bands around 240 nm and 210 nm, which is in accordance with current literature [69,70]. The miRNA-21 imprinted nanoMIPs alone do not show a signal, acting like a baseline as expected, due to their non-uniform structure. The miRNA-21 sequence was dissolved in the miRNA-21 imprinted nanoMIPs solution, and the signal density between 200 nm and 215 nm did not change with this equivalent amount of miRNA-21 (Fig. 4a). However, the peak positions at 270 nm slightly shift to the left, and more importantly the intensity decreased. Similarly, the signal density between 215 nm and 230 nm decreased and the peak significantly shifted right, and the signal density between 230 nm and 250 nm decreased and the peak shifted to the left. Although, the positions and amplitudes of CD bands depend on the sequence, the variations in amplitudes can be related to conformational changes of oligonucleotides [69]. The changes in the signal intensity and the peak locations for miRNA-21 alone and miRNA-21 imprinted nanoMIPs with miRNA-21 indicated clear interactions between miRNA-21 target and miRNA-21 imprinted nanoMIPs, suggesting a gradual interaction between miRNA-21 and nanoMIPs similar to base-base interactions. The change in signal intensity, which decreased upon addition of the imprinted nanoMIPs for both the positive and negative peaks, is indicative of a conformational change arising from encapsulation of the miRNA-21 molecule. Similarly, the UV-vis absorbance of miRNA-21 at 260 nm decreased with the partially capsulation/interaction effect (Fig. 4b). Furthermore,

nanoparticle size change and zeta potential change can give information about material interactions due to the change in hydrodynamic size and electrokinetic potential directly related to colloidal behaviours and interactions [71]. This data is in accordance with the hydrodynamic size and zeta potential change, again suggesting an interaction.

Furthermore, the effect of the miRNA-21 imprinted nanoMIPs on miRNA-21 was investigated using the CD temperature ramp study between 20 – 90 °C (Fig. S1). As a well-known phenomenon, the secondary structures of biological macromolecules such as proteins, nucleotides alter depending on the temperature change and they lose their structural confirmation after the melting point. This can be seen using CD, as the degree of unfolding can be measured, however it can also be observed by the changes in the bands, which no longer show the intense, distinct peaks once the melting point is reached [72]. According to Fig. S2, the miRNA-21 structure started to change with the temperature change just after 25 °C and this trend continued with the temperature increase and miRNA-21 displayed the melting point at 48 °C. However, after the interaction/complexation between miRNA-21 and miRNA-21 imprinted nanoMIPs, the structure of miRNA-21 changed rapidly, and the melting point shifted to 38 °C. This significant melting point change and unfolding trend indicated the clear interaction and penetration between the miRNA-21 and miRNA-21 imprinted nanoMIPs.

An SPR study was used to investigate to explain the binding affinity and specificity of miRNA-21 to miRNA-21 imprinted nanoMIPs (Fig. 5). The imprinted nanoMIPs contain a high amount of amine functionality due to the monomers used, specifically NAMPa, TBAm and NIPAm [61]. A gold chip pre-functionalised with a carboxymethyl dextran hydrogel layer was used, which therefore contained -COOH on the surface. These two factors allowed for NHS/EDC coupling chemistry to activate the surface and covalently bind the nanoparticles to the surface of the gold chip. Ethanolamine was used after immobilisation to quench any unreacted carboxylic groups and unbound MIPs remaining on the surface of the chip. This quenching step prevents any cross reactions between the carboxylic groups and therefore decreased the possibility of non-specific binding. The representative SPR spectra for miRNA-21 imprinted nanoMIPs immobilization was given in Fig. S3.

After the immobilization process of miRNA-21 imprinted nanoMIPs, a concentration range of miRNA-21, random RNA and ssDNA sequence binding affinity were conducted in PBST and in FBS to determine the binding affinity and selectivity of the nanoMIPs, the sequences of which are given in Table 1. The representative SPR raw data for miRNA-21 (0, 2, 4, 8, 16, 32 and 64 nM analyte concentrations in PBST) rebinding to nanoMIPs immobilized sensors, five minute dissociations with only PBST, and a regeneration cycle after each concentration (10

mM Glycine-HCl, pH 2 for one minute), and a final stabilisation cycle (PBST for 1 minute) were given in Fig. S4. Binding affinities K_D values were calculated using a spread of six concentrations (Table 2).

Table 1. Nucleotide sequence of the target molecule miRNA-21 and the random RNA and ssDNA sequences used to test for selectivity.

	Nucleotide Sequence
miRNA-21 Sequence	5'-UAG-CUU-AUC-AGA-CUG-AUG-UUG-A-3'
Random RNA Sequence	5'-CUG-UGC-GUG-UGA-CAG-CGG-CUG-A-3'
ssDNA Sequence	5'-TAG-CTT-CCT-GAT-3'

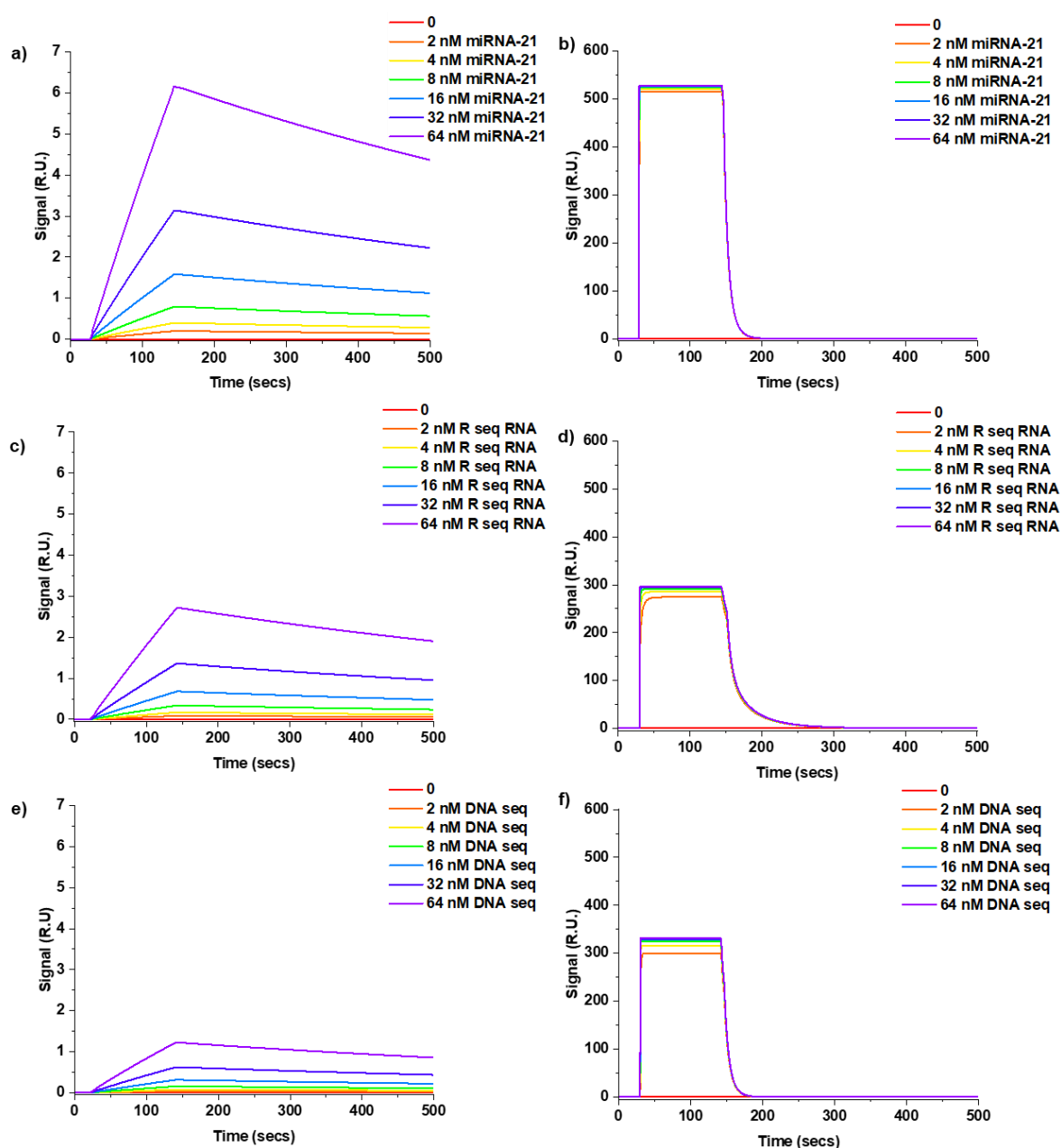


Figure 5. Representative SPR curves showing the rebinding of analytes to: (a) the miRNA-21 molecularly imprinted nanoMIPs in PBST, (b) the miRNA-21 molecularly imprinted nanoMIPs in FBS, (c) the random sequence RNA in PBST, (d) the random sequence RNA in FBS, (e) the ssDNA sequence in PBST, (f) the ssDNA sequence in FBS.

Table 2. Calculated equilibrium dissociation constants (K_D) from the data presented in Figure 5. All experiments were repeated in triplicate. Note the standard deviations presented showing binding ranges.

Sequence (Table 1)	K_D Values / M (St Dev)	
	PBST	FBS
miRNA-21	$2.79 \times 10^{-9} (\pm 1.67 \times 10^{-9})$	$4.95 \times 10^{-11} (\pm 5.25 \times 10^{-12})$
Random RNA	$1.87 \times 10^{-6} (\pm 1.03 \times 10^{-6})$	$9.21 \times 10^{-8} (\pm 3.68 \times 10^{-9})$
Random ssDNA	$1.02 \times 10^{-7} (\pm 2.63 \times 10^{-9})$	$1.04 \times 10^{-7} (\pm 3.11 \times 10^{-9})$

From this SPR data, the calculated K_D value of miRNA-21 binding to its specific nanoMIPs was 2.79 nM in PBST. The random sequence RNA and ssDNA sequence K_D values were calculated as 1870 nM (644-fold) and 102 nM (36-fold) in PBST, respectively. This shows selectivity comparable to other nanoMIPs systems, so we are confident that imprinting has been observed, through the nucleobase face interactions and potentially interactions with the ribose backbone. The polymer surface outside of the binding pocket will be charged, given the monomers used, which will offer non-selective binding, so here we must use the selectivity as a demonstration of imprinting. In a traditional system a non-imprinted polymer would be used to show “imprinting”, however the solid-phase methodology does not allow for a non-imprinted polymer (NIP) to be created. The recent development of the nanoMIP technology via a solid phase synthesis approach requires a nucleation site, in this case the covalently affixed template to glass bead (our miRNA-21 template), for the synthesis of such recognition materials to proceed. However, because of this the synthesis of a NIP is not possible in the same way. Without the template present this nucleation site cannot form and therefore no comparative polymer (size, morphology and composition) will be generated. As such we have used selectivity factor (SF – Equation 1) to measure imprinting performance. This explores a comparator ratio of target to non-target. The larger the number, the better the imprint.

Eq. 1:

$$SF = \frac{K_D \text{ MIP}_{off\text{-}target}}{K_D \text{ MIP}_{target}}$$

where: SF = selectivity factor; K_D = equilibrium dissociation constant

When the working media was changed to FBS from PBST, the signal intensity increases significantly, and the trends of SPR sensorgraphs are altered. This change in intensity is due to the difference in the density and optical properties of PBS and FBS, causing change in the refractive index and therefore causing an increase in intensity. In the real-world applications of detection system, serum could cause matrix effect and so artificial effect can limit or alter detection limits. Although FBS used without dilution or treatment, nanoMIPs system displayed sensitivity and selectivity for miRNA-21 target.

The curves produced also presented lower calculated K_D values, in the pM range. When tested in FBS, miRNA-21 binding to the nanoMIPs has a calculated K_D of 49 pM in FBS, approximately 20-fold improvement in affinity. The same order of magnitude (20-fold) increase was seen for the random RNA sequence binding suggesting that the environment was responsible for the increase, potentially offering conditions that favour RNA-polymer interactions. Interestingly the random ssDNA sequence was comparable between buffer and serum. Given that both ssDNA and RNA will be similarly charged (phosphate backbone and bases) this is interesting, potentially highlighting that the sugar is important to the formation of the imprint. DNA having an extra OH which will alter its charge and shape. This difference is currently under study in our lab and out of scope of this work. This data does however reinforce imprint formation with SF's of 1860 (RNA) and 2040 (ssDNA) demonstrating selectivity and hence effectiveness of our polymer.

The respective curves for each sequence were generated for each analyte in PBS and FBS (Fig. S5), enabling the theoretical limits of detection (LOD) to be calculated. For miRNA-21 in FBS, this was calculated using *standard deviation of sample blank (SD_b)* and *slope of the calibration curve (m)* as approximately 0.5 pM using Equation 1. This detection limit is comparable to the electrochemical system presented by Kranz [53], and it is within the levels expected in disease states and comparable to other published systems [73,74]. These curves are linear for PBS and curves for FBS. This is not surprising given the differences in complexity of the two matrices, increased non-selective binding of other components in FBS and the optical nature of the SPR.

Eq.2:
$$LOD = 3.3 \times \left(\frac{SD_b}{m} \right)$$

4. Conclusions

MiRNA-21 imprinted nanosized MIPS were prepared by solid-phase polymerisation using the relatively long nucleotide sequence miRNA-21 as the target sequence. Hydrodynamic size and size distribution indicated that nanoMIPs were prepared approximately 200 nm in aqueous media and PBS. The zeta potential and CD studies indicated that the miRNA-21 sequence can interact with imprinted MIPS, and this interaction can alter the surface charge, and molecular structure of miRNA-21. The synthesised nanoMIPs displayed high affinity at pM level (approximately 50 pM) and expected selectivity for miRNA-21 sequence compared to non-specific molecules.

Current methods which use ligands and small molecules as therapeutics binding to miRNA-21 have been recorded, showing the K_D values in the μM range for the target-receptor interactions [53,58,75]. Our materials offer improved affinity to this which suggest they may be superior choice moving forward.

We have however, used the SPR system to its limits of detection, mainly due to the optical properties of the matrix which significantly effects signal acquisition. While being able to measure direct in a complex matrix as we do here, without any sample preparation, the complexity of the environment limits capability. The theoretical LOD is ~ 0.5 pM in FBS, however realistically, given expected changes to the matrix between real samples this, and subsequently the LOQ will be higher. As such we will need to expand the study with a variety of matrices and a broader sampling study, though we expect to be working in the pM range. It is also acknowledged that other sensor platforms may offer greater sensitivity, and our next steps are focused on translating these imprinted materials to electrochemical and fluorescent techniques that will allow lower levels of detection, that are less dependent on the matrix effect and therefore enable utilisation of the exceptional recognition properties of the generated nanoMIPs for direct disease detection.

Conflicts of Interest

The authors declare there are no conflicts of interest associated with this work.

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Declaration on Generative AI Use

No AI tools were used in the preparation of this manuscript, or acquisition of data.

Data Availability

The data that support the findings of this study are available direct from the corresponding author upon reasonable request.

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