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Affimer-based impedimetric biosensors for fibroblast growth factor receptor 3 (FGFR3): a novel tool for detection and surveillance of recurrent bladder cancer

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Highlights

- The first electrochemical impedance biosensors to detect FGFR3 have been developed.
- Affimers, synthetic binding proteins, were used as bioreceptors for sensor fabrication.
- A decrease in impedance was observed from the sensors with increased FGFR3.

Abstract

Fibroblast growth factor receptor 3 (FGFR3) is a transmembrane tyrosine kinase protein in the fibroblast growth factor receptor family, which plays a key role in many biological processes. Over-expression and activating mutations in FGFR3 are frequent in non-invasive bladder cancer, highlighting this protein as a potential biomarker for recurrent bladder cancer detection. Affimer reagents isolated against recombinant FGFR3 were assessed for their affinity using double-sandwich ELISA and SPR. Anti-FGFR3-14 and FGFR3-21 Affimer proteins showed strong binding to FGFR3 and were used for fabrication of impedimetric electrochemical biosensors. A decrease in impedance was observed when the sensors were exposed to increasing concentrations of FGFR3. The successful impedimetric biosensors were capable of detecting sub-pM to nM concentrations of recombinant FGFR3 protein in phosphate buffered saline as well as in synthetic urine.

Keywords: Affimer; FGFR3; impedimetric biosensor; bladder cancer

1. Introduction

Bladder cancers form two major groups that are clinically and molecularly distinct [1]. The first group is non-muscle invasive bladder cancers (NMIBC) which do not invade the muscle wall of the bladder and are either confined to the epithelial layer or invade only into the underlying stroma. NMIBC, which constitutes the majority of bladder tumours at presentation (~70%) rarely progresses and 5-year survival is >90%. The second group is muscle-invasive tumours, which have much poorer prognosis, with 5-year survival rates of 60% for patients with organ-confined disease and <10% for patients with metastases [2, 3]. In 2018, bladder cancer was ranked the tenth most common cancer detected worldwide [4]. The GLOBOCAN survey estimated 549,000 newly diagnosed bladder cancer patients and around 200,000 deaths during that year [4]. The incidence rate of this cancer was highest in Southern Europe, Western Europe and Northern America [4]. In NMIBC patients, the recurrence rate after transurethral resection is high (60-80%). Therefore, long-term disease monitoring is required, with associated patient morbidity and high healthcare costs [5].

Several techniques are available for detecting bladder cancer with cystoscopy being the current gold standard [2, 3, 6]. However, this method is invasive and flat lesions such as carcinoma *in situ* may not be detected [3, 6]. Urinary cytology is also used to detect bladder cancer cells. This approach is non-invasive and highly specific [6], but its low sensitivity is a major disadvantage, especially for low-grade NMIBC detection. Urine biomarker-based methods including bladder tumour antigen test (BTA), nuclear matrix protein 22 test (NMP22), ImmunoCyt and UroVysion have also been developed to overcome the limitations of cystoscopy and cytology [6, 7]. All four techniques achieve higher sensitivities than urinary cytology, but they are still less specific compared to cytology and sometimes suffer from false positives. Therefore, there are opportunities for newly developed biomarker-based platforms for bladder cancer monitoring.

Fibroblast growth factor receptor 3 (FGFR3) is a receptor tyrosine kinase in the FGFR family that plays an important role in many biological processes including cell proliferation, survival, migration and differentiation [8]. The basic structure of this protein consists of an extracellular domain, three immunoglobulin (Ig)-like domains, a hydrophobic transmembrane domain and an intracellular tyrosine kinase domain. FGFR3 is expressed at a higher level than the other FGFRs in normal urothelial cells, implying a significant role of FGFR3 in homeostasis of the urothelium [9]. In bladder tumours, mutation and over-expression of FGFR3 is common [1, 9-12], particularly in NMIBC [11, 13]. Several studies have explored FGFR3 mutation detection in urine DNA as a means of bladder cancer detection and disease monitoring [14, 15]. As tumours with FGFR3 mutation and some tumours with wildtype FGFR3 show markedly upregulated expression, detection of FGFR3 protein in the urine represents an alternative approach. Indeed, FGFR3 protein has been detected by western blot analysis in urine samples collected from NMIBC patients [12, 16].

Generally, immunohistochemical staining (IHC) is utilised to detect the expression of FGFR3 in bladder tumours in clinical settings [11, 17-19]. However, sample collection is invasive and the procedure is relatively insensitive and requires trained users. Recently, electrochemical impedance biosensors have been developed for a wide range of biomedical analytes including biomarker proteins [20-24]. These biosensors, as a subtype of electrochemical biosensors, monitor electrical impedance across the transducer surface as the bioreceptor binds its target molecule [25]. Moreover, a very wide range of targets can be detected by impedance changes which occur on binding of the analyte to its bioreceptor, which may be an antibody, Affimer, aptamer or other binding agent. Using this approach, many different analytes have been detected such as bacteria, viruses, proteins and small molecules (Table 1). In contrast, amperometric and potentiometric biosensors require the analyte to be the substrate (or an inhibitor) of a specific enzyme [26-28].

Table 1 Targets successfully detected by impedimetric biosensors

Analyte	Receptor	Reference
Bacteria		
Streptococcus pyogenes	Antibody	[29]
Escherichia coli, *Salmonella typhimurium*	Antibody	[30]
Listeria monocytogenes	Leucocin A	[31]
Escherichia coli O157:H7, Salmonella typhimurium	Magainin I	[32]
Viruses		
Dengue virus	Alumina membrane	[33]
Adenovirus	Reduced antibody fragment	[34]
Influeza A virus	Antibody	[35]
Proteins		
Prostate specific antigen	Antibody	[36]
Myoglobin	Reduced antibody fragment	[23]
Amyloid-β oligomer	Cellular prion protein fragment	[37]
HER2/ErbB2	Aptamer	[38]
C-reactive protein	Affimer	[39]
Anti-myc tag antibody	Affimer	[40]
Human interleukin-8	Affimer	[41]
HER4	Affimer	[42]
Small molecules		
Uranyl ions	Uranyl binding protein	[43]
Ochatoxin A	Antibody	[44]
Progesterone	Aptamer	[45]
Diazinon	Lipases	[46]

More recently, impedimetric biosensors have exploited Affimers, which are small synthetic binding proteins [47, 48] as biorecognition elements. Proteins including C-reactive protein [39], anti-myc tag antibody [40], human interleukin-8 [41] and human epidermal growth factor receptor 4 [42] have all been measured. In this report, we have characterised anti-FGFR3 Affimers and used them to develop an Affimer-based impedimetric biosensor for this biomarker.

2. Experimental section

2.1 Target biotinylation and phage display

Recombinant FGFR3 protein (Genscript) was biotinylated by mixing 100 µl of 0.1 mg/ml FGFR3 with 8 µl of a 0.5 mg/ml EZ-Link™ NHS-SS-biotin (Pierce) for 1 h at room temperature. Excess biotin linker was removed by using a Zeba™ spin desalting column (Thermo Scientific) according to the manufacturer's protocol or by dialysis. Three rounds of phage display panning were performed and a phage ELISA was set up to test binding of randomly picked colonies as described previously [47, 48].

2.2 Affimer protein production

Selected Affimer sequences were amplified by PCR including a C-terminal cysteine for labelling/immobilisation using Forward primer (5'-ATGGCTAGCAACTCCCTGGAAATCGAAG-3') and Reverse primer (primer 5'-TTACTAATGCGGCCGCACAAGCGTCACCAACCGGTTTG-3'). The purified PCR products were subcloned into pET11a and subsequently used for protein production as described previously [47, 48].

2.3 Characterisation of Affimers by ELISA

Double-sandwich ELISA was used to assess Affimer clones. Initially, Affimer proteins were biotinylated after first reducing any dithiol linked dimers using TCEP reducing gel (Thermo Scientific) for 1 h at room temperature. The reduced Affimers were then mixed with 2 mM biotin-maleimide (Sigma-Aldrich) and incubated for 2 h at room temperature. Then, mixtures were desalted using Zeba™ Spin Desalting Columns (Thermo Scientific). The

successful biotinylation was confirmed by liquid chromatography-mass spectrometry (LC-MS).

A 96-well microplate coated with 5 µg/ml Neutravidin was blocked with 2x casein blocking buffer (Sigma-Aldrich) overnight at 37°C. After washing with 1x PBST comprising 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4 plus 0.05% (v/v) Tween-20, 5 µg/ml of biotinylated Affimer was added to the wells and incubated for 1 h at room temperature. The plate was washed twice in PBST before 10 µg/ml of purified FGFR3 (Genscript) was added into each well and incubated for 1 h. Two µg/ml of anti-FGFR3 antibodies specific to its extracellular domain as primary antibodies was added to the wells, followed by 2 µg/ml of anti-rabbit IgG – HRP conjugate as secondary antibodies. The plate was washed six times with PBST before TMB substrate (SeramunBlau® fast, Seramun) was added and the absorbance at 620 nm was measured after 30 min.

2.4 Determination of kinetic parameters by surface plasmon resonance (SPR)

SPR was performed using a Biacore T200 (GE Healthcare Life Sciences, USA) with a streptavidin-functionalised chip which was washed with 20 mM phosphate buffer with 2.7 mM KCl, 137 mM NaCl and 0.05% (v/v) Tween 20 (1x PBS-P+; GE Healthcare Life Sciences, USA) as running buffer for all experiments. Biotinylated Affimers were attached to the chip on flow cell 2-4 whilst flow cell 1 was left empty as a reference surface. Affimers at a concentration of 16.7 nM were injected into flow cells at a flow rate of 5 µl/min. Kinetic binding data were collected by injecting purified FGFR3 at concentrations between 0 and 1000 nM at a flow rate of 30 µl/min. The association phase was run for 300 s, followed by 900 s of dissociation phase with the running buffer. The surfaces were regenerated using 10 mM glycine

pH 2.5 for 120 s at a flow rate of 30 µl/min. SPR data were finally analysed using GraphPad Prism 7 software.

2.5 Biosensor fabrication

Custom screen-printed gold electrodes (model CX2223AT, Dropsens, Spain) were used for biosensor fabrication. Polymerisation of 25 mM polytyramine in 0.3 M NaOH was performed on a Dropsens chip working electrode via cyclic voltammetry on an AUTOLAB type III electrochemical workstation (Metrohm Autolab B.V.; Utrecht, Netherlands) driven by NOVA 2.0.2 software. The applied potential was cycled twice from 0 to 1.6 V and then back to 0 V at a scan rate of 200 mV/s. After polymer deposition, the electrodes were rinsed in 20 mM boric acid/disodium tetraborate buffer pH 9.0 and incubated in the same buffer for 30 min prior to bioconjugation of Affimers.

The Neutravidin-biotin interaction was utilised to tether biotinylated Affimers to the electrode surface. The electrodes were incubated with 3 mM NHS-biotin for 30 min, followed by 67 nM Neutravidin for 45 min. The modified electrodes were then blocked with blocking buffer for 30 min. Finally, biotinylated Affimer was added onto the working electrode and left for 1 h at room temperature. After rinsing in 100 mM PBS pH 7.2, the fully constructed electrodes were incubated in the same buffer for 1 h prior to electrochemical impedance spectroscopy.

2.6 Electrochemical impedance spectroscopy

Fully fabricated sensors were sequentially incubated with increasing concentrations of FGFR3 from 10^{-14} to 10^{-8} M for 15 min, followed by washing in 100 mM PBS pH 7.2 in between. Impedance measurements were conducted in 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ at a

1:1 ratio, in 100 mM PBS, pH 7.2. The measurements were recorded at an applied potential of 0 V over a frequency range of 2.5 kHz to 250 mHz with a modulation voltage of 10 mV. All of the experiments were replicated in triplicate. To analyse the sensor responses, charge-transfer resistance (R_{ct}) calculated automatically from NOVA 2.0.2 software was used. The R_{ct} of each FGFR3 concentration was normalised as $\Delta R_{ct}(\%)$.

2.7 Optimising parameters affecting sensor response

Essentially, two important parameters involved in the performance of sensors were studied. First, a range of blocking agents was examined. Biosensors were fabricated as in Section 2.5 and the following blocking agents, 6.7 µM bovine serum albumin (BSA), 2x casein blocking buffer (Sigma-Aldrich) and 0.2 mg/ml sodium caseinate, were tested for their ability to minimise non-specific binding effects. The concentration of Affimers used was kept constant at 1 µM. EIS was then performed as described in Section 2.6.

To optimise concentrations of Affimer, sensors were again fabricated according to Section 2.5. The sensors were blocked with the selected blocking agent as mentioned above and Affimer concentrations of 0.3, 1 and 3 µM were tested. The optimal condition was employed for fabricating biosensors and the sensors were then challenged with Surine™ Negative Urine Control (Cerilliant, Sigma-Aldrich) spiked with increasing concentrations of FGFR3 from 10^{-14} M to 10^{-8} M.

3. Results and discussion

3.1 Selection and characterisation of Affimer proteins

Three rounds of phage display were performed and 32 randomly picked colonies were tested for binding FGFR3 using a phage ELISA. In total, 24 positive tested clones were sequenced and revealed ten unique Affimer proteins of which seven were subcloned into pET11a for Affimer protein production.

The affinity of bioreceptors for their targets is the key to their performance in assays such as ELISA. Out of seven Affimers showing positive binding to FGFR3 protein from the phage ELISA, three Affimer proteins, namely FGFR3-8, FGFR3-14 and FGFR3-21, were randomly selected and tested for their affinity to FGFR3 protein. Prior to use, all of the Affimers were biotinylated, allowing use of the avidin-biotin interaction for immobilisation. Biotinylation was carried out via maleimide chemistry as a cysteine had been introduced at the C-terminus of the Affimers, allowing site-directed modification. LC-MS showed successful conjugation of biotin to the Affimers (Supplementary Figure S1) as only monomeric forms of the Affimers could be seen after biotinylation.

To identify which Affimer had the highest affinity to FGFR3 protein, a double-sandwich ELISA was employed. Biotinylated Affimers were captured on Neutravidin-coated 96-well plates before adding FGFR3 protein. To investigate the binding, anti-FGFR3 IgG was applied as the detection reagent, followed by an anti-rabbit IgG – HRP conjugate as the secondary detection reagent. Colourimetric detection after adding the TMB substrate revealed that the FGFR3-14 Affimer showed the highest binding level to FGFR3, followed by the FGFR3-21 Affimer (Figure 1). The FGFR3-8 Affimer showed a lower level of binding to FGFR3, compared to the two previously-mentioned Affimers, whereas the control anti-GFP Affimer did not bind to FGFR3.

SPR was also used to study the binding of the selected Affimers to FGFR3 and confirm the data obtained from ELISA. Biotinylated Affimers were attached to a streptavidin-coated chip before the chip was exposed to 0 to 1000 nM of FGFR3. At each cycle, the association was performed for 300 s, followed by the dissociation for 900 s. Changes in response signal, indicating the binding between captured Affimers and FGFR3, are shown in Figure 2. The SPR data showed that whilst FGFR3-8 Affimer failed to bind FGFR3 protein (Supplementary Figure S2), FGFR3-14 and FGFR3-21 bound strongly to FGFR3 (Figure 2A and 2B). These SPR data supported the ELISA data as FGFR3-14 and FGFR3-21 Affimers showed superior binding to FGFR3, compared to FGFR3-8 although some binding of this Affimer was shown by ELISA.

To determine the dissociation constant (K_D) of each Affimer, the double reference-subtracted SPR data were analysed using GraphPad Prism 7. Here, we assume that each Affimer has one binding site which can interact specifically with an epitope on the analyte. Therefore, a 1:1 binding model was used to analyse the SPR data in order to determine the K_D of Affimers binding to FGFR3. The results showed that the SPR data for FGFR3-14 and FGFR3-21 were fitted well by this model, supporting our assumption of single site specificity (Supplementary Figure S3). The K_D for the interaction between the FGFR3-14 Affimer and FGFR3 protein was 327 pM while the K_D for the FGFR3-21 Affimer and FGFR3 was 18.5 pM (Supplementary Table S1). It was reported earlier that the K_D for monoclonal anti-FGFR3 antibody was 16.2 pM (ab133644, Abcam, UK), indicating that the affinity of selected anti-FGFR3 Affimers (FGFR3-14 and FGFR3-21 clones) is comparable to anti-FGFR3 antibody.

3.2 Biosensor fabrication via Neutravidin-biotin linkage

To fabricate a sensor chip for electrochemical impedance measurement, a layer-by-layer sensor construction protocol previously described elsewhere was modified [29, 37]. As shown in Figure 3, biotinylated Affimers were tethered to a polytyramine-functionalised gold electrode via Neutravidin-biotin interaction. At each step of sensor assembly, polytyramine, NHS-biotin, Neutravidin and Affimer were sequentially deposited onto a working electrode with multiple washes in PBS buffer in between.

The stability of the supporting layer is a key factor affecting the performance of sensors. In this work, the non-conducting polymer, polytyramine, was employed as it offers high stability, minimal conductivity and self-limiting electrodeposition thickness and porosity [29, 49, 50]. Tyramine was dissolved in 0.3 M NaOH in dH_2O prior to electropolymerisation. A small peak during the first cycle was seen at an applied potential of 0.5 V, indicating oxidation of tyramine, and this phenomenon disappeared in the second cycle (Figure 4A). This event shows the self-limiting property of polytyramine during polymerisation. During each step of sensor fabrication, EIS was performed in order to identify successful deposition of material. As presented in Figure 4B, the Nyquist plot for a bare gold surface was the lowest with R_{ct} = 32.3 kΩ. After polytyramine deposition, the impedance increased, indicated by the shift of R_{ct} to 80.3 kΩ. Neutravidin was then used as a bridge to link biotinylated Affimers to the functionalised surface. The immobilisation of NHS-biotin and Neutravidin onto the polytyramine-modified surface caused a significant increase in impedance as seen by the shift of R_{ct} up to 1,610 kΩ. The large increase in resistance and capacitance is because deposition of large molecules such as Neutravidin (Mr = 60 kDa) can potentially hinder the transfer of charged components to the transducer surface. However, subsequent addition of biotinylated Affimers to the functionalised surface caused a decrease in impedance (R_{ct} = 842 kΩ) most likely due to physical effects on the underlying polytyramine. Each Dropsens electrode chip

contains two working electrodes. Therefore, in our study, anti-GFP Affimer was immobilised on working electrode 1 (WE1) as a control bioreceptor and FGFR3-21 Affimer was used on WE2 as the specific bioreceptor for FGFR3 protein. Prior to being challenged with the analyte, stability of the sensors was examined by four consecutive impedance scans (Supplementary Figure S4). The impedance from all four measurements was almost constant, indicating that the sensor surface was stable.

3.3 Electrochemical impedance spectroscopy for detection of FGFR3

Electrochemical impedance spectroscopy (EIS) is a powerful tool to monitor an interaction of a target with its bioreceptor on an interface such as a biosensor surface. The impedance data from the measurement are presented as a Nyquist plot (Figure 4C) with data then fitted to a Randles' equivalent circuit model (Figure 4C, inset) to determine charge-transfer resistance (R_{ct}) [29, 34, 37, 51, 52]. The R_{ct} data were normalised to a percentage and $\Delta R_{ct}\%$ was then calculated.

Affimer-modified sensors from Section 3.2 were incubated with increasing concentrations of FGFR3 protein from 10^{-14} to 10^{-8} M with five washes between additions. Impedance measurements were then performed in 10 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ redox mediator in PBS pH 7.2. As shown in Figure 4C, there was a continuous decrease in impedance as the concentration of FGFR3 increased. Although analyte binding often increases impedance ($\Delta R_{ct}\%$), the presence of larger analytes can cause changes in the nanostructure of the interface, causing a drop in impedance to be observed [25].

The sensors presented in this section were not blocked with any anti-fouling agents and the concentration of Affimers used was kept constant at 1 µM. After EIS measurements, the impedance data were normalised using the following equation;

$$\Delta R_{ct}\% = [(R_{ct}(FGFR3) - R_{ct}(\text{zero FGFR3})) / R_{ct}(\text{zero FGFR3})] \times 100\%$$

The $\Delta R_{ct}\%$ was then presented versus log[FGFR3] (Figure 4D). Although a continuous decrease in impedance (from 0 to -50%) was seen on the anti-FGFR3-21 Affimer sensors, a significant fall in impedance (-20%) was also detected when applying 10^{-14} M FGFR3 to the control, anti-GFP Affimer sensors and this shift remained constant until 10^{-8} M FGFR3. This event was likely due to non-specific binding of the analyte to the sensors. Attempts were then made to minimise this non-specific binding.

3.4 Optimising sensor fabrication parameters to minimise non-specific binding

In order to minimise non-specific binding effects from the target matrix, urine, blocking agents were applied to the sensor prior to testing with biological samples. Dropsens electrodes were modified according to the protocol in Section 2.5. In this work, 6.7 µM bovine serum albumin (BSA), 2x casein blocking buffer (Sigma-Aldrich) and 0.2 mg/ml sodium caseinate were tested for their ability to reduce non-specific binding. The Affimer concentration was fixed at 1 µM. As shown in Supplementary Figure S5, applying BSA as a blocking agent could minimise non-specific binding effects on anti-GFP Affimer sensors (control) to approximately -12% at the highest concentration of FGFR3 whereas a continuous decrease in impedance on FGFR3-21 Affimer sensors was still retained (Supplementary Figure S5A). The use of either casein in blocking buffer or sodium caseinate as blocking agents was ineffective for this sensor platform as significant shifts in impedance on the control sensors were still observed (Supplementary Figure S5B and S5C). For this reason, BSA at the concentration of 6.7 µM was chosen for blocking biosensors.

The optimal concentration of Affimer was also investigated in order for the sensors to achieve the maximum level of analyte binding. Too high packing density of bioreceptors leads

to steric hindrance, which affects the accessibility of bioreceptor binding sites to their analytes [29, 53]. As a consequence, signal generation from the sensors may be reduced or lost. On the other hand, insufficient bioreceptor loading can negatively affect biosensor performance since the signal generated is not high enough to be detected and unoccupied sensor surface may be a source of non-specific background [54]. In this study, three concentrations of Affimers, 0.3, 1 and 3 μM, were examined for the optimum surface loading (Supplementary Figure S6). Although all concentrations of Affimers showed similar patterns of sensor performance, non-specific binding on anti-GFP Affimer (control) sensors at the Affimer concentration of 1 μM was the lowest with 0 – 12% shift of R_{ct} (Supplementary Figure S6B), compared to the Affimer concentration of 0.3 μM (Suplementary Figure S6A) or 3 μM (Supplementary Figure S6C). The R_{ct} response on the FGFR3-21 Affimer sensors decreased continually as expected. Conclusively, 1 μM Affimer was selected as the optimal concentration of bioreceptors for sensor fabrication.

Taking all the data together, biosensors were fabricated following the optimised protocol in Section 2.5. Then, BSA at 6.7 μM was applied to block Neutravidin-modified electrodes before 1 μM Affimer was added to the working electrodes. To confirm whether the positive response on FGFR3-21 Affimer sensors resulted from FGFR3-21 Affimer – FGFR3 protein interaction, FGFR3-14 Affimer, another clone against FGFR3, was used instead of the FGFR3-21 clone whilst the anti-GFP Affimer was used as a control bioreceptor. Compared to the GFP/FGFR3-21 Affimer sensors (Figure 5A), the GFP/FGFR3-14 Affimer sensors showed a similar response. The change of R_{ct} on the anti-GFP Affimer sensors slightly fell between 0% and -10% whilst the R_{ct} on the FGFR3-14 Affimer sensors decreased continually with increasing FGFR3 concentrations (Figure 5B). These data confirmed that the selected Affimers can be used to detect FGFR3 in PBS within an electrochemical sensor platform.

3.5 Testing biosensors with urine samples

Even though Affimer-based sensors succeeded in detecting FGFR3 in PBS buffer, the sensors need to be tested with biological samples as only traces of target analytes are typically present and components other than the target analyte may cause non-specific background and interfere with the specific response. GFP/FGFR3-21 Affimer sensors were prepared according to the optimised protocol in Section 3.4. The fully-constructed sensors were then exposed to 100% (v/v), 10% (v/v) and 3% (v/v) synthetic urine spiked with FGFR3 ranging from 10^{-14} M to 10^{-8} M prior to EIS measurement. Compared to the response of the sensors tested with FGFR3 in PBS buffer (Figure 5A), the sensors exposed to 100% (v/v) synthetic urine spiked with FGFR3 could not distinguish specific interaction between FGFR3 and the Affimer from non-specific background (Figure 6A). When synthetic urine was diluted to 10% (v/v) or 3% (v/v) in PBS buffer before being spiked with FGFR3, a specific response from the binding of FGFR3 to FGFR3-21 Affimer could be detected as shown in Figure 6B and 6C. However, non-specific binding still remained as an approximate -30% shift of R_{ct} on the anti-GFP Affimer sensors, although the sensors had been blocked with BSA which is widely used as an effective blocking agent in many immunoassays. Yet, thanks to its hydrophobic patches that potentially form hydrophobic interaction with other molecules, albumin can interact with many analytes such as fatty acids, hormones, drugs, toxin, metal ions, amino acids and proteins [55-58] and its biological role is to carry these molecules in circulatory system. Therefore, it is possible that BSA may interact with other components in urine, leading to impedance shifts on control sensors.

The work published here is the first impedimetric biosensor that allows the detection of FGFR3 as a promising diagnostic biomarker for recurrence of bladder cancer. However, further optimisation of the sensor fabrication is required in order to eliminate non-specific binding and make sensors work consistently and precisely.

4. Conclusions and future directions

In this study, we present the first impedimetric biosensor capable of detecting fibroblast growth factor receptor 3 (FGFR3) protein. The sensor is label-free and highly sensitive with linear detection range from sub-pM to nM for FGFR3 diluted in PBS buffer and synthetic urine, although non-specific background with urine samples is still an important issue and requires further optimisation. Until now, there have been no reports regarding the pathological levels of FGFR3 protein present in the urine of bladder cancer patients and analysis of patients' samples is also needed.

Nowadays, biosensing research has shifted towards label-free systems as they offer cost effectiveness, simplicity and fewer reagents needed. From a clinical perspective, precision and reproducibility are prerequisites for every sensor. Robotic platforms such as sensor printing will improve sensor reproducibility as they minimise any errors caused by manual sensor assembly [34, 53]. We expect that the sensor platform developed here may offer an effective tool for bladder cancer screening and monitoring and provide a basis for detecting other protein biomarkers of diseases.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflict of interest

The authors declare no conflict of interest.

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Biographies

Pattanapong Thangsunan was a PhD student fully funded by the Royal Thai Government. He received his BSc and MSc in biology from Chiang Mai University, Thailand before participating in the Leeds Bionanotechnology Group in 2014. He was awarded the degree of PhD in Biomedical Sciences from Faculty of Biological Sciences at the University of Leeds in 2018. He is currently working as a researcher at the National Institute of Metrology (Thailand). His research interest is to develop novel bioanalytical methods for detecting a vast range of targets.

Nidhi Lal was a postdoctoral fellow working with Dr. Paul Beales. She gained her PhD degree from Indian Institute of Science, India in 2014 before joining the group. Her PhD work involved electrical biosensor fabrication and cancer biomarker detection using different types of nanostructure. She was funded by Yorkshire Cancer Research to develop diagnostic tools to monitor a biomarker of superficial bladder tumours.

Christian Tiede is the manager of the BioScreening Technology (BSTG) at University of Leeds. He received PhD from Department of Biochemistry, the Charite Universitätsmedizin Berlin, Germany in 2010 and moved to join the BSTG in 2011. His interest is the generation of Affimer reagents against a vast range of biological targets for clinicians, academic researchers and inductrial partners.

Sophie Moul was a Wellcome Trust student working with Dr. Paul Beales. Her research for the first rotation with the BSTG was to develop Affimer-functionalised liposomes capable of specifically binding cells upregulating FGFR3 proteins.

James Robinson is currently a senior research fellow at School of Medicine, Faculty of Medicine and Health, University of Leeds. He received PhD in Immunogenetics from University of Leeds in 2011. His research interests focus on the role of Fc gamma receptors in autoimmunity and discovery of novel ways to treat autoimmunity.

Margaret Knowles is a professor in experimental cancer research and the leader of the Urothelial Cancers group at Leeds Institute of Medical Research, St James's University Hospital. She gained her PhD from the Imperial Cancer Research Fund. Her research interests focus on the investigation of the molecular features of urothelial carcinoma of the bladder and the attempts to translate this information into clinical applications.

Peter Stockley is a professor in biological chemistry. He was awarded PhD degree from the University of Cambridge in 1980. His research interests are involved in protein-nucleic acid interactions, genetic regulatory mechanisms, virus assembly and infection mechanisms in ssRNA virions.

Paul Beales is an Associate Professor in School of Chemistry, University of Leeds. He gained his PhD in Physics from the University of Edinburgh in 2005. His research group, in the field of soft matter and biophysics, are specifically interested in topics related to membrane biophysics, bionanotechnology, nanomedicine and synthetic biology.

Darren Tomlinson is the leader of the BSTG and a professor in School of Molecular and Cellular Biology, University of Leeds. He received PhD in developmental biology from the University of Edinburgh. His research of interest is in the areas of protein engineering, signalling cascades in cancer, inhibition of membrane proteins and anti-bacterial reagents. His expertise is the use of Affimers for a wide range of applications.

Michael McPherson is a professor in biomolecular engineering and the director of BSTG. He was awarded PhD degree from the University of Leeds. The research interests in his group include Affimer binding proteins as alternatives to antibodies, protein engineering, directed evolution, antimicrobial resistance and biosensors.

Paul Beales is an Associate Professor in School of Chemistry, University of Leeds. He gained his PhD in Physics from the University of Edinburgh in 2005. His research group, in the field of soft matter and biophysics, are specifically interested in topics related to membrane biophysics, bionanotechnology, nanomedicine and synthetic biology.

Paul Millner is a professor in bionanotechnology and the leader of the Leeds Bionanotechnology Group. He initially worked as a biochemist and was awarded his PhD in plant sciences from the University of Leeds in 1979. Currently, his interests are in the areas of biosensors and nanoparticles for medical and environmental applications.

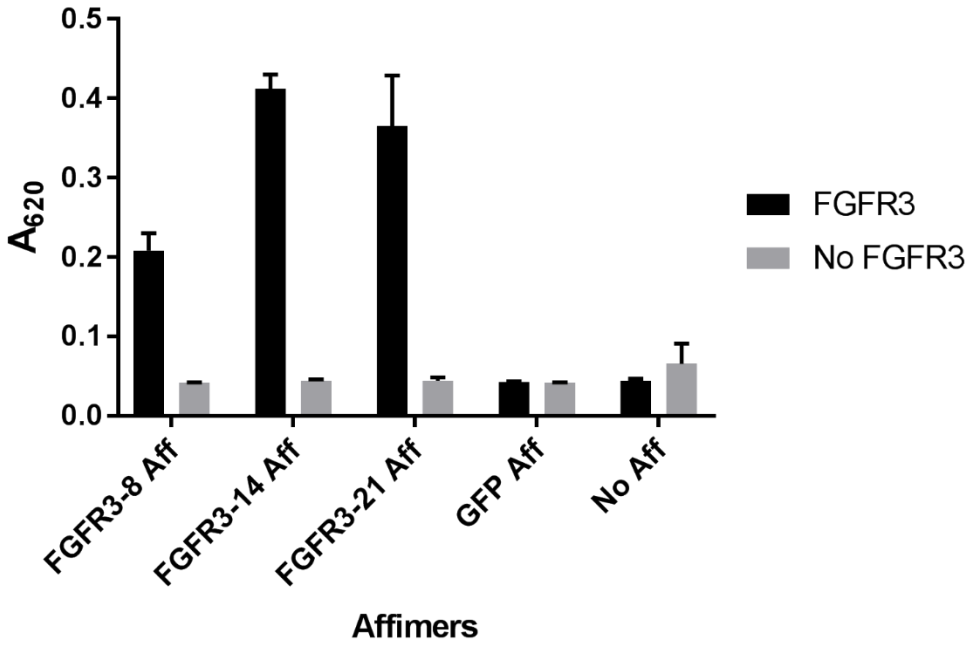

Figure 1 Double-sandwich ELISA to determine the binding of selected Affimers and FGFR3 protein. The Affimers were immobilised onto a 96-well plate via Neutravidin-biotin interaction. Three clones of Affimer (Aff) were tested for their binding to FGFR3 whilst anti-GFP Affimer was used as a negative control. Data are means ± SEM (n = 3).

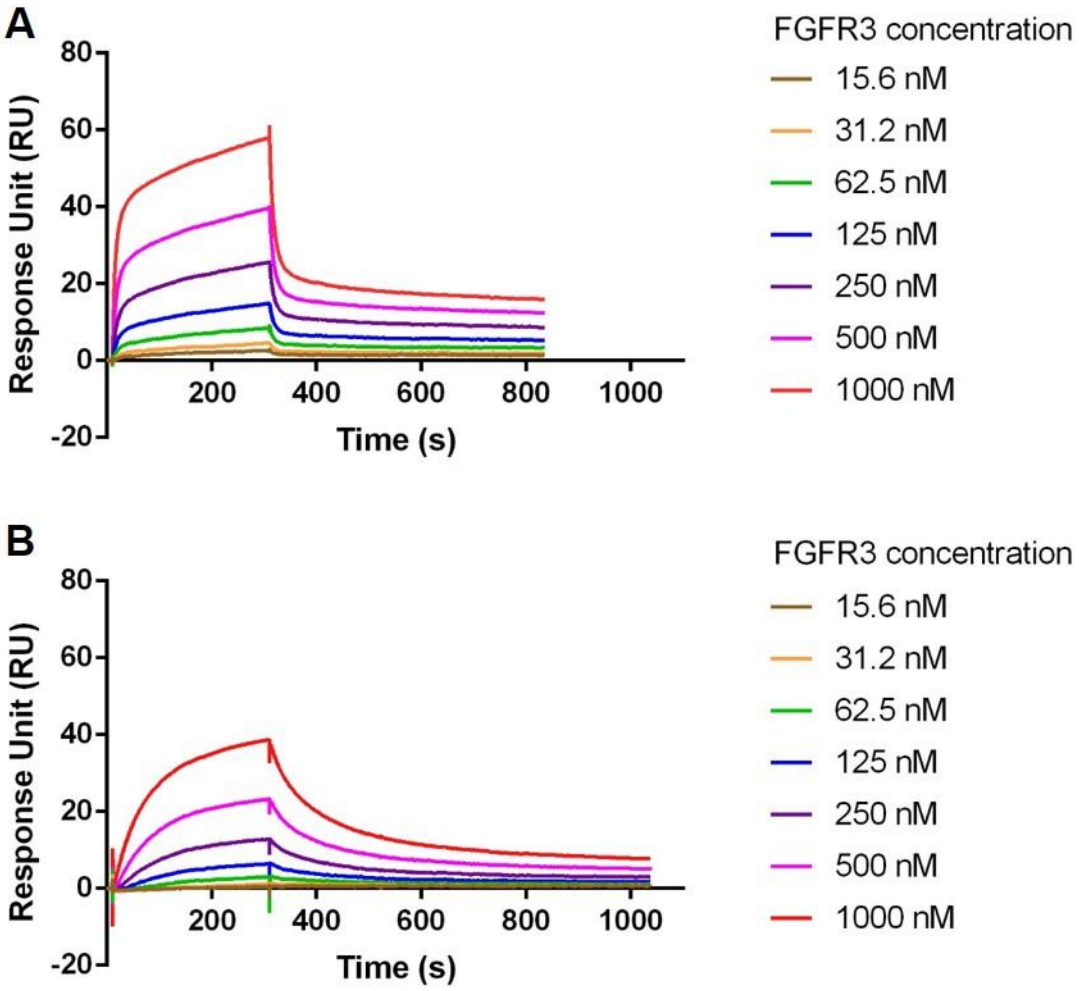

Figure 2 SPR sensorgrams of Affimer interaction with FGFR3. (A), FGFR3-14 and (B), FGFR3-21 Affimers were immobilised onto streptavidin-coated sensors prior to injection of FGFR3 protein at concentrations ranging from 0 to 1000 nM.

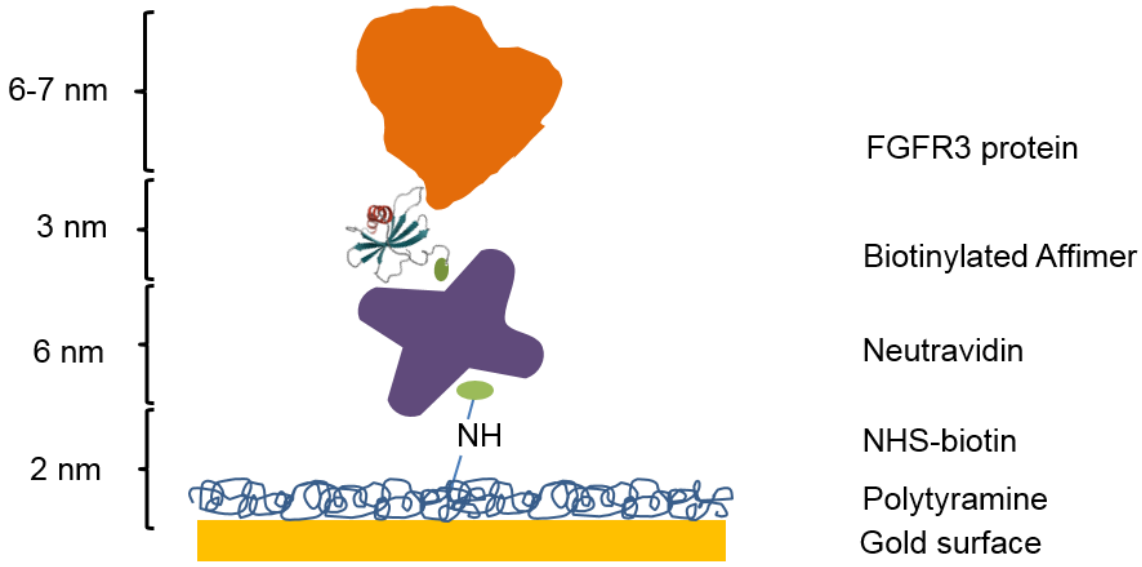

Figure 3 Schematic of a fully-fabricated Affimer-based biosensor. The gold transducer surface on an electrode chip was initially coated with a polytyramine film. A high affinity "Neutravidin-biotin linkage" then allowed a biotin-tagged Affimer to be tethered to the surface in an oriented fashion.

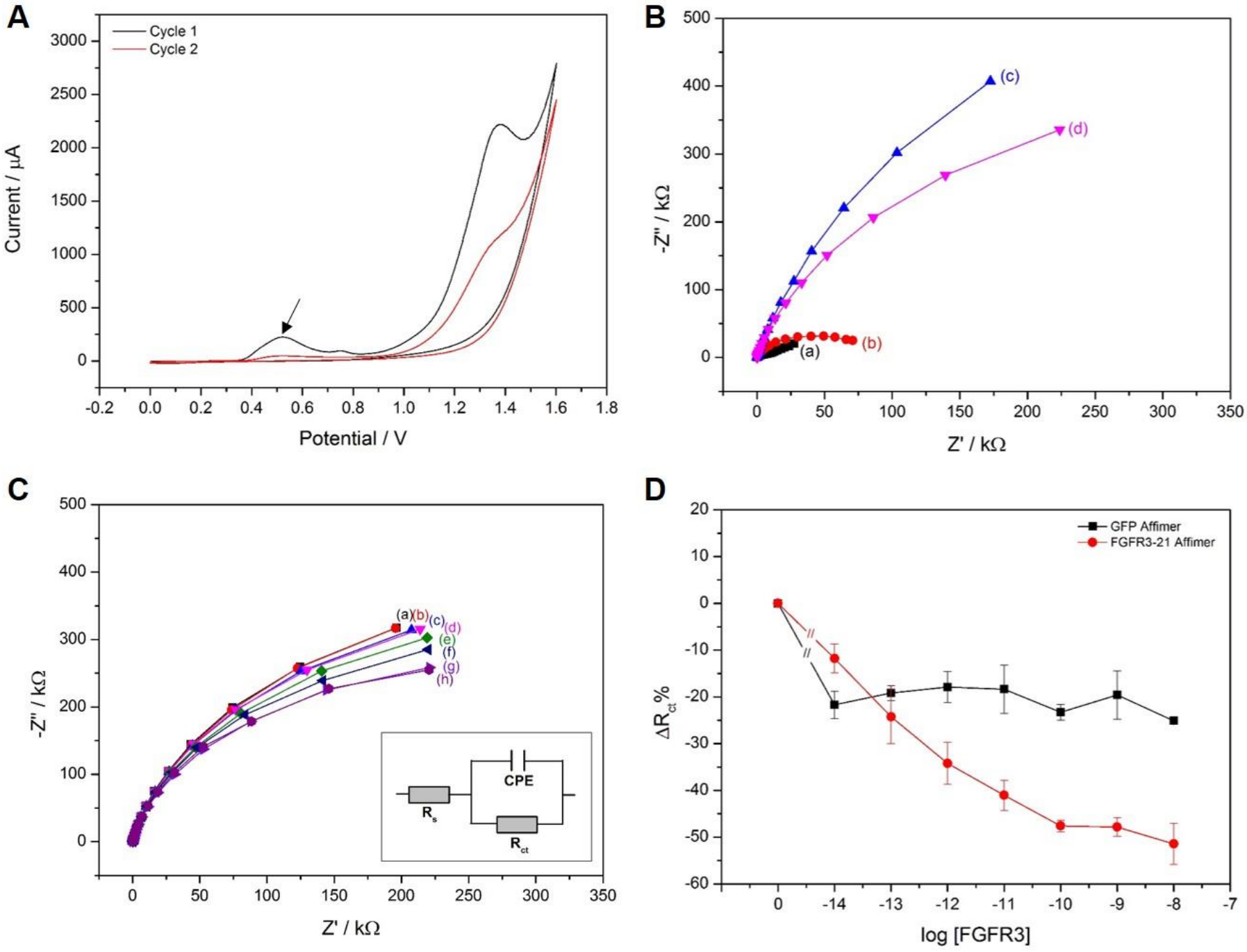
A
Cycle 1
Cycle 2
Current / µA
Potential / V
B
-Z'' / kΩ
Z' / kΩ
(a)
(b)
(c)
(d)
C
-Z'' / kΩ
Z' / kΩ
(a)(b)(c)(d)(e)(f)(g)(h)
CPE
R_s
R_ct
D
GFP Affimer
FGFR3-21 Affimer
ΔR_ct %
log [FGFR3]

Figure 4 Biosensor construction and sensor response when testing with FGFR3 protein. (A) Cyclic voltammogram of polytyramine deposition. Cyclic voltammetry was performed in 25 mM tyramine in dH_2O with 0.3 M NaOH, 2 cycles from 0 - 1.6 V, a scan rate of 200 mV/s. The arrow indicates an oxidation peak during the first CV scan (B) Nyquist plots showing layer-by-layer construction of an anti-FGFR3-21 Affimer biosensor. Each plot is (a), bare gold surface; (b), polytyramine-modified surface; (c), biotin-Neutravidin coated surface and (d), anti-FGFR3-21 Affimer-immobilised sensor. (C) Nyquist plots showing sensor response after adding increasing concentration of FGFR3. The plots represent (a), a baseline (PBS buffer), and (b-h), FGFR3 at 10^{-14} to 10^{-8} M respectively. Inset is the Randles' equivalent circuit model for data fitting, where R_s = solution resistance, R_{ct} = charge transfer resistance, and CPE = constant phase element. (D) Calibration curves of the unblocked Affimer-based sensors for detecting a range of FGFR3 concentrations in PBS buffer. The EIS measurements were performed in 10 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ redox mediator solution. Data points are means ± SEM (n = 3).

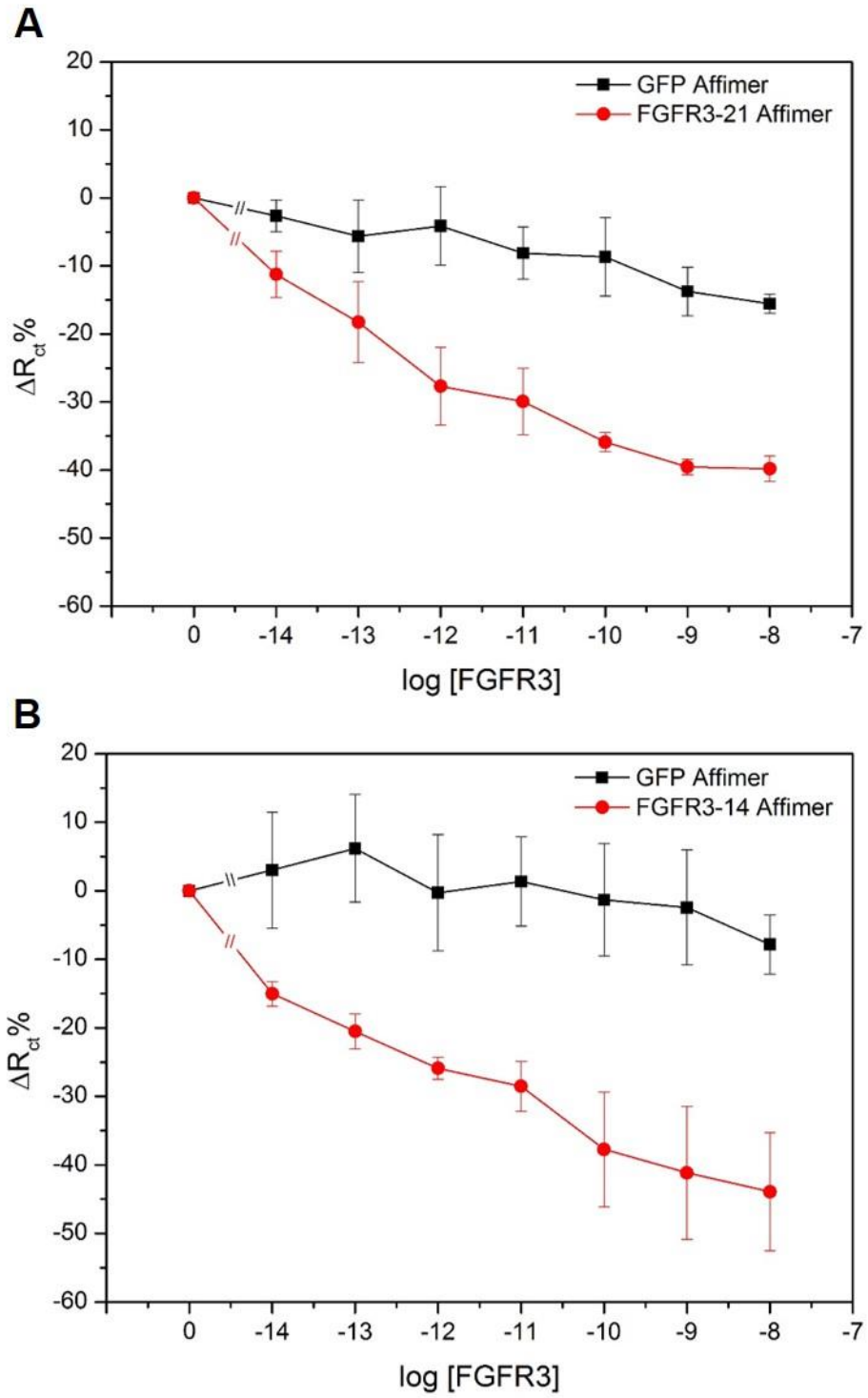

Figure 5 Calibration curves of the Affimer-based sensors tested with increasing concentrations of FGFR3 in PBS buffer. The sensors were blocked with 6.7 µM BSA prior to Affimer immobilisation. The sensors were modified with (A), GFP and FGFR3-21 Affimers, and (B), GFP and FGFR3-14 Affimers. EIS interrogation was performed in 10 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ redox mediator solution. Data are means ± SEM (n = 3).

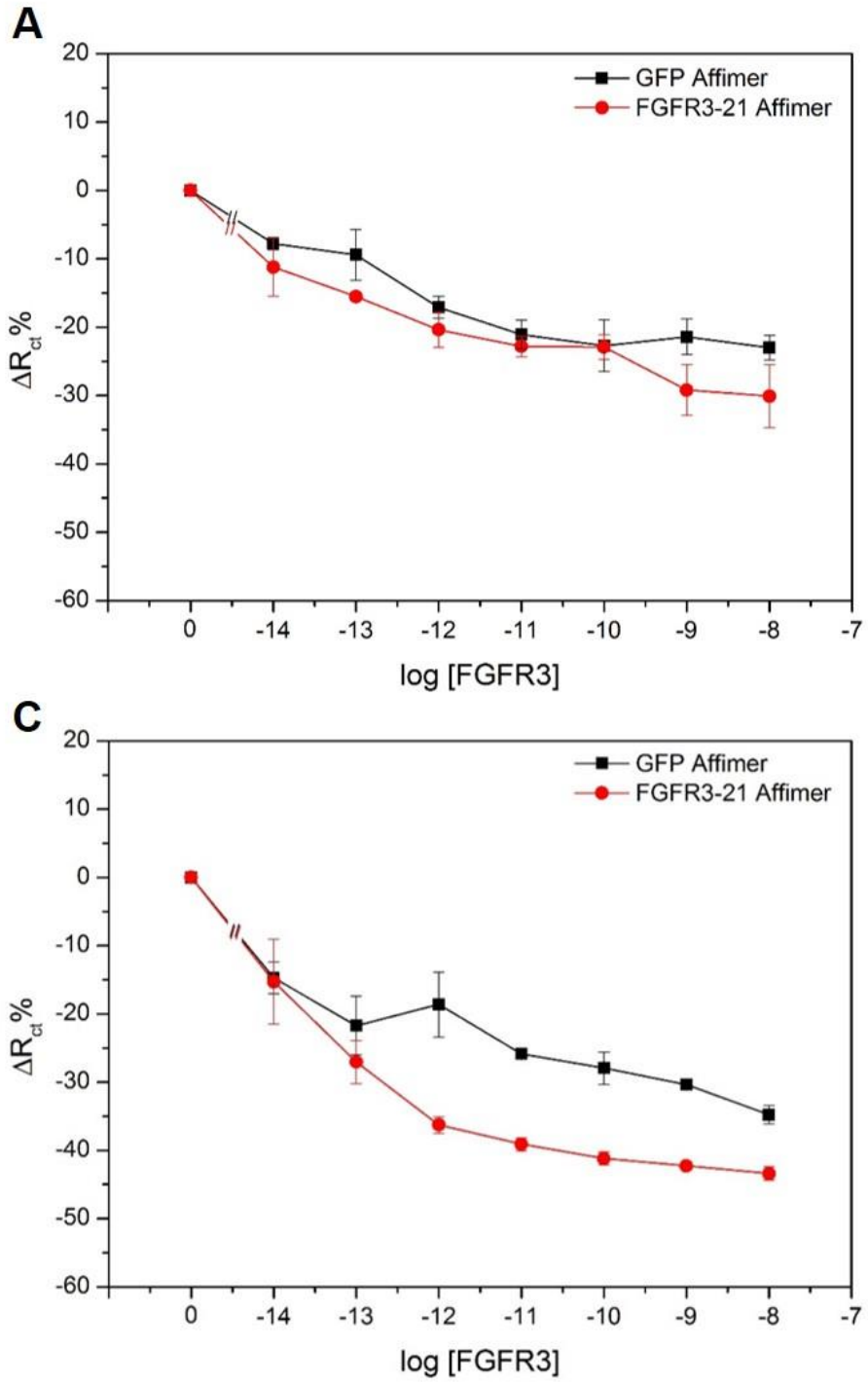

Figure 6 Calibration curves of the Affimer-based impedimetric biosensors for detecting FGFR3 in synthetic urine. The sensors were blocked with 6.7 µM BSA and then were immobilised with 1 µM GFP and FGFR3-21 Affimers. The sensors were tested with FGFR3 diluted in (A), 100% (v/v) synthetic urine; (B), 10% (v/v) synthetic urine and (C), 3% (v/v) synthetic urine. The EIS measurements were performed in 10 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ redox mediator solution. Data are means ± SEM (n = 3).