Electronic Supplementary Material (ESI) for Analyst.

An SPR investigation toward the therapeutic drug monitoring of anticancer drug imatinib with selective aptamers operating in human plasma

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Experimental details

Chemicals. Analytical reference standards of **1** (imatinib mesylate), PBS buffer, magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), formic acid, ammonium acetate, imatinib-d_8, LC-MS grade isopropanol and acetonitrile were purchased from Merk Sigma-Aldrich (Milan, Italy). N-Desmethyl imatinib (**2**) was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Tween20 was purchased from VWR International S.r.l. (Milan, Italy). N-ethyl-N'-(dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), ethanolamine, N-ε-maleimidocaproic acid hydrazide (EMCH), sodium borate buffer, cysteine and HEPES buffer solution (HBS-EP+: 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) were purchased from GE Healthcare Life Sciences (Milan, Italy). Imatinib aptamer (**3a**, 100-mer), 5'biotin imatinib aptamer (**3b**), 5'amino imatinib aptamer (**3c**), 5'thiol imatinib aptamer (**3d**) and 5'biotin immobilization oligomer (**4**, 15-mer) (Aptamer Diagnostics Ltd 2018, UK Patent Application No. 1819580.0) were purchased from Aptamer Group (York, UK). Upon arrival, lyophilized oligomers were solubilized with milli-Q water, aliquoted and stored at -20°C. Co-medications for selectivity tests, in particular telmisartan (**5**), lansoprazole (**6**), tamsulosin (**7**), finasteride (**8**), Lisinopril (**9**), pravastatin (**10**) were purchased from Merk Sigma-Aldrich, Merk group. Amlodipine (**11**), paracetamol (**12**), furosemide (**13**), enalapril (**14**), hydrochlorothiazide (**15**) and allopurinol (**16**) were provided by the pharmacy of National Cancer Institute (Aviano, Italy). Control human plasma stabilized with K_2EDTA for the preparation of daily standard calibration curves and quality control (QC) samples was obtained from healthy volunteers and was provided by the Transfusion Unit of the *Centro di Riferimento Oncologico di Aviano*, Italy.

Aptamer selection. Selection of the aptamers against imatinib was carried out by Aptamer Group (York, UK) according to the company's 'Displacement Selection' approach, which was extensively developed and automated, based on previously reported methods.[1,2] Briefly, iterative cycles of selection and amplification of aptamers targeting imatinib were conducted starting from a degenerate library of ssDNA oligonucleotides (synthesized by Integrated DNA technologies). In each round, the aptamer library was immobilized on streptavidin coated Dynabeads (Invitrogen/Life Technologies) modified with 5′-biotin immobilization oligo (**4**). Imatinib mesylate (20 µM) in 50% human plasma buffered at pH 6 (10 mM PBS containing 137 mM NaCl, 2.7 mM KCl, 2mM $MgCl_2$, 1 mM $CaCl_2$ and 0.01% Tween 20) was then incubated with the aptamer-conjugated magnetic beads. Interaction with the imatinib results in a conformational change in the aptamer, causing them to be displaced from the magnetic beads and released into solution. The displaced aptamers were then recovered and amplified by PCR, using the primers (synthesized by Integrated DNA technologies) specific to the 5′ and 3′ ends of the aptamer library. In the first round, 3 nmol of library was used; in subsequent rounds, the enriched fraction of oligonucleotides selected from each round were used as ssDNA

pool. The amount of aptamer released from the beads during the Imatinib incubation was determined by fluorescence measurements and used as a means of monitoring the refinement of the aptamer library. In total, 10 cycles of aptamer selection were performed to enrich the population with imatinib-specific aptamers.

The pool of selected aptamers obtained after the 10th selection round was amplified with aptamer specific primers and subsequently cloned into the pJET sequencing vector according to manufacturer's protocols (ThermoFisher Scientific). Chemically competent NEB 5-alpha E. coli were transformed according to manufacturer's protocols (New England BioLabs). Once the plasmid DNA was isolated, several clones were sequenced by Sanger Sequencing methods by DNASEQ (University of Dundee). Aptamer **3a** was identified through individual binding analysis of the obtained sequences, using a displacement assay adapted for bio-layer interferometry on the Octet QK system (ForteBio, Molecule Devices).[3] Aptamer **3a** was then modified at the 5′-end with a 6-carbon linker to provide the desired functional groups and related functional aptamers, namely: 5′-biotin (**3b**), 5′-amino (**3c**), 5′-thiol (**3d**) imatinib aptamers.

SA chip functionalized with 3c. The immobilization of 3c onto the CM5 chip was carried out following the manufacturer instructions. After activating the surface of a CM5 chip with a mixture of EDC (400 mM) and NHS (100 mM) in miliQ water, 3c (25 μM) in acetate buffer (50 mM, pH 5) was injected for 1000 s at 5 μL/min over FC2 only. Finally, ethanolamine 1M was injected as the blocking reagent. The immobilization process yielded 136 RU of 3c (Fig. S4).

SA chip functionalized with 3d. The immobilization of **3d** onto the CM5 chip was carried out following the manufacturer instructions. After activating the surface of a CM5 chip with a mixture of EDC (400 mM) and NHS (100 mM), EMCH (50 mM in 10 mM sodium borate containing 1 M NaCl at pH 8.5) was injected to provide active maleimide groups on the chip surface. Ethanolamine 1M (pH 7 in 0.1 M sodium phosphate) was then injected to prevent the remaining carboxylic groups to further react. Aptamer **3d** (25 μM) in acetate buffer (50 mM, pH 5) was injected for 1000 s at 5 μL/min over FC2, followed by cysteine (50 mM in 1 M NaCl, 0.1 M sodium acetate, pH 4.0) as the blocking reagent. The immobilization process yielded 48 RU of **3d** (Fig. S5).

Validation study

This study was carried out by taking into account FDA recommendations for ligand-binding assays (LBA) validation.[4] The following parameters were evaluated: recovery, calibration curves, intra- and inter-day precision and accuracy, reproducibility, lower limit of quantification (LLOQ), selectivity and matrix effects.

Recovery. The recovery of extraction/microfiltration procedure was determined at three concentrations of **1** in quality control (QC) samples at low (QCL), medium (QCM), and high (QCH) concentrations (Table S2). Two set of QC samples were prepared using the same working solutions of **1** for each concentration level: in the first set **1** was spiked in plasma, which was then diluted (1:15) with incubation buffer and microfiltrated following the procedure described in Scheme S1; in the second set **1** was spiked in the diluted (1:15 with incubation buffer) and microfiltrated plasma, which provided the reference for 100% recovery. Measurements were carried out in triplicate and the results were compared to provide the recovery %.

Calibration curves. The closeness of the four parameter logistic (4PL) model (eq. 1) to fit the calibration curves was evaluated over six different working days with calibration standards prepared as described in the main text. For each standard point, a background corrected sensorgram was obtained firstly by subtracting the response of FC1 (reference cell) from FC2, and secondly by flipping data (after blank subtraction) to plot the aptamer dissociation as a positive response. The 4PL regression analysis was performed with OriginPro 2019b (OriginLab, Northampton, MA, USA). The RUs for each standard point were taken 200 s after the end of sample injection and plotted against the nominal concentration of the sample. To meet FDA requirements, the accuracy of back-calculated values had to be within 80–120% of the theoretical concentration for a minimum of five standards (75–125% for the lowest calibrator). The equation for the 4PL model is:

$$y = \alpha + \frac{\beta - \alpha}{1 + \left(\frac{x}{\gamma}\right)^{\delta}} \qquad \text{(eq.1)}$$

Where: *x* is the independent variable, *y* is the dependent variable, α is the maximum response value that can be obtained (i.e. infinite concentration), β is the minimum response value that can be obtained (i.e. at concentration = 0), γ is the point of inflection and δ is the slope of the curve at point γ.

Intra-day and inter-day precision and accuracy and reproducibility. Precision and accuracy were evaluated on six different days by measuring **1** at three QC levels (QCL, QCM, QCH Table S2) in triplicate, as summarized in Table S2. The SPR responses of QC samples were analyzed using different standard calibration curves prepared on each day of the validation study. The precision of

the method at each concentration was provided as the coefficient of variation (CV%) and the accuracy was determined as per cent of the nominal concentration. In each set of measurements, the back-calculated concentration for at least six out of nine QC samples had to be within 20% of the nominal value with one QC sample that could be excluded for each concentration level.

$$CV\% = (SD/mean)\cdot 100 \qquad \text{(eq.2)}$$

$$accuracy\% = (BC/NC)\cdot 100 \qquad \text{(eq.3)}$$

where: SD is the standard deviation, BC and NC are the determined value and nominal concentration at each standard/QC.

Lower Limit of quantification (LLOQ) and matrix effect. To evaluate LLOQ, eight replicates of each calibration point were independently prepared and analyzed. According to guidelines, LLOQ is the lowest point in which the bias is within 25%. Matrix effects were investigated using eight independent sources of blank human plasma, which were individually analyzed and evaluated for interference: a single 23.75 μL-aliquot from each matrix was spiked with 1.25 μL of working solution "a" (see Table S2) and processed as described in the main text. The back-calculated concentration values had to provide acceptable precision (25%) and accuracy (between 75% and 125%).

Table S1. List of co-medications associated with the administration of imatinib

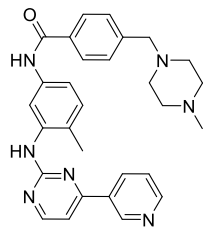
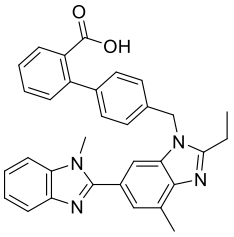
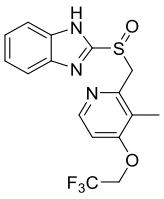
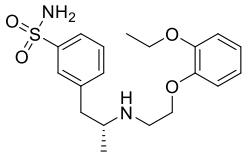
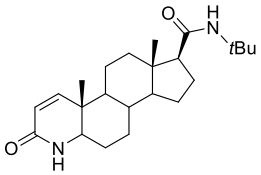
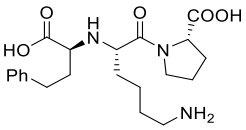
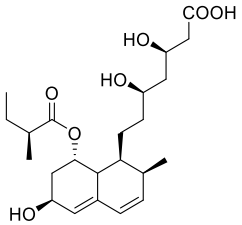
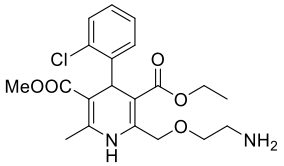
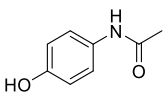
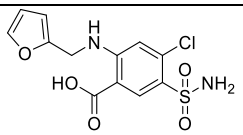
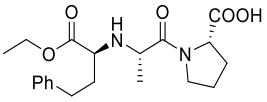
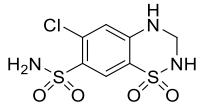
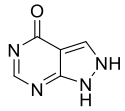
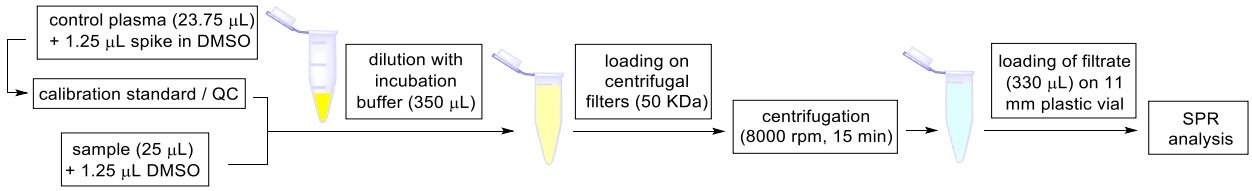
 Imatinib (**1**)	 Telmisartan (**5**)	 Lansoprazole (**6**)	 Tamsulosin (**7**)
 Finasteride (**8**)	 Lisinopril (**9**)	 Pravastatin (**10**)	 Amlodipine (**11**)
 Paracetamol (**12**)	 Furosemide (**13**)	 Enalapril (**14**)	 Hydrochlorothiazide (**15**)
 Allopurinol (**16**)			

Table S2. Concentrations of imatinib mesylate (1) in working solutions (WS) and final concentrations (C_{fin}) in calibration and QC standards.

	Concentration (μg mL^{-1})									
	a	b	c	d	e	f	g	QCL	QCM	QCH
WS	8.0	20	40	60	80	100	120	15	50	90
C_{fin}	0.4	1.0	2.0	3.0	4.0	5.0	6.0	0.75	2.5	4.5

Scheme S1. Schematic procedure for detection of **1** in human plasma samples.



Immobilization scheme of 3a and 4 on SA chip

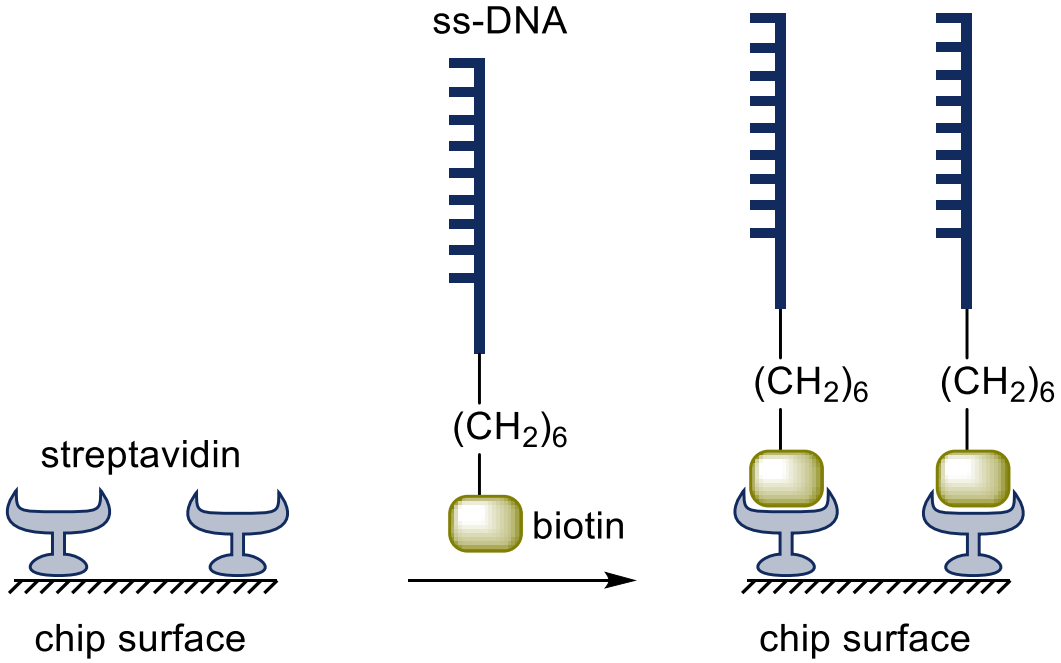


Fig. S1. Immobilization of **3a** and **4** on a chip surface modified with streptavidin (SA chip, GE) is spontaneously occurring due to the strong affinity between streptavidin and biotin.[5]

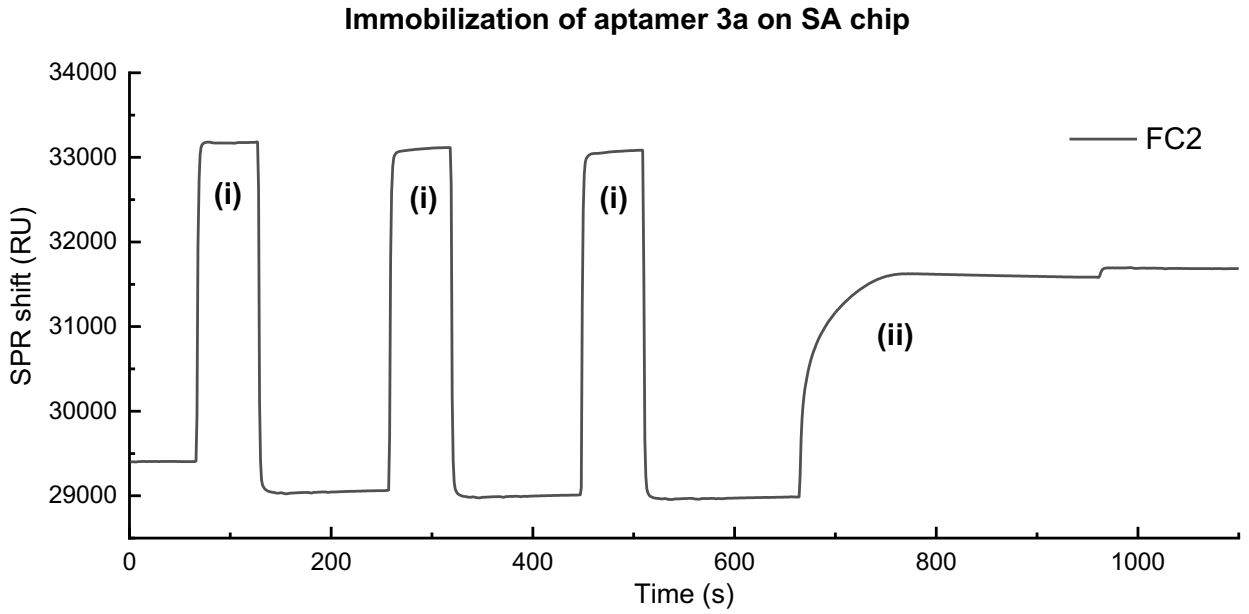


Fig. S2. Sensorgram showing immobilization of **3a** (5 µM) in PBS buffer (25 mM, pH 7,2) on FC2 using a SA chip. Flow rate: 10 µL/min, injection time: 240 s. Immobilization yield: 2713 RUs of **3a**. (i) Conditioning the surface of a SA chip with a mixture of 50 mM NaOH and 1M NaCl (3 x 1 min). (ii) Injection of **3a**.

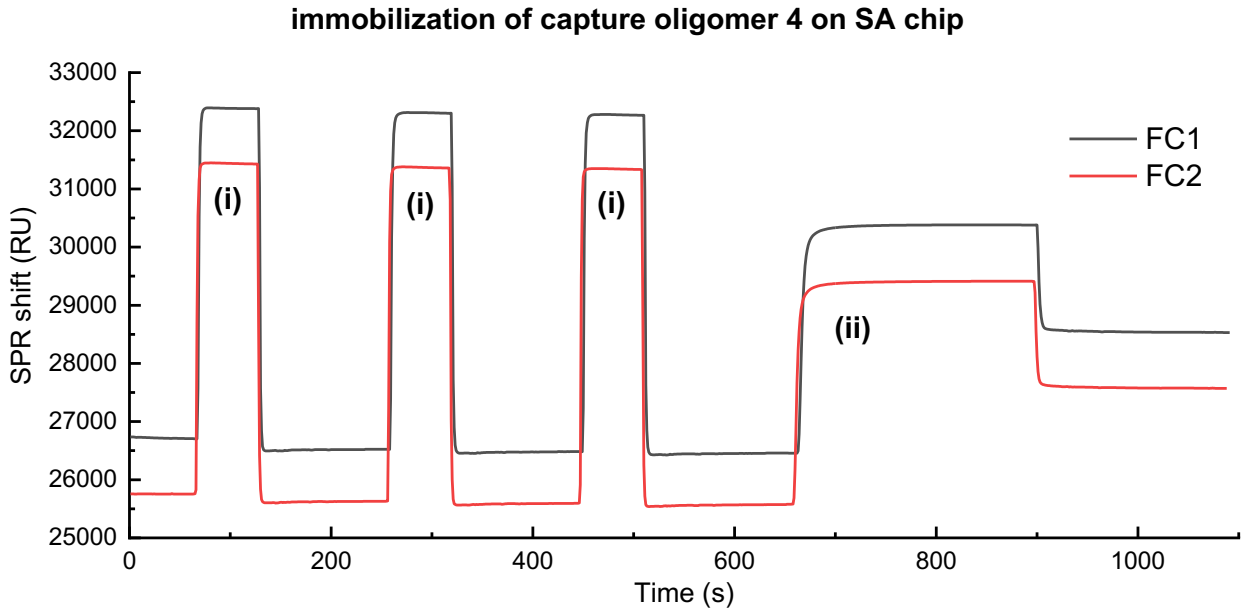


Fig. S3 Sensorgram showing immobilization of **4** (5 µM) in PBS buffer (25 mM, pH 7,2) on both FC1 and FC2 using a SA chip. Flow rate: 10 µL/min, injection time: 240 s. Immobilization yield: 2084 and 2007 RUs of **4** in FC1 and FC2 respectively. (i) Conditioning the surface of a SA chip with a mixture of 50 mM NaOH and 1M NaCl (3 x 1 min). (ii) Injection of **4**.

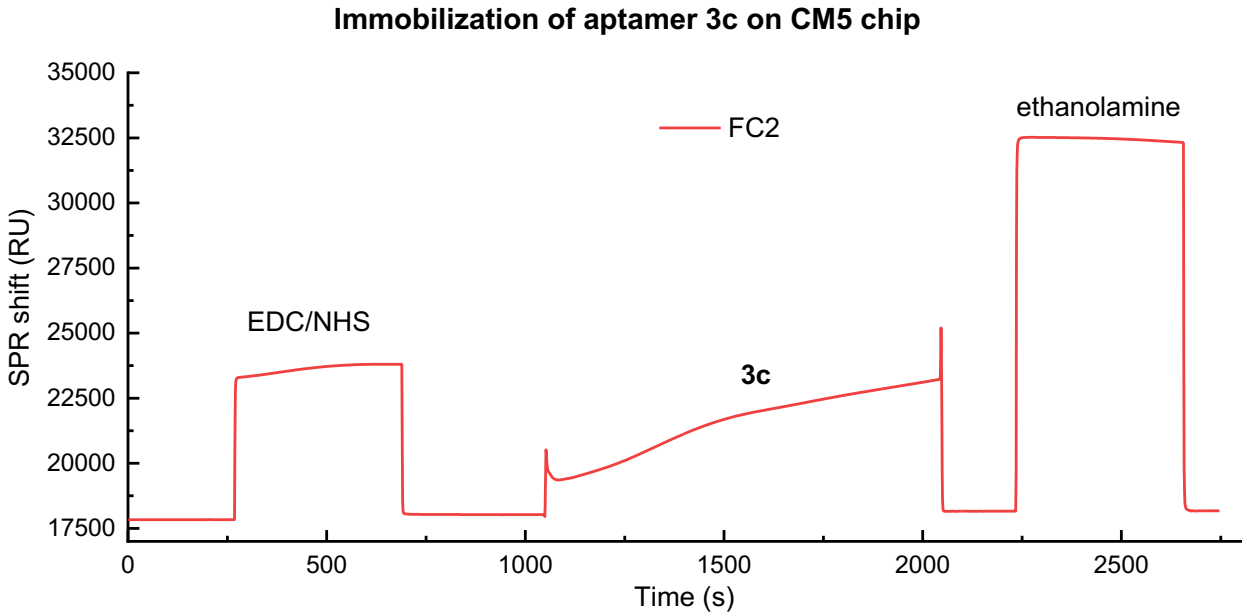


Fig. S4 Sensorgram showing immobilization of **3c** (25 µM) in acetate buffer (50 mM, pH 5) on FC2 using an activated CM5 chip. Flow rate: 5 µL/min, injection time: 1000 s. Immobilization yield: 136 RUs of **3c**.

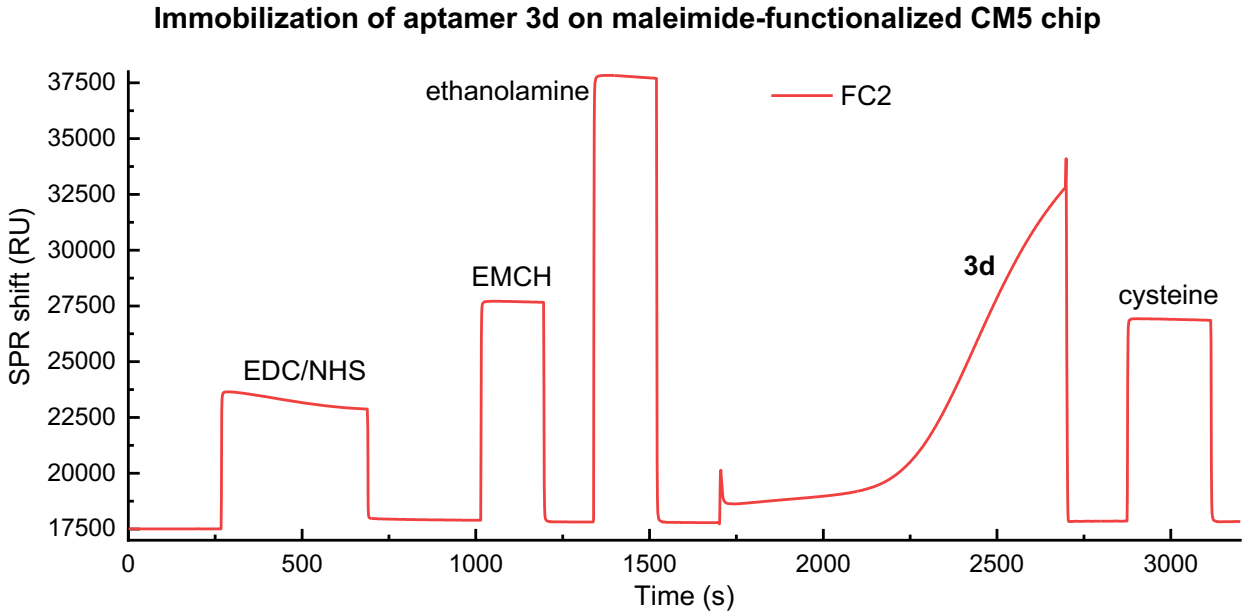


Fig. S5 Sensorgram showing immobilization of **3d** (25 µM) in acetate buffer (50 mM, pH 5) on FC2 using a maleimide-functionalized CM5 chip. Flow rate: 5 µL/min, injection time: 1000 s. Immobilization yield: 48 RUs of **3d**.

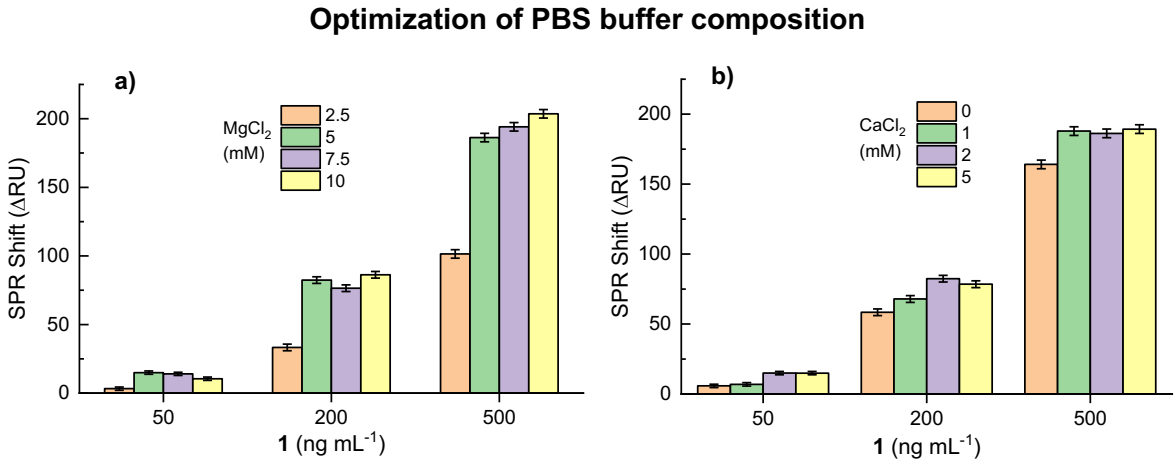
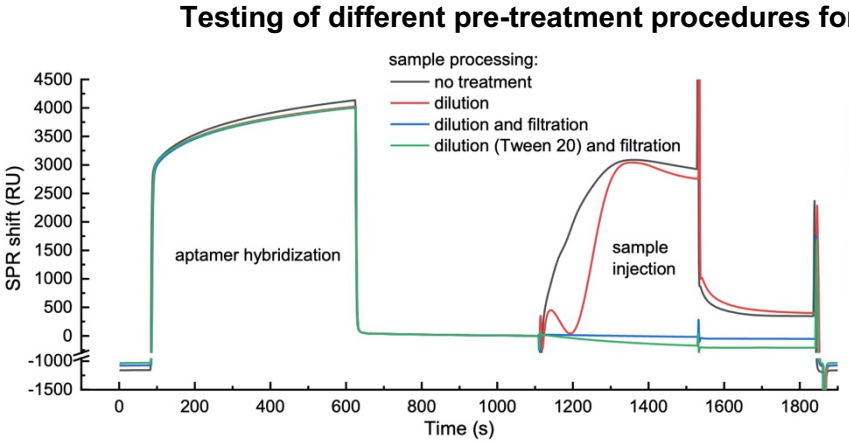


Fig S6. Histograms showing the aptamer dissociation level at 50, 200 and 500 ng mL^{-1} of **1** in PBS 10 mM at pH 6 varying the concentrations of $MgCl_2$ and $CaCl_2$. (a) Variation of the concentration of $MgCl_2$ in the range 2.5-10 mM while keeping the concentration of $CaCl_2$ at 2 mM. (b) Variation of the concentration of $CaCl_2$ in the range 0-5 mM while keeping the concentration of $MgCl_2$ at 2 mM.



Treatment	Response (RU)
No treatment	352.8
1:10 dilution	430.3
1:10 dilution, microfiltration	-51.3
1:10 dilution, addition of Tween20 (0.05%), microfiltration	-207.4

Fig S7. Referenced (FC2-FC1) senorgrams obtained by testing different pre-treatment procedures for plasma. SPR response levels after the sample injection can be found in the included table. Concentration of **1** was 1000 ng mL^{-1}. Dilution buffer was PBS (10 mM, pH 6,0) containing NaCl (100 mM), KCl (2 mM) $MgCl_2$ (6 mM) and $CaCl_2$ (2 mM). Response was referred to the baseline level before sample injection.

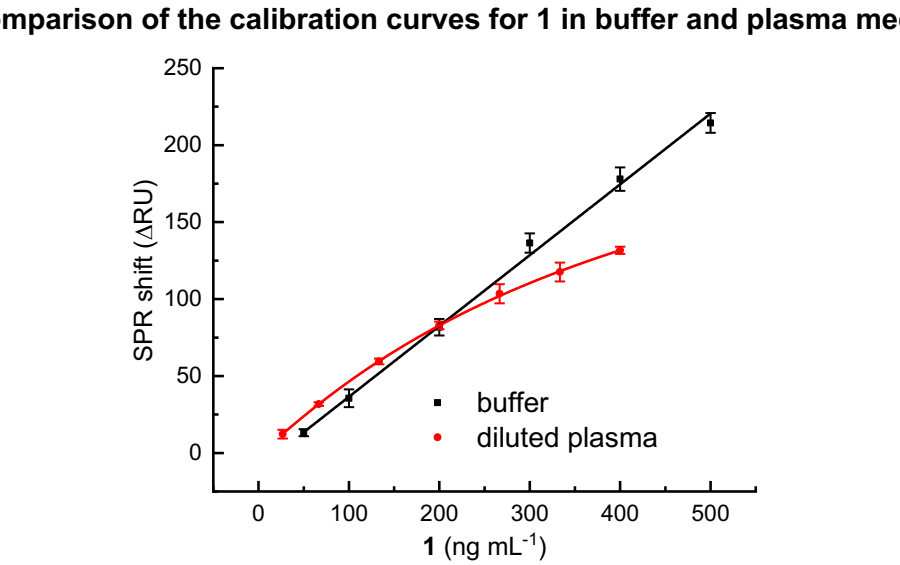


Fig. S8. Comparison of the calibration curves for **1** in buffer and plasma media. The concentrations in plasma were corrected to take into account the dilution factor (1:15).

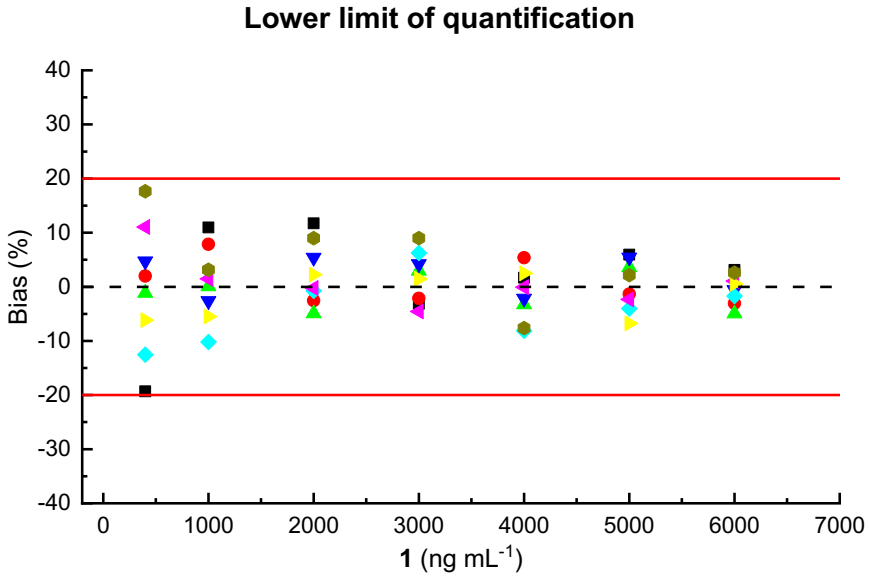


Fig. S9 Biases obtained by analyzing the SPR response of independent replicates of each calibration standards with a 4PL fitting model.

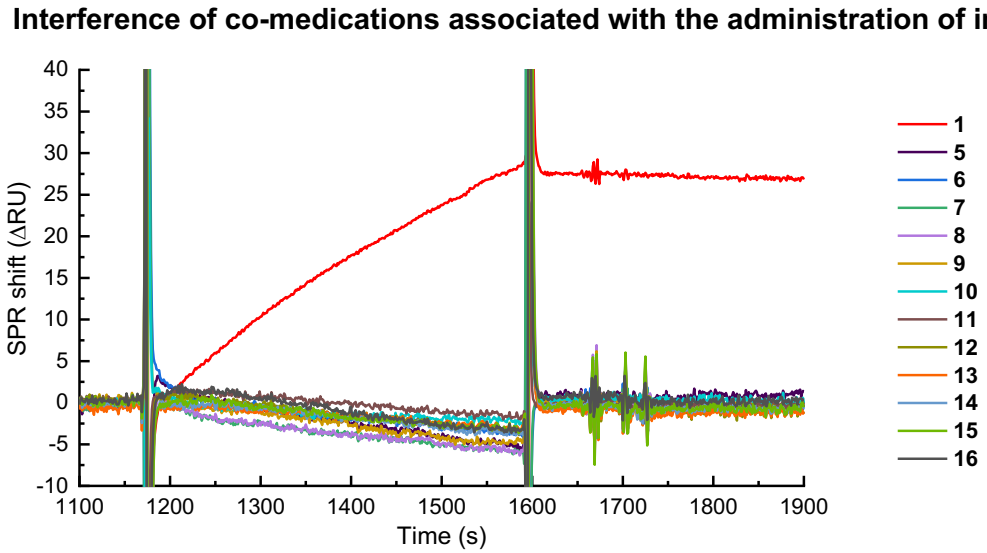


Fig. S10 Blank subtracted SPR response of imatinib at 1 μg mL^{-1} and co-medication listed in Table S1 (all 10 μg mL^{-1}) spiked in human plasma, diluted with incubation buffer, microfiltrated and injected over the aptamer hybridized on the chip surface.

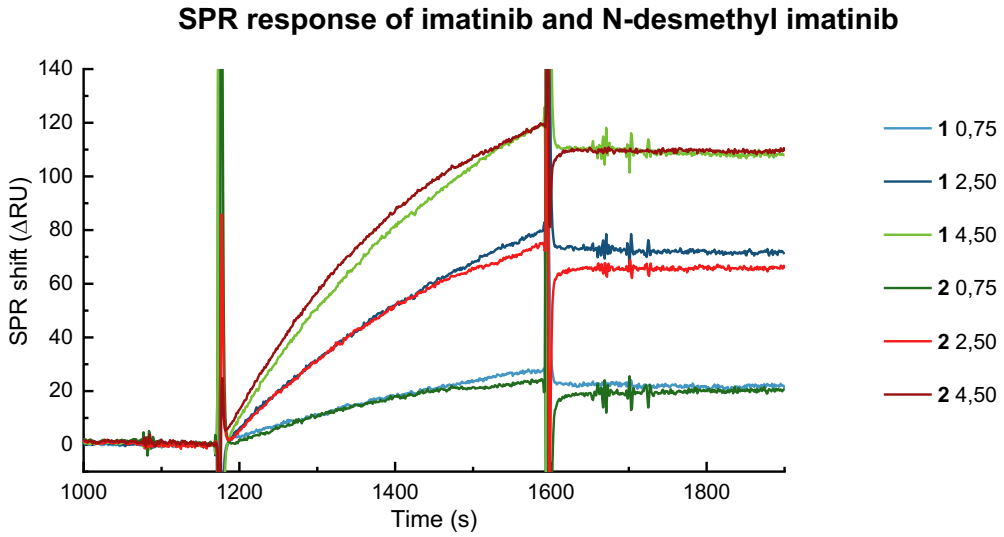


Fig. S11 Blank subtracted SPR response of imatinib (**1**) and N-desmethyl imatinib (**2**) spiked in human plasma, diluted with incubation buffer and microfiltrated at QCL, QCM and QCH concentration levels (Table S2). Average of three measures.

References

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