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## Development of an assay for label-free high-throughput screening of thrombin inhibitors by use of reflectometric interference spectroscopy

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**Abstract** We describe the development of, and analytical conditions used for, parallel affinity assay for thrombin inhibitors adapted to the first label-free optical screening HTS detection set-up fully integrable into a screening platform. To achieve compatibility with pharmaceutical libraries, an HTS-transducer was realized by gluing the bottomless scaffolds of 96- and 384-well plastic microplates on to transducer slides. The transducer are coated with a dextran, to ensure biocompatibility and functionality, and a known thrombin inhibitor was attached covalently to it. By adapting reflectometric interference spectroscopy for simultaneous reading of the whole transducer plate we were able to detect the binding of thrombin in all the wells of the microplates on-line, in parallel, and time resolved. By using an inhibition assay, the screening of 384 substances for thrombin activity can be performed within an assay time of less than 15 min. We also show that the data quality is high enough for parallel quantification of the IC<sub>50</sub> values of the library substances.

**Keywords** High throughput screening · Label-free detection · Thrombin · Reflectometric interference spectroscopy · Assay development

### Introduction

In recent years pharmaceutical research has been searched for new ways to discover future drugs and agrochemical products and has developed high-throughput screening (HTS). The objective of this field of research is to screen enormous numbers of compounds for activity against a target of interest without the need for knowledge about the structure of the target or the mechanism of the action

of these substances. The substances are pooled in substance-libraries which often contain 10 to 300 thousand, mostly low-molecular-weight, compounds of high structural diversity [1].

One of the targets is the intensively investigated enzyme thrombin. Thrombin is a serine protease with a molecular weight of 34 kDa (40 kDa in cows) which consists of two subunits. It catalyses the splitting of fibrinogen to active fibrin and is the main component in the last step of the blood-coagulation cascade. Exact regulation of the blood coagulation is of special importance and determines the balance between bleeding and thromboses. This makes thrombin an important target in pharmaceutical drug screening and much effort is being devoted to finding effective thrombin inhibitors.

Whereas nowadays the number of interesting targets is numbered in hundreds only, the human genome project will lead consistently to the discovery of new targets which, although not attributed to a discrete function, are of great importance for the pharmaceutical industry. The main targets actually tested are enzymes in the classes kinases [2] or proteases [3] and membrane-bound or nucleoplasmatic receptors [4]. Screening of these targets is realized either by functional assays or by binding assays. Functional assays correlate the function of the target directly towards the concentration of the test substance. This function can be as complex as the modulation of gene expression or as simple as the activity of an enzyme. Binding or affinity assays determine the biomolecular interaction of two substances. These assays are less complex and easier to perform but the target function cannot be predicted. On the other side, binding assays supply additional information if a competitive assay is used. For binding assays the availability of very pure targets, e.g. chromatographically purified, is necessary.

Among the physical methods used for HTS experiments, imaging and scanning optical detection systems are particularly widely used. The most important strategies are fluorescence polarisation spectroscopy [5, 6], fluorescence correlation spectroscopy [7], and fluorescence resonant energy transfer [8].

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Although labelling of the binding partners is not necessary and the binding data provide supplementary information about affinity constants and kinetic data [9], highly parallel label-free optical detection systems are still not commercially available. Intensive work was undertaken to adapt surface plasmon resonance [10] or reflectometric interference spectroscopy [11] to the demands of HTS on stability, automation, and parallelisation.

The RfS screening arrangement presented is the first label-free optical screening HTS detection system fully integrable in a screening laboratory. It was used to develop a binding assay to screen for compounds which reduce or inhibit thrombin activity. Parallel binding studies were performed in special RfS microplates with the standard 96- or 384-well format. These plates are coated with a thin  $\text{SiO}_2$  layer which is required for signal detection and on to which a dextran polymer layer was attached covalently. This amino functionalised dextran layer provides biocompatibility [12]. In addition, the shield effect of this polymer layer ensures that non-specific binding in the microplates was suppressed maximally. A thrombin inhibitor similar to the inhibitor thrombostop [13] was covalently attached to the dextran layer and enabled detection of interaction of thrombin at the surface for screening substance libraries by means of a binding inhibition test for thrombin activity.

## Experimental

### Equipment and reagents

Thrombin and common chemicals and biochemicals were purchased from Sigma (Deisenhofen, Germany) and Fluka (Neu-Ulm, Germany). A thrombostop-analogous thrombin inhibitor with a free, reactive amino group was supplied by Dr Peter Eckard, Knoll AG (Ludwigshafen, Germany).

Partially amino-functionalized dextran with a average molecular mass of 260 kD was obtained from dextran by diol oxidation and reductive amination as described elsewhere [14]. By this method, approximately 10% of the anhydroglucose units were functionalised.

Interference transducers were produced by Schott (Mainz, Germany) by means of an ion-plating process.

The arrangement for optical detection and for gluing of the RfS microplates was performed by Carl Zeiss Jena GmbH, (Jena, Germany). The parallel sample handling system for 96- and 384-well plates was realized by CyBio (Jena, Germany).

Manual liquid handling for surface modification in the microplate wells was achieved by use of an eight-channel piston stroke pipette (25–200  $\mu\text{L}$ ) from Eppendorf (Hamburg, Germany).

### Preparation of AMD RfS microplates

AMD was covalently attached to glass transducers by use of a modification of the procedure of Piehler et al. [14]. The interference transducers (73 mm $\times$ 108 mm) were cleaned in freshly prepared, hot  $\text{H}_2\text{SO}_4\text{--H}_2\text{O}_2$ , 6:4, rinsed with water and dried in a nitrogen stream. The activated transducers were silanised with glycidoxypolytrimethoxysilane (400  $\mu\text{L}$ ) for 1 h. AMD (2.5 g) was dissolved in water (5 mL) and the solution was dropped on the pretreated transducers to furnish a uniform coating. Two such transducers were arranged in a sandwich. After a reaction time of 14 h in a saturated-water atmosphere the sandwich was separated and both transducers were rinsed with water. After drying the plates in

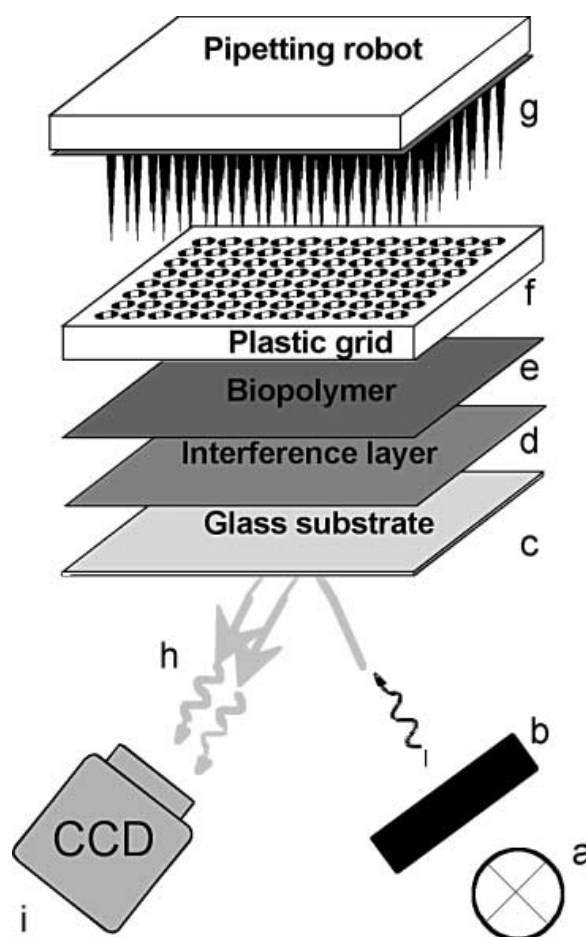


**Fig. 1** Exploded schematic diagram of the detection unit. From the white light source (a) six wavelengths are separated (b) sequentially and illuminate the RfS microplate from underneath. The light passes through the bulk carrier glass slide (c) and is reflected partially at the bottom and the top of the thin interference layer (d, e) consisting of a 330 nm silicon dioxide layer and a functionalized dextran layer. The reflected light (h) beam results in a typical interference pattern, depending of the wavelengths of the light. The intensity of the reflected beams is recorded by the CCD camera (i) and enables the calculation of the optical thickness of the interference layer. The plastic grid (f) has the dimensions of a standard 96-well microplate. Liquid handling is realized by use of a 96-pipette robot (g)

a nitrogen stream, they were glued to 96- or 384-well polystyrene microplate grids.

### Immobilization of thrombin inhibitor

The wells of the microplates were filled with solid glutaric acid anhydride and the maximum amount of water was also added. The plates are covered and shaken gently for 150 min. The wells were rinsed thoroughly with water. A freshly prepared aqueous solution of EDC (9.6 mg  $\text{mL}^{-1}$ , 0.05 mol  $\text{L}^{-1}$ ) and NHS (5.9 mg  $\text{mL}^{-1}$ , 0.05 mol  $\text{L}^{-1}$ ) was pipetted into the microplate wells. The plates were covered and shaken gently for 20 min. The plates were washed with water and immediately filled with 70  $\mu\text{L}$  (96-well plate) or 30  $\mu\text{L}$  (384-well plate) of an aqueous inhibitor solution (1.5 mg  $\text{mL}^{-1}$ ; 3.8 mmol  $\text{L}^{-1}$ ). The covered plates were shaken for 14 h, washed with water and dried in a nitrogen stream. The mod-

ified plates were stored at room temperature. The plates were stable for months.

#### Detection method

Parallel reflectometric interference spectroscopy (RIfS) enables the optical on-line detection of specific biomolecular interaction in special 96- and 384-well RIfS microplates without the need for labelling techniques. A schematic diagram of the detection and pipetting unit is shown in Fig. 1. Basic information about single-channel RIfS is available elsewhere [15, 16].

The parallel RIfS is based on the spectral distribution of reflectance from thin transparent layers on a glass substrate. The beams reflected at both sides of an interference layer (330 nm thickness) coated with a biopolymer adlayer lead to an interference pattern from which the optical thickness,  $nd$ , of this thin layer may be calculated. Changes in the optical thickness,  $\Delta nd$ , of the layer caused either by changes in the reflective index or in the physical thickness of the adlayer, e.g. because of specific binding to this surface, leads to a shift of the interference pattern which is evaluated in real-time.

The new parallel screening system was realized by a simultaneous illumination of the whole RIfS microplate glass bottom with a halogen-light source. As a certain spectral resolution of the reflected light is necessary for signal evaluation, a monochromator system was placed directly behind the light source. The monochromator consists of a filter wheel which enables the subsequent passage of monochromatic light of seven different wavelengths (516.0, 543.5, 572.3, 606.8, 643.3, 685.9, and 707.0 nm). A total turn of the filter wheel takes 18 s and determines the time resolution of the measurement. Diffusion and binding of protein to the transducer surface normally occurs much more slowly. If the number of filters can be reduced to three; this leads to better time resolution and increased signal noise.

The signal was recorded by means of a 12-bit CCD camera, EEV type CCD05–20, which traps the reflected light with the interference information. The beam illuminates half of the CCD chip. Simultaneously a reference beam was detected with the other half of the chip. This reference beam enables correction for light inhomogeneities caused by temporal intensity fluctuations of the light source and by spatial differences in illumination intensity.

An automatic well mask generation program assigns the pixels on the CCD chip to the wells of the microplate and accumulates the signals corresponding to one well. Because the well mask generation tool is very flexible, masks can be created for standard 96-, 384-, and 1536-well microplates and for individual plate formats.

From these data the software calculates the average change in optical thickness for each well and each measurement point in real time. The data are saved in common file types. Signals from the larger wells are calculated from a larger number of CCD camera pixels; this results in a reduced noise. The root-mean-square noise (RMS) is proportional to  $N^{-0.5}$ , where  $N$  is the number of illuminated CCD camera pixels.

Liquid handling was achieved by means of a modified pipetting robot IGEL 250/96 from CyBio (Jena/Germany). Samples can be transferred between four standard microplates and one RIfS microplate. The robot was modified in such a way that liquid handling was possible without lifting the RIfS microplate from the detection position. This enables continuous data recording totally independent of the pipetting steps.

#### Changes in the refractive index

To examine the effect of changes in the refractive index, water and glycerine solution (10% in water) were alternately measured in a 96-well microplate. Liquid handling was performed by means of an automated pipettor control to ensure the plate was repeatedly filled with both liquids for 600 s alternately. In this and all further experiments data accumulated when the plates contained no liquid were not used for evaluation.

#### Protein interaction

Protein adsorption was measured with the same pipetting program for 96- and 384-well microplates, with the exception of the sample volume. In the following, sample volumes are given for measurements in 96-well plates, volumes for 384-well plates are given in parentheses.

Before starting a measurement the plate was filled with PBS for at least 10 min to prevent swelling effects within the data-recording time. The plates were depleted and data recording and the pipetting program were started simultaneously. First the RIfS microplate was filled with 70  $\mu\text{L}$  (60  $\mu\text{L}$ ) PBS and the baseline was recorded for 300 s. The PBS was then removed and 70  $\mu\text{L}$  (40  $\mu\text{L}$ ) protein sample was pipetted from one of the storage microplates into the RIfS microplate. After a binding time of at least 900 s, the sample was replaced by 80  $\mu\text{L}$  (60  $\mu\text{L}$ ) hydrochloric acid to regenerate the plate. This enables re-use of the same plate for more than 100 screening runs.

#### Screening experiments and evaluation of IC 50 value

For the binding inhibition test 96 samples of 100  $\mu\text{L}$  PBS buffer solution containing thrombin (6 units  $\text{mL}^{-1}$ ) and chicken ovalbumin (500  $\mu\text{g mL}^{-1}$ ) were pre-incubated with the inhibitors in a standard polystyrene microplate. Ovalbumin was added in large excess to prevent adsorption of thrombin within the pipettes and microplates. These pre-incubated mixtures were measured with the RIfS arrangement as described above.

As the binding event is controlled by diffusion of antibodies to the surface, the calibrated signal is proportional to the concentration of free antibody in the solution at each time. After the binding event the sensor was regenerated by pipetting hydrochloric acid (pH 1.7) into the wells to prepare it for the next cycle.

For Screening experiments high, standardized inhibitor concentrations (60  $\text{ng mL}^{-1}$ ,  $1.5 \times 10^{-7} \text{ mol L}^{-1}$ ) were used beside samples without added inhibitor.

For the determination of the IC 50 value, inhibitor concentration was varied between  $4 \times 10^{-9} \text{ mol L}^{-1}$  and  $1 \times 10^{-7} \text{ mol L}^{-1}$ . Evaluation occurs by plotting the calibrated signal after a binding time of 13 min against the inhibitor concentration.

## Results and discussion

### Signal drift and noise

As this is a new arrangement for parallel screening, fundamental data analysis was performed to specify the signal noise and the temporal signal drift. Both examinations were conducted using plates with and without aminodextran coating. Measurements are performed with dry plates and with plates filled with water. Aminodextran is a hydrogel

**Table 1** Data for drift and RMS noise

| Plate type                   | RMS in air (pm)  | RMS in liquid (pm) | Drift in air ( $\text{nm h}^{-1}$ ) | Drift in liquid ( $\text{nm h}^{-1}$ ) |
|------------------------------|------------------|--------------------|-------------------------------------|----------------------------------------|
| 96-Well aminodextran         | 1.62 $\pm$ 0.003 | 19.3 $\pm$ 5.1     | 0.02 $\pm$ 0.02                     | 0.40 $\pm$ 0.26                        |
| 96-Well without aminodextran | 1.41 $\pm$ 0.003 | 23.6 $\pm$ 0.9     | 0.04 $\pm$ 0.02                     | 0.29 $\pm$ 0.52                        |

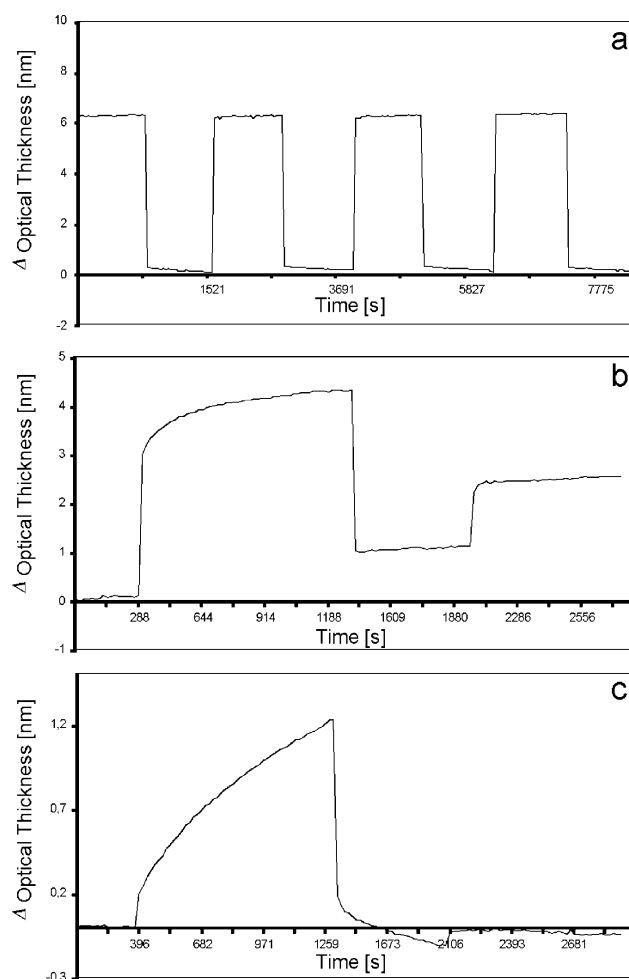


**Fig. 2** Signals arising from: (a) changes in refractive index, (b) non-specific adsorption of calf serum, and (c) specific binding of thrombin, with subsequent surface regeneration. One of 96 registered curves is given as an example

with the property of swelling in aqueous solution. To obtain results unaffected by the swelling processes the plates were equilibrated with water for at least 20 min before the start of liquid measurements. For both types of plate re-

sults are shown in Table 1. The quality of the data was not affected by the biopolymer adlayer in air or in an aqueous environment. Both drift and root-mean-square noise (RMS) were at such a low level that they do not limit detection of biomolecule adsorption.

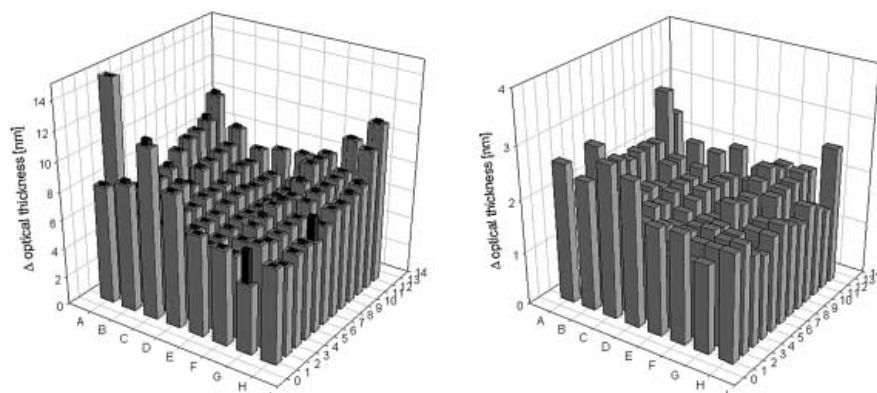
#### Effect of changes in the refractive index on sensor signals and signal calibration

The binding signal depends on the physical thickness,  $\Delta d$ , and on the changes in the refractive index,  $\Delta n$ . Measurements of liquids with different refractive indices were used to characterize the sensor signals. During data registration the liquid in the RIfS microplate was changed between glycerine solution (10% in water;  $n=1.345$ ) and water ( $n=1.333$ ). A signal curve for one well of a 96-well microplate is given in Fig. 2a. The signal for the glycerine solution is 6.3 nm higher than that for pure water. Data from repeated measurements were highly reproducible. This enables the direct comparison of sequentially recorded plate screens in HTS experiments. Signal differences arising from the change in refractive index are given in Fig. 3 for the complete 96-well plate. Error bars are plotted as above the columns. The errors result mainly from the pipetting process. From this plot it is also apparent that the responses differ from well to well. To standardise the signals calibration was undertaken for each plate and all further measurements were normalized relative to this calibration measurement. The calibrated signals for the measurements in Fig. 3a are shown in Fig. 4a. The same calibration was performed during the protein absorption measurements.

#### Specific and non-specific binding of protein

Non-specific binding was examined with calve serum, bovine albumin, and chicken ovalbumin. Because of the shield effect of the aminodextran coating, non-specific binding of bovine albumin and ovalbumin could be successfully prevented. Even at high protein concentrations of  $1 \text{ mg mL}^{-1}$  non-specific binding signal vanishes in the baseline noise. Only with calve serum at very high con-

**Fig. 3** Signal arising from changes in refractive index (left) and from non-specific adsorption of calf serum (right)



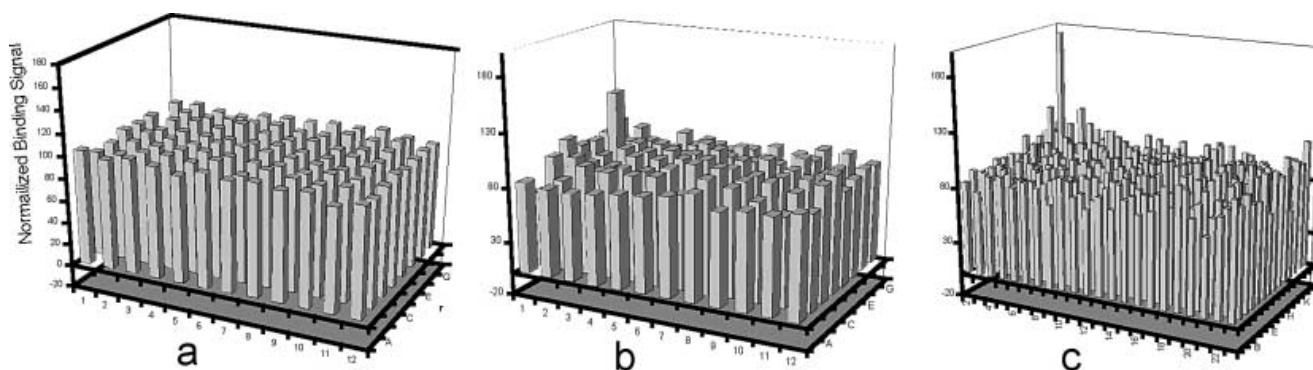


**Fig.4** Normalized signals for glycerine solution (a) and for the specific binding of thrombin in a 96-well microplate (b) and in a 384-well plate (c)

centrations (1:10 diluted in water) non-specific binding was detected. One binding curve for a 96-well plate is shown in Fig. 2b. The high protein concentration results a rapid coverage of the surface by protein absorption. Such non-specific interaction often is characterized by irreversible binding. We found, in fact, that the baseline signal in Fig. 2b could not be reached again. This showed that the non-specific interaction of calve serum could not be regenerated under acidic conditions under which specific thrombin binding could be regenerated.

For the specific binding of thrombin, the plates were covalently modified with a thrombin inhibitor. Thrombin concentration was  $6 \text{ units mL}^{-1}$ . A single binding curve for a 96-well plate is shown in Fig. 2c. The binding of thrombin could be detected with sufficient time resolution and the surface was completely regenerated with hydrochloric acid. The sensor surface was not affected by the regeneration solution and stayed stable for more than 80 measurement cycles. Because the detected signals represent changes in the optical thickness, they are independent of the size of the microplate wells and signals from 96-well plates can be compared with those from 384-well plates without further conversion. Calibrated binding curves for both types of plate are shown in Figs 4b and 4c. Each well was filled with a thrombin solution of 6 units

**Fig.5** Screening signals on 96- and 384-well plates. *Left*, non-calibrated binding curves of screening runs for thrombin inhibitors; *right*, calibrated screening data; *black*, samples containing inhibitor. For reasons of clarity, only 96 binding curves (systematically distributed on the plate) are plotted for the 384-well microplate

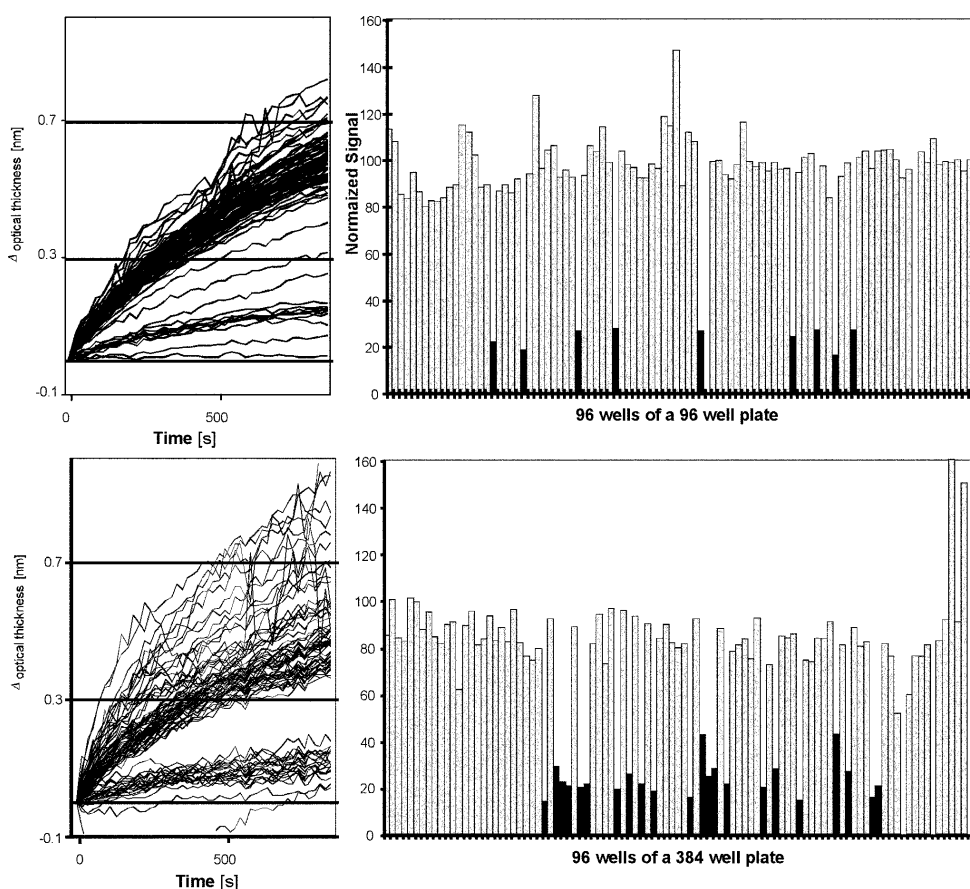


**Table 2** Screening results obtained with the thrombin–thrombin inhibitor system with different decision criteria

|                             | Re-found activity | Re-found inactivity | False negative | False positive |
|-----------------------------|-------------------|---------------------|----------------|----------------|
| 96-Well plate 60% criteria  | 9 of 9            | 85 of 87            | 0              | 2              |
| 96-Well plate 50% criteria  | 9 of 9            | 85 of 87            | 0              | 2              |
| 384-Well plate 60% criteria | 87 of 88          | 288 of 296          | 1              | 10             |
| 384-Well plate 50% criteria | 86 of 88          | 291 of 296          | 2              | 6              |

mL<sup>-1</sup>. With few exceptions the signals are very homogeneous for both types of plate and enable well to well comparison. The system is, therefore, suitable for binding-inhibition high-throughput screening assays.

The better results from the 96 plates are a consequence of the lower signal noise, because of the greater well area. Signal derivation results mainly from the manual surface modification chemistry and might be prevented by an automated preparation process; this is recommended when many plates are needed for screening of whole substance libraries.

### Screening experiments

To show the suitability of the parallel RfS system thrombin binding was examined for an unknown number of samples spiked with thrombin inhibitors. The results of this screening are shown in Fig. 5. For the 384-well plate signals for every fourth microplate position are shown. Samples without inhibitor give binding signals of more than 0.3 nm whereas samples with inhibitor added resulted in signals <0.2 nm. For most of the positions, the inhibited samples could be re-found even without a calibration step. The few curves which resulted in signals which could not be attributed with certainty could be assigned after performing the standard calibration presented above. These results are shown in Fig. 5 as bar chart. The samples with additional thrombin inhibitor are plotted in black. For samples without inhibitors binding signals of 100% were expected whereas inhibited thrombin samples should result in obviously distinguishable smaller signals. For both plate types, nearly all of the pure thrombin samples gave binding signals of 80–120% whereas the samples incubated with inhibitors mostly gave signals of <40%. Re-finding rates are given in Table 2. To identify a hit, two criteria of 40% and 50% signal inhibition were stipulated. Except for two false-positive predictions, all samples are classified correctly on the 96-well plate – in particular all the inhibitor samples were identified as hits, which means that no false negative hits were found. This is of primary importance, because each false negative hit leads to the loss of a potential drug candidate. False positive hits in the 96-well plates occurred in wells in which surface damage as a result of the manual surface coating had previously been detected. In summary, the 96-well plate gave absolutely stable and reliable results for the inhibitor screening.

The smaller well-size of the 384-well plates resulted in a higher signal noise but did not affect either the absolute

binding signal or the calibrated binding signal, as is apparent from Fig. 5. Depending on the criteria for hit identification 86 (50% criteria) or 87 (86% criteria) of the 88 inhibitor samples were identified on the 384-well plate. As mentioned above, false negative hits are most problematic in drug screening. The higher number of false negative hits should, therefore, be accepted and the more generous 60% criterion should be used so that as few hits as possible are missed.

In addition to the experiments with the thrombostop-analogous thrombin inhibitors, three other inhibitors of unknown structure were examined; the results confirmed the high assay stability. Screening was also performed with 5% DMSO added to the samples. No influence of DMSO was detected. Because library substances are normally stored in DMSO, stability of the assay against DMSO is essential even when a stabilisation of the assay compounds is not necessary.

### Evaluation of IC 50 value

In addition to the determination of hit compounds, quantitative results such as IC<sub>50</sub> value or binding constants of drug candidates are of interest. The stability and reliability of binding signals with the parallel RfS-screening system enable such examination. A binding inhibition test was performed to determine the IC<sub>50</sub> value of the thrombostop-analogous thrombin inhibitor (Fig. 6). In this assay

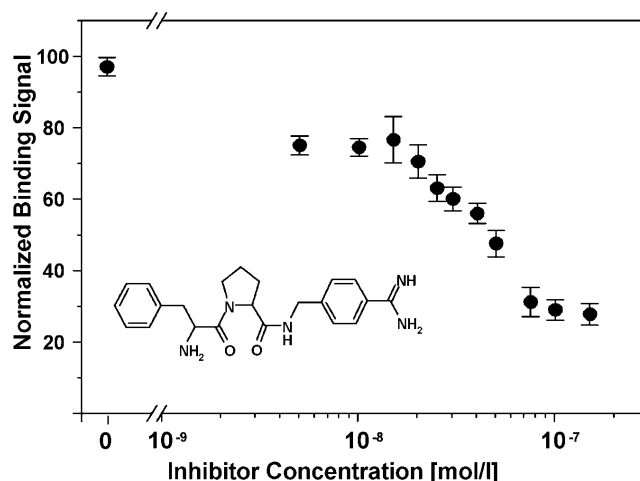


**Fig. 6** Determination of the IC<sub>50</sub> value of a thrombin inhibitor by simultaneous detection of 96 different samples within less than 30 min. *Inset*, the structure of the thrombostop analogue inhibitor

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the IC 50 value represents the concentration at which half of the original binding signal was obtained. Thrombin samples ( $20 \mu\text{g mL}^{-1}$ ) were incubated with inhibitor at concentrations of  $2 \times 10^{-9} \text{ mol L}^{-1}$  to  $2 \times 10^{-7} \text{ mol L}^{-1}$ . Each sample concentration was detected eightfold and simultaneously in one 96 RfS microplate. After data calibration, an IC 50 value of  $50 \text{ nmol L}^{-1}$  could be determined from the binding inhibition curve for this thrombin concentration (Fig. 6).

## Conclusion

We have reported the development of a new binding assay to screen for thrombin inhibitors with HTS methods. We used a new, parallel, label-free detection method based on the RfS technique. With label-free optical methods it is not necessary to wait for equilibrium binding to determine the binding characteristic [17]. This enables shorter assay times. Although we used binding times of 900 s, noticeable and evaluable signals are available after <250 s.

The quality of screening data for 96- and 384-well plates are excellent and promise successful further parallelisation on denser plates, e.g. 1536-well plates. The imaging system based on a CCD camera is not restricted to a certain plate density and enables much denser plates with marginal changes in the evaluation software. Because the signal does not depend on the well area and the noise can be reduced by use of a high pixel-to-well ratio in the CCD camera, even a chip-based arrangement can be realised. The throughput of the system presented is high enough to fulfill the specification of HTS and even ultra-HTS which demands screening of 100,000 samples per day [18].

Limitations of this new approach are the low sensitivity and therefore the restriction on sample molecules with a high molecular mass, e.g. enzymes or receptors. Small molecules, which are of main interest in drug discovery, can be examined with an indirect binding-inhibition test.

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