DNA-intercalation on pyrene modified surface coatings

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Utilising the strong affinity between nucleic acids and an intercalating pyrene derivate, a novel efficient method for unspecific immobilisation of double-stranded DNA on to solid support for applications in bioanalytic, biophysics and microbiology is presented.

Considerable effort has been made to investigate methods and techniques to immobilise and attach nucleic acid probes to various support or substrate materials for the usage in biomedical and biophysics studies.[1–4] Short oligonucleotides are commonly attached covalently by their modified extremities, whereas for long oligos and double-stranded DNA non-covalent immobilisation methods are preferred.[3,5,6] Usual hydrophobic or electrostatic interactions between substrate and nucleic acid have been used to bind DNA non-covalently, but they are susceptible to DNA removal from the surface by pH, salt concentration and temperature changes.[5,7]

In this study we report a novel efficient method for a unspecific binding of double-stranded DNA (dsDNA) on to support matrices. Thereby the non-covalent binding properties between nucleic acids and a surface-tethered intercalator are adopted for DNA immobilisation. By insertion of a positive-charged, planar ring-system between two base pairs intercalating substances can bound to nucleic acids with very high affinity constants and resistance against competitor salt concentrations.[8–13] Häfliger *et al.* synthesised an amine-modified pyrene derivate that intercalates DNA with a stability constant of 2.7×10^6 M^{-1} (Fig. 1, compound **A**).[14] Aminopyrene can be covalently immobilized on solid substrates by molecular tethers.[15,16] Here, the derivate was attached covalently by its terminal NH_2-group to a 3-aminopropyltrimethoxysilane-coated glass slide plus 1,4-phenylenediisothiocyanate (Fig. 1). Therefore a spotting solution (Genorama, Asper Biotech, Estonia) containing an appropriate concentration of compound **A** was reacted on Genorama SAL slides (Asper Biotech) for 3 h and exchanged against a 1% ammonia solution (Suprapur, Merck, Germany) for 30 min to block extant functional surface-groups. After fixation of the intercalator, the BOC (*tert*-butoxycarbonyl) protecting groups of the derivate were removed by CF_3COOH–CH_2Cl_2 (1:1) treatment for 3 h. As reference, slides without pyrene derivate were also prepared and both kind of slides, modified and unmodified, were washed several times with bi-distilled water and stored under vacuum until usage. Owing to the fact that the absorption spectra of the pyrene derivate shows a specific shape at higher wavelength than the UV-permeability for glass,[14] successful surface binding of the intercalator was checked by UV/Vis spectroscopy.

Especially when minute amounts of material need to be analysed, fluorescence detection is often the method of choice to measure the interaction between a surface bound receptor and a ligand. Therefore the successful attachment of Cy5-labelled, 2 kb (kilo-base-pair) long dsDNA fragments on to the pyrene modified coating was tested with an optical biosensor, that has been proven for highly selective and real-time fluorescence measurements at water/glass interfaces.[17–20] Supercritical angle fluorescence (SAF) biosensors collect only fluorescence emitted in to the angular region above the critical angle of refraction, thereby the detection volume in to the aqueous probe is rigorously reduced and bulk fluorescence is rejected for the most part. With this microscopy technique the surface confinement is even more efficient than achieved with common biosensors based on total-internal-reflection fluorescence (TIRF).[19,21]

Fig. 2 shows the immediate increase of surface-generated fluorescence in real-time, after addition of 150 µl of 10^{-10} M Cy5-labelled DNA fragments in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.1) on to the coated glass slide. For better demonstration in Fig. 2 the background obtained by the pure buffer solution was subtracted from the data and start time was set to zero.

Addition of dye-labelled dsDNA to the reference support caused a rapid increase of the fluorescence to 16 ± 2 kHz and the signal remained at this level afterwards. This count rate can be attributed to non-specific intercalation of the Cy5-labelled DNA

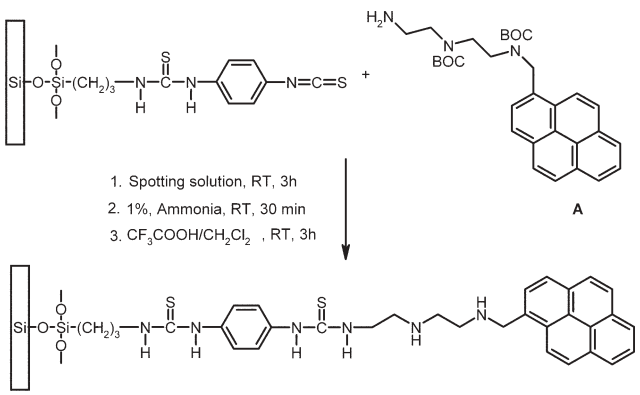

Fig. 1 Reaction scheme for the coupling of amine modified pyrene **A** to a coverslip coated with 3-aminopropyltrimethoxysilane plus 1,4-phenylenediisothiocyanate.

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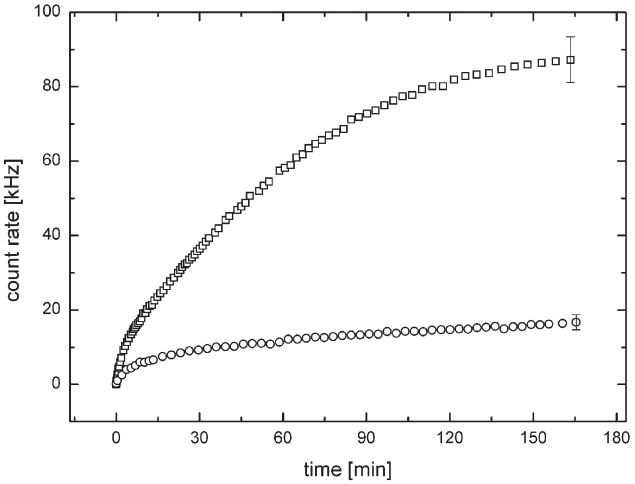

Fig. 2 Binding curves of 10^{-10} M Cy5-labelled dsDNA (2 kb) on pyrene modified SAL surface (squares) and on unmodified SAL surface (circles). The plots give the average fluorescence intensity of three independent experiments minus background count rate.

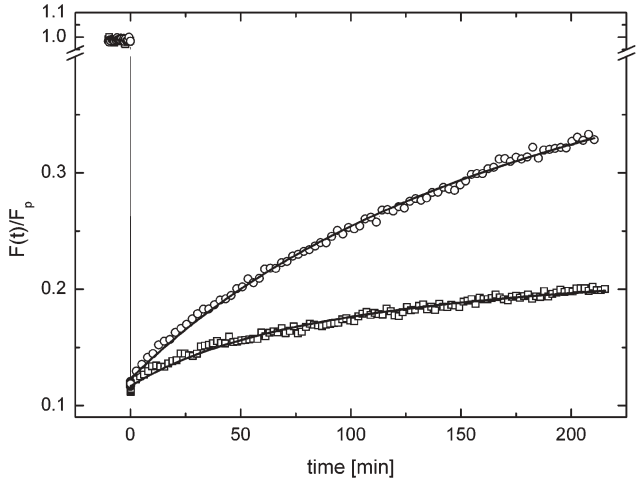

Fig. 3 Fluorescence recovery after photobleaching of Cy5-labelled dsDNA fragments on pyrene modified SAL (squares) and on unmodified SAL surface (circles). Measured fluorescence intensity is plotted as a function of time $F(t)$ relative to the prebleach fluorescence F_p (data points) and the data were fit to eqn (1) (lines).

fragments with the unmodified SAL surface. Addition of the probe solution to the pyrene-modified surface leads to a substantial increase in the fluorescence signal of more than 80 ± 7 kHz (Fig. 2). To remove electrostatically bound fragments from the surface, both slides were rinsed rigorously with TE-buffer for several minutes and the surface-generated fluorescence was detected again by the SAF-biosensor. The average count rate of six detection spots on the slides show a only 15% weaker signal for the pyrene-surface and about 70% weaker signal for the reference coating after removal of unspecific bound components. Thus, a difference of about one order of magnitude between both coatings indicates a fundamentally stronger DNA-binding at the pyrene modified surface. At the described reaction conditions the pyrene derivate carries two positively charged amines, which can electrostatically increase the interaction to the negatively charged DNA phosphate backbone. However, reducing the positive charge of the surface by protecting the NH_2 groups results in similar fluorescence augmentation, so electrostatic interaction can not be responsible for the strong DNA affinity of the surface.

A more detailed study of surface binding mode was done by fluorescence recovery after photobleaching (FRAP, also known as fluorescence photobleaching recovery FPR). Thereby fluorescent molecules in a region of interest are first bleached by a laser beam and afterwards the fluorescence increase from the same region is monitored by the same laser in an attenuated form. Adopted from diffusive studies of molecules in living cells, the technique can be used to estimate the exchange rates of bleached and unbleached molecules on a surface area.[22–25] For the FRAP experiments both slides were treated with 10^{-7} M Cy5-labelled DNA solution and the coupling was checked after 6 h. The unattenuated laser of the SAF-biosensor was used to illuminate the detection area for a specific time and post-bleaching fluorescence changes were obtained immediately after the high-intensity illumination. The fluorescence of the bound Cy5-DNA dropped to 10% of the maximum signal and after photobleaching the surface-generated fluorescence increased owing to the diffusion of unbleached molecules in to the illuminated region (Fig. 3). Kwon *et al.* have presented a useful empirical equation that can be directly fitted to FRAP data:[24]

$$F(t) = F_0 + \frac{(F_\infty - F_0)t}{t + \tau_{1/2}} \quad (1)$$

where t is the time after bleaching, $F(t)$ is the fluorescence as a function of t, F_0 is the fluorescence immediately after bleaching, F_∞ is the fluorescence at infinitive time, and $\tau_{1/2}$ is the time for half-maximal recovery. Typically the fluorescence does not recover to its initial pre-bleach fluorescence intensity F_p in FRAP measurements owing to the presence of a immobile population of fluorophores.[23] Therefore the fractional recovery of fluorescence was calculated from the expression $(F_\infty - F_0)/(F_p - F_0)$ as 50 ± 5% on unmodified SAL slides and of only 11 ± 2% on the modified coatings. The time for half-maximal recovery, that is indirectly proportional to the diffusion constant, was estimated as 231 ± 10 min for the unmodified and 104 ± 2 min for the modified surface, and so molecule movements are similar on both surfaces.

Recovery of surface-generated fluorescence only takes place when photo-bleached molecules desorb from the surface and non-bleached fluorophores absorb at the surface. The same molecule mobility on both surfaces indicates the exchange of electrostatic Cy5-labelled DNA fragments, but a lower fractional recovery of fluorescence at the pyrene surface consequently points to a lower amount of electrostatic-bound molecules. Thus, the majority of the DNA-fragments tethers with a higher affinity at the pyrene-modified slides than obtained from electrostatic interactions. Due to the strong intercalating properties of the modified pyrene, we assume an intercalation binding between the surface-tethered pyrene derivate and Cy5-labelled dsDNA fragments.

The presented study clearly demonstrates the improved binding efficiency of unmodified DNA fragments to a surface induced by an immobilized pyrene intercalator. Intercalation is unspecific, but with stability constants of about 10^6 M^{-1} strong enough to bind DNA effectively. There is no need to modify the DNA, so that this

method could be a very sensitive application in different types of DNA and intercalation studies in biophysics and bioanalytics.

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