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REVIEW

Sensitive bioanalysis—combining single-molecule spectroscopy with mono-labeled self-quenching probes

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Abstract Fluorescence single-molecule spectroscopy is an appropriate tool for modern bioanalysis. This technique enables the development of ultra sensitive assays, especially when combined with self-quenching probes. In this review we report novel DNA, enzyme, and antibody assays based on mono-labeled fluorescent probes that are quenched by photoinduced electron transfer (PET).

Keywords Single-molecule spectroscopy · Photoinduced electron transfer (PET) · Self-quenching probes · Ultra-sensitive bioanalysis

Introduction

Modern medical diagnostics without bioanalytical methods could not be imagined. Efforts to develop more sensitive methods for detection of biomolecules such as DNA or enzymes have been rapidly increasing for the last decade. Since their development in the late 1980s, single-molecule fluorescence spectroscopic (SMS) techniques have proved to be ideal tools for accurate and sensitive detection of biomolecules. With SMS one can detect single fluorescent molecules in solution and on surfaces. The substantial progress made in this field has been described in recent reviews [1–3].

A confocal microscope is usually used for investigation of fluorescent single molecules in solution. The detection volume is defined by the laser focus and a small pinhole. In most experiments the concentration of the investigated molecules is so low that, on average, much less than one molecule is located in the detection volume at a time. Consequently, each detected photon burst arises from a single molecule passing the detection volume. The necessarily low concentration of the probe molecules can also be disadvantageous. For example, higher concentrations of DNA probes would lead to more efficient hybridization but would also result in a higher background, precluding observation of individual molecules. Diffusion of each single molecule through the detection window limits its observation time to the order of several milliseconds. Immobilization of molecules on a surface enables investigations on a longer timescale with a similar microscope arrangement. A single molecule can be located directly by scanning the surface in the laser focus and the fluorescence of this particular molecule can be recorded as a function of time until the fluorophore is bleached; this enables observation times of several seconds and even minutes if quantum dots are used instead of conventional fluorescent dyes. It is also possible to attach two chromophores forming a fluorescence resonance energy transfer (FRET) pair to a molecule [4]. Monitoring the FRET efficiency enables, for example, investigation of the conformational dynamics and cleavage mechanisms of individual enzymes (staphylococcal nuclease) [5]. As a complement to the confocal single-molecule arrangement, other systems, for example total internal reflection fluorescence microscopy (TIRFM), can illuminate a larger surface area to detect many individual molecules simultaneously, or follow the

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movement of a single dye-labeled molecule using sensitive CCD camera-based imaging systems [6]. In sensitive bioanalysis, fluorescence correlation spectroscopy (FCS) was one of the first approaches to combine confocal microscopy with single-photon sensitive detectors to develop very sensitive assays of biomolecules. In this method fluctuations in detected fluorescence intensity are analyzed to characterize samples. In contrast with other SMS techniques, several molecules are in the detection volume during the measurement. In bioanalytical assays FCS exploits differences in size and thus in diffusion velocity to identify molecules, thus only mono-labeled probes that bind to the target molecule, e.g. DNA or proteins, are required to detect bioanalytes. To clearly distinguish between molecules a tenfold size differential, at least, is necessary. Detection sensitivity is further limited because a relatively large amount of the fluorescently labeled probes must be bound to the target molecule to enable detection of a change against a large background signal of the free probes. Conventional FCS usually affords sensitivity to a nanomolar concentration of the target molecule [7].

The challenge of detecting lower concentrations of analyte is posed in the identification of only a small fraction of bound probes in a huge excess of free probes. If the target molecule (e.g. DNA) can bind two probe molecules, it is possible to use two different probes labeled with different dyes, which can be detected individually. If both probes are bound to the target DNA, they pass the detection volume simultaneously. These signals can be separated, with high accuracy, from signals that originate from free probe molecules diffusing through the laser focus [8, 9]. Another possible assay is to design the two probes in a way that they form a FRET system after hybridizing to the target DNA [10]. A high concentration of free fluorescent probes raises the background signal, however, so several efforts have been made to develop self-quenching systems that generate fluorescent light only after binding target molecules. The most popular quenching systems are the so-called molecular beacons developed in 1996 [11]. These are hairpin-structured oligonucleotides labeled at the 5′ end with a fluorescent dye and at the 3′ end with a quencher molecule, for example DABCYL. When the hairpin is closed both molecules are in juxtaposed proximity and the fluorescence is quenched. On hybridization to the target DNA the hairpin probe is opened and the fluorescence intensity increases.

Most quenching assays used for DNA, protein or enzyme detection are based on probes or substrates labeled with a fluorescent dye and a quencher molecule or two chromophores, forming a FRET system. In this brief review we focus on novel self-quenching probes for bioanalytical applications that contain only one fluorophore and are suitable for single-molecule experiments. In this method the fluorescence quenching is achieved not by tagging the probe with a second fluorophore or quenching molecule but rather by natural quenchers, for example guanine or tryptophan residues, via photoinduced electron transfer (PET).

Fluorescence quenching via photoinduced electron transfer

The property that some fluorophores can be quenched by different synthetic quenchers (e.g. DABCYL, Black Hole Quencher) or natural biomolecules (e.g. guanosine, tryptophan) can be utilized in single-molecule spectroscopy. Very often the quenching is caused by a dipole–dipole interaction between the chromophore and the quencher, which can be another chromophore. This phenomenon, fluorescence resonance energy transfer (FRET), first described by Förster in 1948 [12], is often used in single-molecule spectroscopy for investigation of conformational changes in biomolecules, e.g. proteins or DNA [13–15], because the FRET efficiency depends on the distance between the chromophores over a 10-Å to 100-Å range.

Other possibilities for efficient fluorescence quenching, e.g. the formation of non-fluorescent H-type dye dimers, are based on electronic interactions and require contact to occur. In this review we focus on the photoinduced electron transfer (PET) that can occur between the first excited singlet-state of a fluorophore and the ground-state of a quenching molecule. Quenching of fluorophores in the first excited singlet state by electron donors, for example tryptophan or guanosine, usually leads to formation of a radical ion pair $A^{-\bullet}/D^{+\bullet}$, which returns to the ground state via radiationless charge recombination. The PET efficiency depends on the free energy of the reaction, the reorganization energy, and the distance between fluorophore and quencher [16]. To estimate the free energy change for the charge separation (ΔG_{cs}), which must be negative for efficient quenching via PET, Weller's equation can be used [17].

$$\Delta G_{cs} = E_{ox} - E_{red} - E_{0,0} + C$$

where E_{ox} and E_{red} are the first one-electron oxidation potential of the donor and the first one-electron reduction potential of the acceptor in the solvent under consideration. $E_{0,0}$ is the energy of the zero–zero transition to the lowest excited singlet state of the electron acceptor (fluorophore) and C is the solvent-dependent Coulombic attraction energy, which is small in polar environments and can be usually neglected if aqueous solvents are used. For example, the charge separation in the complex of the excited fluorescent dye MR121 (E_{red}=−0.5 V relative to the SCE (standard calomel electrode); $E_{0,0}$=1.9 eV) [18] with guanosine or tryptophan is estimated to be −0.2 eV and −0.5 eV, respectively.

Because PET is based on contact formation, only small distances (~0.4 nm, van der Waals contact) between dye and quencher are needed. PET reactions between the natural nucleobase guanine and a fluorescent dye have been reported for coumarin [19], rhodamine [20–22], oxazine [23, 24], and bora-diaza-indacene [25] dyes. This property makes these dyes ideal for analytical applications, for example specific detection of DNA or RNA at the single-molecule level [23, 26]. It has been known since 1977 that the fluorescence intensity of fluorescein can be quenched by tryptophan [27]. Tryptophan has an oxidation potential of ~1.00 V relative to the NHE (normal hydrogen electrode) at pH 7 [28, 29] and is, therefore, a better electron donor than guanine, with an oxidation potential of 1.25 V relative to the NHE [19]. The red-absorbing oxazine MR121, ATTO 655, and ATTO 680, which are also supposed oxazine derivatives [30], are very well quenched by tryptophan, whereas all other amino acids have no quenching effect [31]. The quenching mechanism is mainly caused by the formation of weak or non-fluorescent ground-state complexes, i.e. they are statically quenched, with association constants, K_{ass}, from 96–206 mol^{-1} L. Fluorescein, rhodamine, and bora-diaza-indacene derivatives are also often quenched by the amino acid tyrosine, mainly by a dynamic (collisional) quenching mechanism. Quenching by the amino acid tyrosine has been successfully used for probing the conformational dynamics of individual proteins on the angstrom-scale [32].

Detecting biomolecules at the single-molecule level

With SMS techniques it is possible to rapidly and directly measure low concentrations of biomolecules without the need for separation or target amplification. These methods are, therefore, especially attractive for qualitative and quantitative analysis of biomolecules. We will describe three examples of how mono-labeled probes can be used in combination with single-molecule spectroscopy to assay DNA, antigens, and proteases. The fluorescence of these single dye-labeled probes is quenched by either an intrinsic guanosine or a tryptophan residue. The fluorescence quenching is caused by a photoinduced electron-transfer reaction that occurs if dye and quencher are in close proximity because of the conformation of the probe. On interacting (e.g. hybridization, cleavage) with the target molecule the fluorescence intensity increases significantly as the dye is separated from the quencher. For development of a successful assay, the interaction and, therefore, the fluorescence increase, should last for some milliseconds, at least, which is the time the complex needs to pass the detection volume.

Highly sensitive detection of specific DNA sequences using self-quenching probes

In 1996 Tyagi and Kramer described the first hairpin-structured self-quenching DNA probes, the so-called molecular beacons [11]. These probes are based on oligonucleotides labeled with a fluorescent dye and a quencher molecule. The last 4–6 nucleic bases at each end of the oligonucleotide are complementary to each other. Thus the probe forms a hairpin-structure in which the fluorophore and the quencher molecule are in close proximity, resulting in efficient quenching of the fluorescence intensity. The loop sequence of the hairpin-structured probe is complementary to that of the target DNA. If hybridized to the target, a rigid double helix is formed and the hairpin is opened. Thus, dye and quencher are separated and the fluorescence intensity increases. For the first molecular beacons, EDANS and DABCYL were used as the fluorescent dye and quencher, respectively. Since then many different pairs of dye and quencher molecules have been implemented. The fluores-

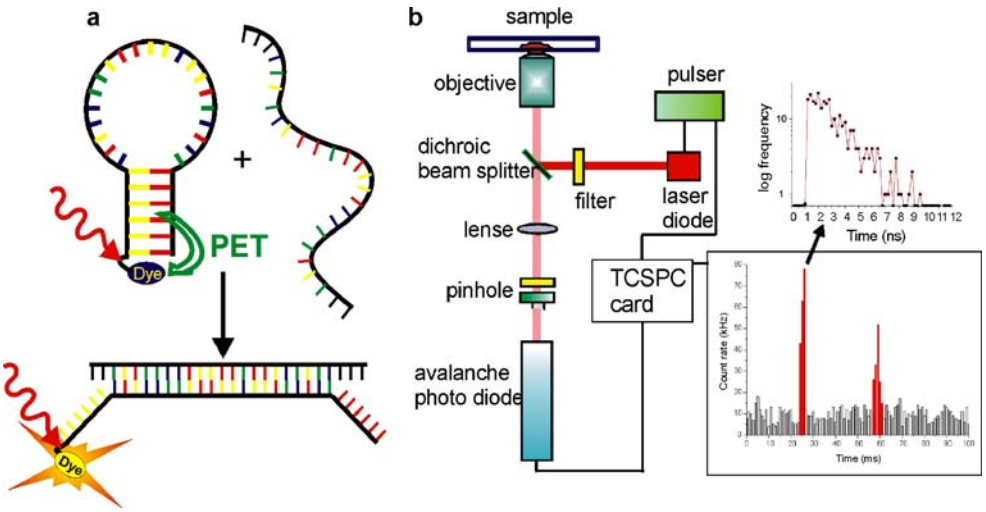

Fig. 1 (**a**) Working mechanism of smart probes. The probe oligonucleotide is labeled at one end with a fluorescent dye (e.g. the oxazine derivative MR121) and contains four to six guanosine residues at the opposite end. Because of hairpin formation, dye and guanosine residues are in close proximity and fluorescence is quenched via a photoinduced electron-transfer reaction. On hybridization to the matching target sequence the spatial contact is lost and the fluorescence intensity increases. (**b**) Confocal microscope arrangement for time-resolved single-molecule spectroscopy

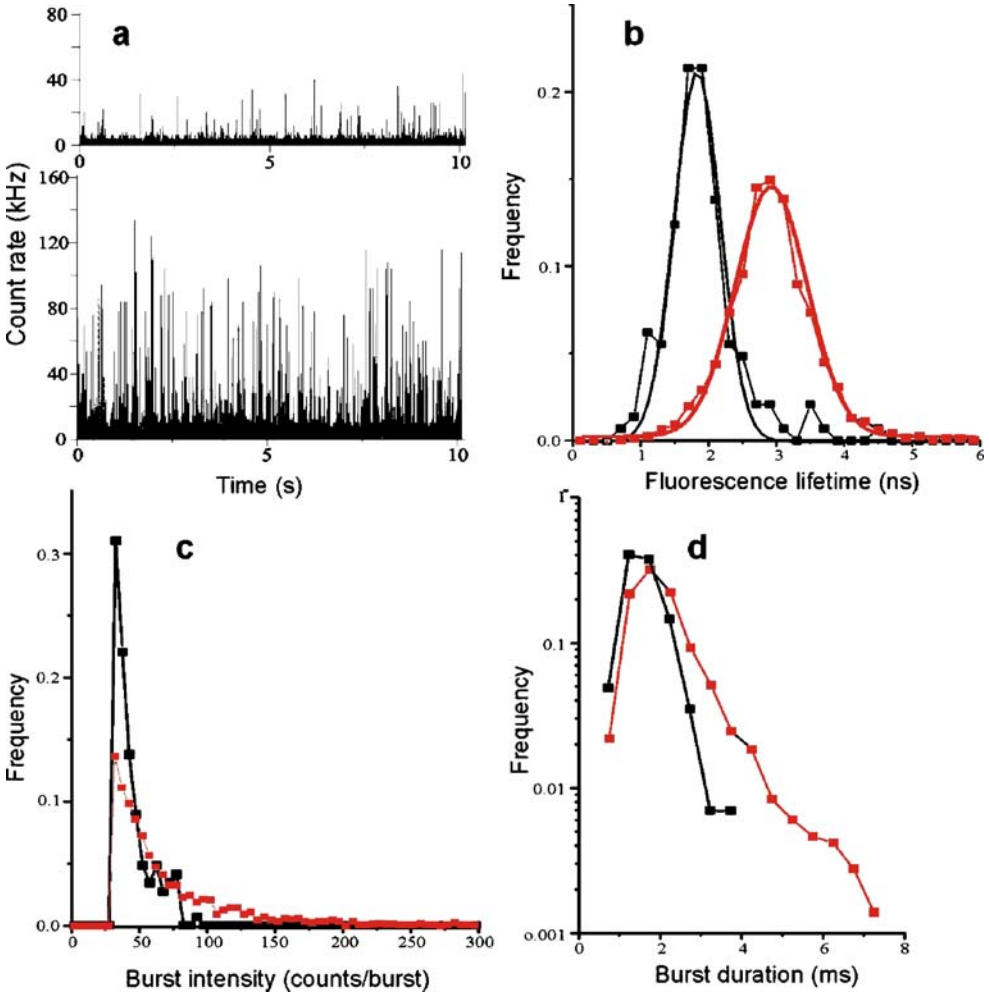

Fig. 2 (**a**) Fluorescence signals obtained from a 5×10^{-10} mol L^{-1} solution of smart probes in the absence (*top*) and presence (*bottom*) of 10^{-9} mol L^{-1} of the respective target DNA. (**b**) Fluorescence lifetime distribution, (**c**) normalized burst size, and (**d**) burst duration of the closed smart probe (*black*) and of the hybridized probe (*red*)

cence quenching is because of FRET or electronic interactions between the two chromophores. Although these probes are well established, and used for routine analysis, for example online monitoring of PCR (polymerase chain reaction) or for single nucleotide polymorphism (SNP) analysis, molecular beacons have (rarely) been used in combination with single-molecule spectroscopy [33, 34]. We recently used two identical fluorophores to design hairpin-structured probes in which the fluorescence quenching is achieved by formation of H-type dye dimers. As excitation theory predicts [35, 36], this type of dimer has no significant fluorescence. The major advantage of these probes is their simple and efficient one-step synthesis.

Fluorescent dyes with a high aggregation tendency (e.g. TMR, DY-636) [37] are usually very hydrophobic, which limits their use in single-molecule spectroscopy, especially in homogeneous solutions. We synthesized a DNA probe from the red-absorbing oxazine derivative MR121 that also forms

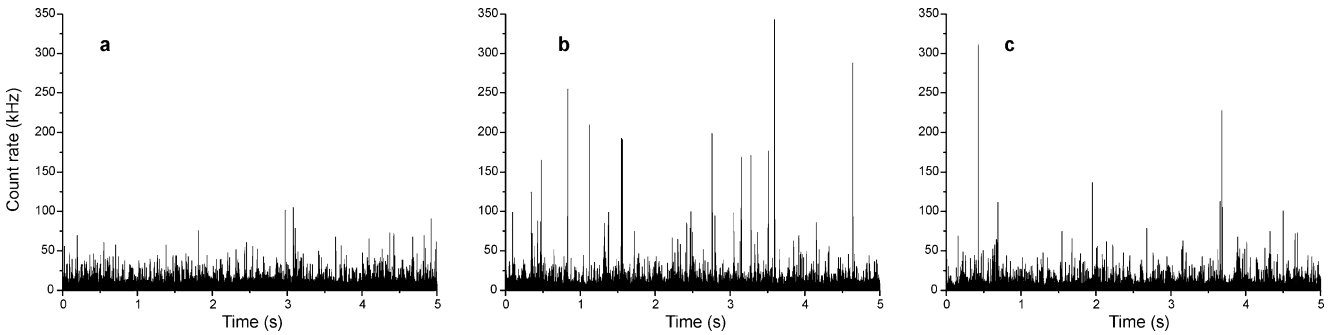

Fig. 3 Fluorescence traces obtained from a 5×10^{-10} mol L^{-1} solution of the smart probe-M before (**a**) and after addition of a tenfold excess of the matching PCR-M (**b**) and PCR-WT (**c**) products

nonfluorescent dye dimers [38] but is less hydrophobic than the aforementioned dyes. This DNA probe can be used to distinguish between the DNA of two different mycobacteria and can be used in single-molecule techniques [39].

We have also shown that hairpin-structured probes with a single dye label can be used, if the fluorescence quenching of the natural guanosine residue is exploited [23, 24, 26]. These so-called smart probes are usually labeled at the 5′ end with a fluorophore (e.g. oxazine and rhodamine derivatives) that can be efficiently quenched by several guanine residues located on the 3′ end of the probe (Fig. 1). Depending on the sequence (e.g. length of the stem and the loop), relative fluorescence quantum yields from 0.1–0.4 were observed for these probes. The fluorescence decay time is also substantially reduced. For example, MR121-labeled smart probes have monoexponential decay times of approximately 3 ns if they are hybridized to a target DNA whereas lifetimes of 1 to 2 ns were measured when the hairpins are closed. This corresponds to a partly static and a partly dynamic quenching mechanism. Static quenching means that dye and guanosine form nonfluorescent or only weakly fluorescent complexes with fluorescence lifetimes that cannot be measured with our equipment (<0.1 ns). Dynamic quenching is because of collisions between quencher and fluorophore while it is in the excited state, resulting in a shorter fluorescence lifetime.

In 2000 it was demonstrated, for the first time, that smart probes can be used with confocal time-resolved fluorescence single-molecule spectroscopy for ultra-sensitive detection of DNA sequences in homogeneous solutions [23]. In ensemble measurements averaged fluorescence intensity and lifetime only can be measured. For such measurements with standard fluorescence spectrometers a concentration of 10 to 100 nmol L^{-1} is needed to obtain a fluorescence signal. In the experiment with this particular smart probe we used a 50 nmol L^{-1} concentration and achieved a detection limit of 2×10^{-7} mol L^{-1} target DNA. An approximately tenfold excess of the target DNA is necessary to open 50% of the smart probes, because the hairpin probe is more stable (T_{melt}=65 °C) than the probe–target complex (T_{melt}=55 °C). Other smart probes with different sequences can open more efficiently. At least 10% of the smart probes must be opened by a matching target sequence to effect a significant increase of the fluorescence intensity, however, so the detection limit is usually in the nanomolar range.

The arrangement used for the single-molecule experiments is shown in Fig. 1b. A pulsed laser diode with a wavelength of 635 nm and a repetition rate of 80 MHz is used as an excitation source. The laser beam is coupled to an oil-immersion objective and focused in the sample solution. Fluorescence light emitted by the sample is collected by the same objective focused through a 100-μm pinhole and detected by an avalanche photodiode. Because of the small detection volume and the low concentration of probe, less than one probe molecule at a time is located in the detection volume and thus the fluorescence light (burst) of individual smart probes passing the laser focus volume can be

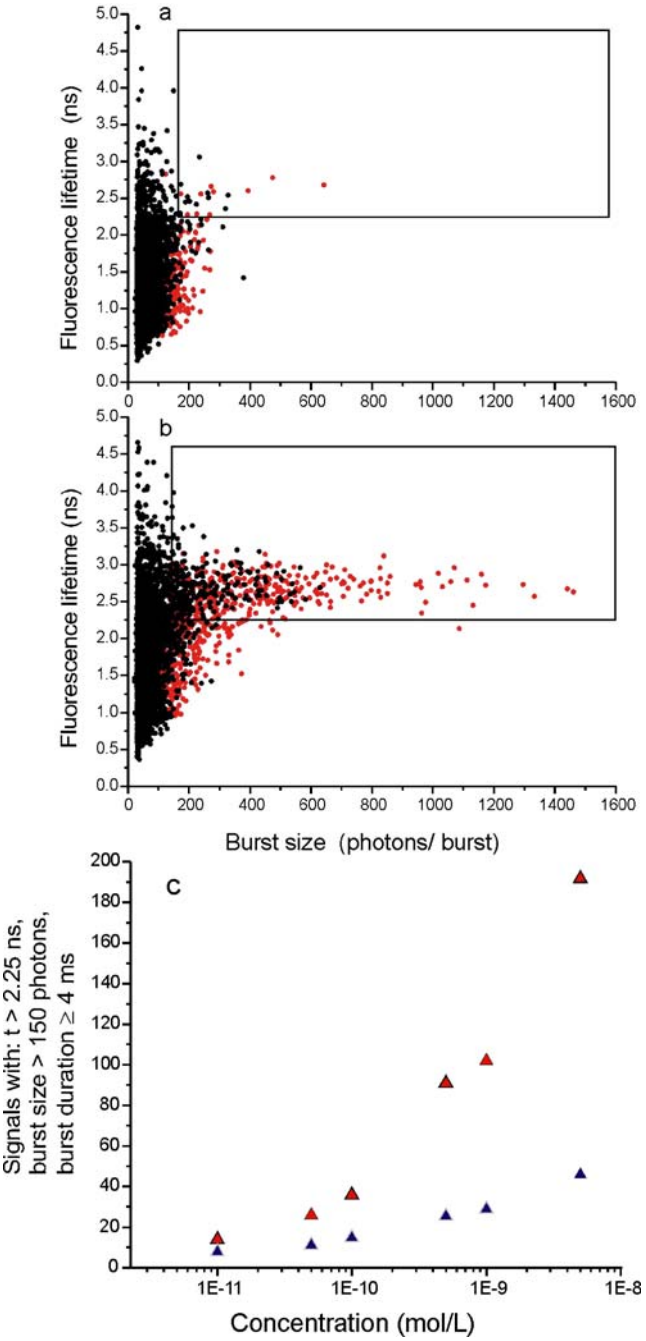

Fig. 4 Fluorescence lifetime of each burst obtained from a 5×10^{-10} mol L^{-1} solution of the smart probe-M (**a**) before and (**b**) after addition of 2×10^{-8} mol L^{-1} of the matching PCR-product is plotted against the respective burst intensity. Signals with a burst duration >3 ms are plotted *red*. (**c**) Dependence on the concentration of the added PCR-M (*red*) or PCR-WT (*blue*) of the number of signals with a burst size >150 photons, a fluorescence lifetime >2.25 ns, and a burst duration >3 ms for a 5×10^{-10} mol L^{-1} solution of the smart probe-M

analyzed. The count rate (fluorescence intensity), fluorescence lifetime, and burst duration can thus be determined for single probes. These properties differ substantially for closed and smart probes hybridized to the target DNA (Fig. 2). Whether the passing smart probe is closed or hybridized to the target DNA can be determined very accurately by invoking all these single-molecule specific properties. This multiparameter analysis enables identification of target DNA-bound smart probes in the presence of a huge excess of free fluorescently labeled DNA probes. The detection limit of this method is in the picomolar range; it is, therefore, 3 to 4 orders of magnitude more sensitive than the respective ensemble experiment.

Smart probes have been used to distinguish between DNA of different mycobacteria [40] and for identification of a single nucleotide polymorphism (SNP) in the DNA of *Mycobacterium tuberculosis* [41]. Exchange of a specific single nucleotide is responsible for the rifampicin resistance of this bacterium. Rifampicin is one of the most common antibiotics for treatment of tuberculosis. The experiments were performed with PCR products obtained from this mycobacteria. This particular SNP of *M. tuberculosis* is difficult to access by use of the smart probe, because even the single-stranded PCR product (after denaturing and crushing on ice) forms many secondary structures. This problem was overcome by using additional unlabeled ("cold") oligonucleotides that hybridize to the PCR product and partly break up the secondary structure. Smart probe experiments with standard fluorescence spectrometers and 10–100 nmol L^{-1} PCR product lead to poor results, however, whereas single-molecule detection is suitable for analysis of these PCR products even at subnanomolar concentrations [41].

Figure 3 shows the fluorescence signals obtained from a 5×10^{-10} mol L^{-1} solution of the smart probe-M (complementary to the mutant and thus the resistant form of the bacterium) in the absence and presence of the matching PCR-product (PCR-M) at a concentration of 5×10^{-9} mol L^{-1} (Figs. 3a,b). It is apparent that the number of bursts and their intensities are substantially enhanced. As a control experiment the PCR product of the wild type (PCR-WT), which differs from (b) in one nucleotide (A–T replacement), was added to the smart probe (Fig. 3c). Further burst analysis shows that not only burst intensity but also the fluorescence lifetime and the burst duration increase. The burst duration is important in comparison with experiments with short artificial target sequences. Because of the high mass of the PCR product, the diffusion time of the smart probe becomes 3–4 times longer upon hybridization to the PCR strand. Accurate discrimination between both PCR products can be achieved by counting all fluorescence bursts with >150 photons, a fluorescence lifetime >2.25 ns, and a burst duration of >3 ms (Figs. 4a,b). With this method the specific PCR product can be clearly identified even at a concentration of 10^{-10} mol L^{-1} (Fig. 4c).

Sauer and coworkers have recently shown that smart probes can also be used in heterogeneous assay formats. In these the 3′ end is biotinylated, so the probe can bind to a streptavidin-coated glass surface. By analyzing the fluorescence signals of the individual probes by scanning confocal microscopy a concentration of 10^{-13} mol L^{-1} of artificial target DNA was detected [26].

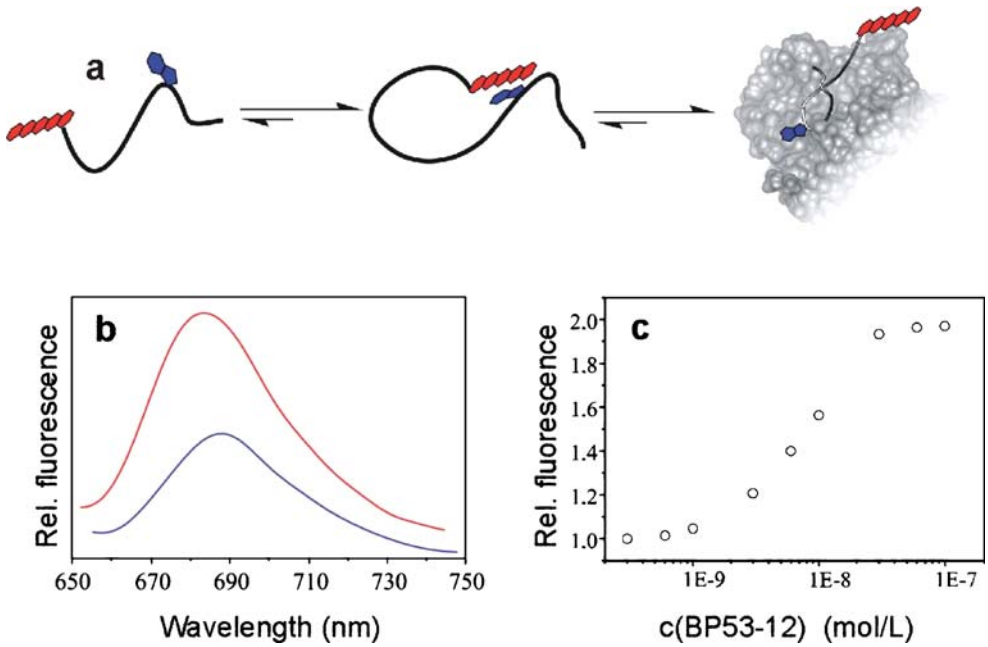

Fig. 5 (**a**) Working mechanism of the peptide-based probe used for identification of anti-p53 autoantibodies. The fluorescently labeled probe is quenched because of close contact between the dye and the tryptophan residue. Binding to the anti-p53 autoantibody prevents the contact and so the fluorescence increases. (**b**) Fluorescence spectra obtained from a solution (10^{-8} mol L^{-1}) of the probe before and after addition of a 100-fold excess of the anti-p53 autoantibody. (**c**) Dependence of relative fluorescence on BP53-12 concentration. Adapted from diagrams in [42]

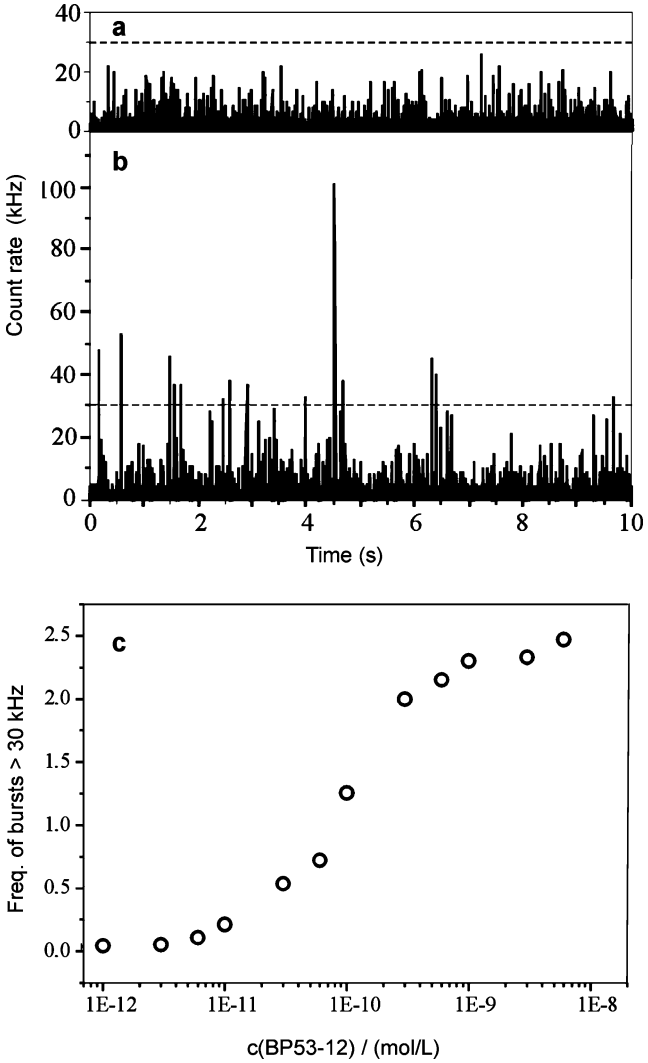

Fig. 6 Fluorescence signals obtained from a 5×10^{-11} mol L^{-1} solution of the MR121-labeled epitope in the absence (**a**) and presence (**b**) of 10^{-10} mol L^{-1} of the BP53-12 antibody. (**c**) Dependence on antibody concentration of the number of fluorescence bursts over 30 kHz. Adapted from diagrams in [42]

Identification of antibodies

The efficient fluorescence quenching of the oxazine derivative MR121 by the amino acid tryptophan has been used to develop peptide-based molecular probes for detection of the anti-p53 autoantibody in a homogeneous assay format [42]. In modern cancer diagnostics anti-p53 autoantibodies are used as independent and highly specific tumor markers [43]. They are directed against the protein p53, which is a transcription factor that regulates the cell cycle and hence functions as a tumor suppressor.

Because it has been known that small linear peptide epitopes (parts of a macromolecule that are recognized by the immune system) located in the N-terminal region of the p53 sequence are responsible for the immune response [44], the probes consist of a N-terminal dye-labeled peptide of 14 amino acids. This peptide sequence contains one tryptophan residue and thus the fluorescence intensity of the MR121 is reduced by a factor of two, because of contact between the chromophore and the tryptophan. After binding to the anti-p53 autoantibody formation of this contact is hindered and the fluorescence intensity increases (Fig. 5).

With a standard fluorescence spectrometer a detection limit of approximately 10 nmol L^{-1} is achieved. Similar to descriptions of smart probes for ultra-sensitive DNA detection, use of confocal fluorescence single-molecule spectroscopy enables an increase in sensitivity of 2 to 3 orders of magnitude. Figure 6 shows the fluorescence signal observed from a 5×10^{-11} mol L^{-1} solution of the peptide probe before and after addition of 10^{-10} mol L^{-1} of a model antibody (BP53-12). It is apparent that signals with a count rate over 30 kHz occur only after binding of the probe to the antibody. The dependence on concentration of the number of bursts over the threshold of 30 kHz is depicted in Fig. 6c.

One important advantage of single-molecule confocal spectroscopy is that the fluorescence signals (bursts) from individual dye molecules can be easily separated from the background. In combination with red-emitting diode lasers it is possible to identify single anti-p53 autoantibodies directly in blood serum of cancer patients, because at these wavelengths the auto-fluorescence of other biomolecules is relatively low [42].

Sensitive methods for detection of proteolytic enzymes

In recent years interest in rapid, sensitive assays for proteolytic enzymes, i.e. enzymes that cleave peptide bonds, has increased substantially. There are two main classes of proteolytic enzyme, endopeptidases and exopeptidases. Whereas endopetidases cleave specific peptide bonds within a peptide or protein, exopeptidases digest peptides stepwise either from the N-terminus (aminopeptidases) or from the C-terminus (carboxypeptidases). Both types are becoming increasingly important targets in bioanalysis. Because of their involvement in tumor progression and metastasis, many proteases, for example matrix metalloproteinases [45, 46] urokinase plasminogen

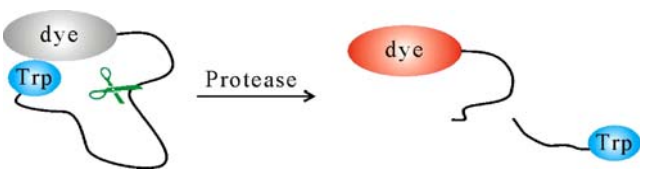

Fig. 7 Working mechanism of mono-labeled self-quenching peptide substrates. The fluorescence intensity of the dye is quenched via photoinduced electron transfer (PET) because of conformations in which the tryptophan residue is directly adjacent to the chromophore. Hydrolysis by the target enzyme removes the tryptophan and the fluorescence is no longer quenched

activator (uPA) [47], or cathepsins [48] (e.g. cathepsin B, cathepsin D) are extremely important in cancer research. Proteases are also important targets in research on many other diseases, for example type-2 diabetes mellitus and AIDS. For example, the HIV protease (HIV-PR), which is not present in noninfected persons, has specific cleavage sites. Thus, a very sensitive HIV-PR assay could be used for diagnosis of HIV.

Most commercially available fluorescence-based protease assays use short peptide substrates labeled with two fluorescent dyes that form a donor–acceptor pair, so that a FRET occurs. The specific cleavage site of the respective target enzyme is located between both chromophores. On digestion the dyes are separated and thus no FRET is possible; this results in a measurable increase of the donor fluorescence intensity. Instead of two different dyes, two identical chromophores can also be used. Because of hydrophobic interactions in aqueous solutions, the fluorophores form nonfluorescent or weakly fluorescent ground-state complexes (H-type dimers) [49–51]. Both methods enable direct monitoring of protease activity by measuring the increase in fluorescence intensity by standard fluorescence spectrometry. As far as we are aware, none of these substrates has yet been used in combination with single-molecule spectroscopy. Substrates containing two chromophores can usually be used for detection of endoproteases only, because most exopeptidases are unable to cleave dye-labeled amino acid residues.

We developed novel self-quenching substrates labeled with one dye only. In these the fluorescence quenching is caused by a tryptophan residue in the substrate sequence (Fig. 7) [52]. The cleavage site of the target enzyme is located between the tryptophan residue and the dye label. Flexibility of the peptide chain and hydrophobic interactions induce contact formation between tryptophan and the chromophore. This leads to the formation of ground state complexes and thus to fluorescence quenching via photo-induced electron transfer. On hydrolysis of a peptide bond in the cleavage site by the respective target enzyme the spatial proximity of the dye and the tryptophan residue is lost and the fluorescence intensity is no longer quenched.

We have demonstrated this method works for different endopeptidases and exopeptidases [52–54]. Several dyes have been tested to investigate the interaction with the amino acid tryptophan. We found the oxazine derivative MR121 and Atto 655 are very suitable chromophores for these substrates in terms of efficient fluorescence quenching, stability, and nonperturbative effect on enzyme activity.

Figure 8 shows the recorded fluorescence intensity of different MR121-labeled substrates after addition of the respective target enzymes. For assay of carboxypetidase A (CPA) the dipeptide lysine–tryptophan is used. Because the tryptophan residue is directly adjacent to the dye-labeled lysine, efficient fluorescence quenching occurs. The quantum yield relative to the free dye is 0.1 in PBS buffer and 0.15 in pure water. The measurements were conducted in pure water to reduce adsorption of the CPA. Consequently, a 6.5-fold increase in fluorescence intensity can be observed after digestion. Even an enzyme concentration of 10^{-13} mol L^{-1} can be rapidly detected (Fig. 8a). Figure 8b shows the fluorescence increase of a substrate designed for the HIV protease [53]. Here the increase was approximately 3-fold only, which corresponds to a relative quantum yield of 0.3. The fluorescence quenching is less efficient than that for the dipeptide, because nine amino acid residues are located between dye and the tryptophan. The data show that the HIV-PR can be detected within 1 h, even at nanomolar concentrations. Figure 8, middle, (c), shows results from a control experiment in which the protease was mixed with an inhibitor (indinavir). An example of the detection of aminopeptidases is shown in Fig. 8c. In this work we designed a substrate for dipeptidylpeptidase IV (DPP IV),

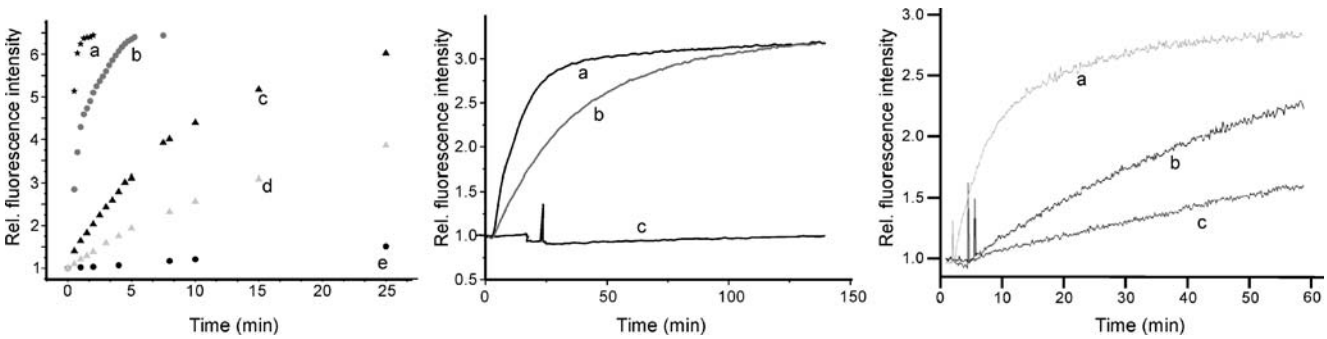

Fig. 8 Plots of relative fluorescence signal against time for different substrates. The fluorescence was detected at 680 nm (excitation wavelength 650 nm). **Left**: Signal from (MR121)KW (10^{-7} mol L^{-1}) in the presence of (*a*) 10^{-6} mol L^{-1}, (*b*) 10^{-9} mol L^{-1}, (*c*) 10^{-11} mol L^{-1}, (*d*) 10^{-13} mol L^{-1}, and (*e*) 10^{-15} mol L^{-1} carboxypeptidase A. **Middle**: Relative fluorescence signal from the HIV-PR substrate Ac-K(MR121) CGSQNYPIVW (10^{-6} mol L^{-1}) after addition of HIV-protease at a concentration of (*a*) 10^{-8} mol L^{-1} and (*b*) 10^{-9} mol L^{-1}. (*c*) Control experiment with enzyme inhibitor Indinavir. **Right**: Signal from WPSGTFTKC(MR121) (10^{-7} mol L^{-1}) in the presence of (*a*) 10^{-7} mol L^{-1}, (*b*) 10^{-8} mol L^{-1} and (*c*) 10^{-9} mol L^{-1} DPP IV. (*K* = lysine, *W* = tryptophan, *C* = cysteine, *G* = glycine, *S* = serine, *Q* = glutamine, *N* = asparagine, *Y* = tyrosine, *P* = proline, *I* = isoleucine, *V* = valine, *T* = threonine, and *F* = phenylalanine). Adapted from diagrams in [52]

which is important in type-2 diabetes mellitus. Because of steric effects, several amino acids must be placed between the N-terminal tryptophan and the dye-labeled lysine, so the increase in fluorescence intensity is only approximately twofold (unpublished data).

In contrast with the DNA or antibody assay described above, use of single-molecule techniques to enhance the sensitivity of the enzyme assay leads to several problems, particularly for homogeneous assays. Because the quenched substrates still have some fluorescence—the relative quantum yield varies, depending on substrate sequence, from 0.1 to 0.4—the concentration of substrate must be reduced to approximately nanomolar levels to observe fluorescence signals from single molecules. This means fewer substrate molecules must be digested to result in an increase in fluorescence. The low substrate concentration also leads to a lower turnover of the enzyme, however, as predicted by Michaelis–Menten kinetics. Consequently, a specific amount of enzyme does not hydrolyze a smaller amount (lower concentration) of substrates much faster than a larger amount (higher concentration) if the substrate concentration is below the saturation limit, which is always true for the single-molecule experiments described.

An important advantage of single-molecule spectroscopy is that different properties, e.g. fluorescence lifetime or burst intensity, can be determined for each signal arising from the individual molecules. This often enables identification of bursts that indicate, for example, binding of a DNA probe or an antigen, even if only a small fraction of the bursts are positive signals. Unfortunately, neither the fluorescence lifetime nor the burst intensity of the MR121-labeled substrates changes substantially after hydrolysis by the target enzyme; instead only the number of burst signals increases. The number of signals correlates with the average fluorescence intensity measured in conventional fluorescence spectrometers. Consequently, use of the MR121-labeled substrate in single-molecule experiments has no significant advantages compared with standard spectrometry. We therefore developed a substrate labeled with BODIPY 630/650 that is also efficiently quenched by tryptophan. In contrast with MR121, the BODIPY-dye is quenched via a mixed static and dynamic mechanism. This means that the substrate has a shortened fluorescence lifetime before digestion by the HIV-PR. Furthermore, perhaps because of higher hydrophobicity, the BODIPY 630/650 substrate is quenched three times more efficiently than the MR121-substrate [54]. Figure 9 shows there are clear differences between cleaved and uncleaved substrates at the single-molecule level. The fluorescence intensity trace for the substrate after digestion shows that fluorescence bursts are significantly more intense. The maximum of the fluorescence lifetime distribution also changes from 1.5 to 4.5 ns. The changes in these values are an important

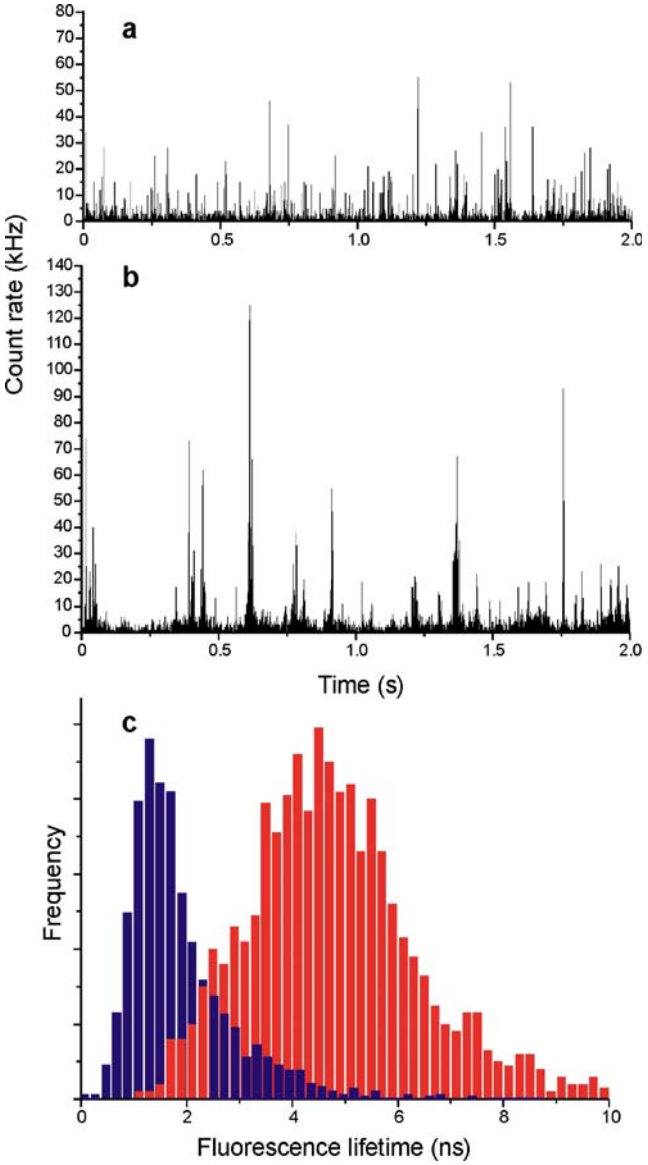

Fig. 9 Fluorescence signal obtained from HIV-PR substrate Ac-K (Bodipy 630/650)CGSQNYPIVW (10^{-10} mol L^{-1}) after incubation for 24 h and dilution (1:100) of samples containing (**a**) no HIV-PR and (**b**) 8×10^{-10} mol L^{-1} HIV-PR. (**c**) Fluorescence lifetime distribution of the signals detected for the substrate before (*blue*) and after (*red*) cleavage

condition for use of multiproperty analysis to enhance sensitivity, as described in the section "Highly sensitive detection of specific DNA sequences using self-quenching probes". This substrate has serious disadvantages, however. The high hydrophobicity results in severe adsorption effects and the fluorescence quenching is highly dependent on the molecular environment, which complicates handling and reduces reproducibility. Despite this, we were able to achieve a detection limit of 10^{-11} mol L^{-1}, which is 10–100 times better than that for standard spectroscopy [54].

These mono-labeled self-quenching substrates are usually also appropriate for heterogeneous surfaced-based assays. We

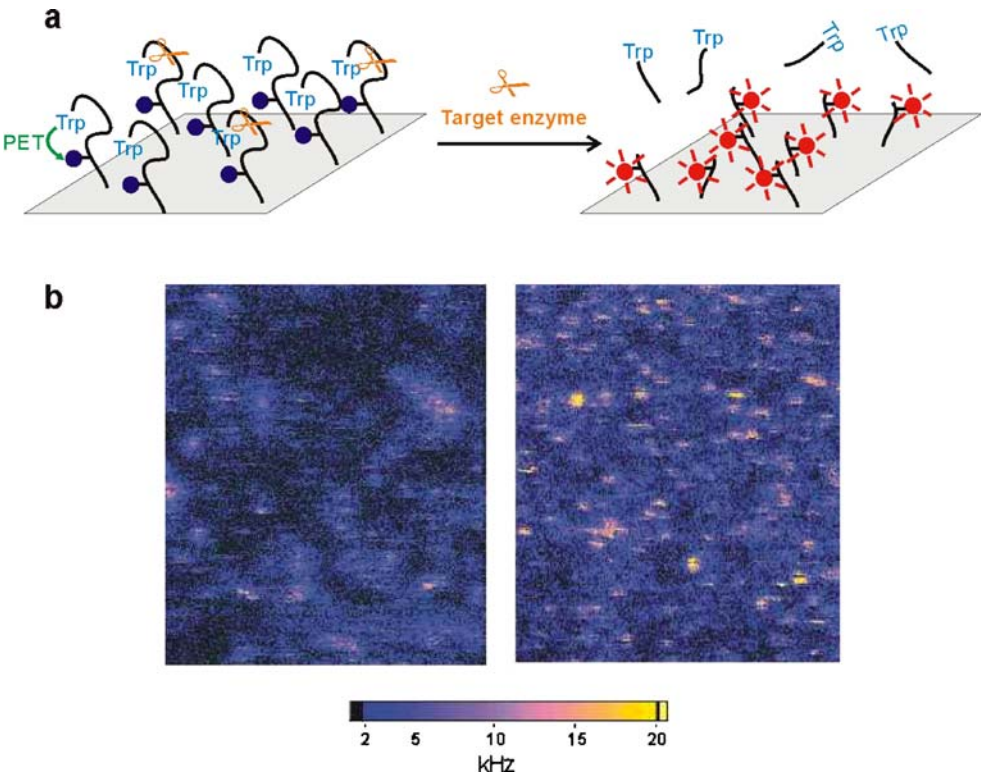

Fig. 10 (**a**) Working mechanism of the surface-based system for assay of carboxypeptidase A. Dye-labeled peptide substrates (CGGK(MR121)W) are covalently linked to a PEG-coated glass surface via the SH group of cysteine. (**b**) Fluorescence scanning images of the substrate loaded surface before (*left*) and after (*right*) addition of 10^{-10} mol L^{-1} CPA

demonstrated proof of principle for carboxypeptidase A [55]. The substrate CGGK(MR121)W (cysteine–glycine–glycine–lysine–tryptophan) is covalently linked to the amino groups of a PEG-modified glass surface via the thiol group of the cysteine (Fig. 10a). Measurements must be performed under aqueous conditions, otherwise the dye interacts not with the tryptophan residue but rather with the surface and no fluorescence quenching occurs. On digestion by CPA the tryptophan residues are removed and the dyes remain on the surface. Figure 10b shows a 15 μm×20 μm confocal fluorescence scanning image of the PEG-coated surface on which the quenched substrates are bound. The left image was taken before addition of 10^{-10} mol L^{-1} CPA; that on the right 15 min after. In the left image, count rates are below 10 kHz for most of the fluorescence spots and few spots have count rates above 15 kHz. The latter might be because of substrates for which quenching is prevented by strong interaction of the fluorophore with the glass surface, enabled by coating defects. The image on the right shows the number of spots with intensity above 10 kHz is approximately ten times greater. This corresponds to measurements in solution, where an up to ninefold increase in fluorescence was detected. Although the sensitivity achieved with these surface-based assays is currently less than that for the respective assay in homogeneous solution using standard fluorescence spectroscopy, it might be the basis for developing chips which enable the simultaneous detection of many different proteases. The sensitivity of this assay format could, furthermore, be significantly improved by use of optimized substrates and coating strategies.

Conclusion

Fluorescence single-molecule spectroscopy in homogeneous solutions and on surfaces is an appropriate basis for development of ultra-sensitive assays for a wide range of biomolecules, for example DNA, RNA, proteins, or enzymes. These methods usually require well-designed probes that contain at least one fluorescent dye. Many modern methods use probes designed in such a way that the presence of the respective target molecule is apparent from an increase in fluorescence intensity. The probes therefore need a quenching moiety that reduces the fluorescence of the fluorophore until the probes interact (e.g. hybridization, binding, cleavage) with the target enzyme. In this review we have described some examples that utilize quenching of mono-labeled probes by an intrinsic quenching moiety, for example guanine or tryptophan, via a photo-induced electron transfer reaction. The synthesis of such probes is usually less complicated and less expensive than synthesis of probes containing two labels, for example probes based on a FRET mechanism. Indeed, the range of appropriate dyes that are efficiently quenched by guanine or tryptophan is limited but many of these dyes (e.g. rhodamine and oxazine derivatives) absorb light above 600 nm and are therefore ideal for single-molecule experi-

ments. The quantum yields are sufficient, the molecules can be excited by use of inexpensive diode lasers, and autofluorescence in this spectral region is very low, so the dyes are easily detected, even in untreated blood serum [56]. Many problems must still be overcome, however, if specific, sensitive, and highly reliable assay methods, that can be used in conventional quantitative routine laboratories, are to be achieved. Use of single-molecule detection techniques, in particular, requires relatively complicated experimental equipment and personal skills. In addition to these technical aspects the experiments themselves suffer from several experimental factors that are difficult to control. For example, dealing with low probe concentrations requires excellent coating techniques to minimize errors arising from nonspecific adsorption. Although it is impossible to use single-molecule spectroscopy for quantitative analysis without control over such conditions, we believe further research will lead to many analytical applications and that mono-labeled self-quenching probes, especially, could make an important contribution.

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