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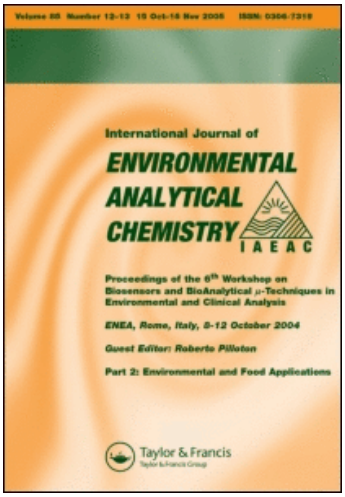

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A fluorescence-based assay for exopeptidases using self-quenching peptide probes with single-molecule sensitivity

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In recent years, the interest in assaying exopeptidases has become increasingly important because they are significantly involved in many diseases like cancer. To date, no generally applicable fluorescence-based detection method has been developed because commercially available doubly-labeled substrates are not always digested by exopeptidases. In this article we present a new method for the sensitive detection of exopeptidases based on fluorescently-labeled substrates containing only one fluorophore that is efficiently quenched by an adjacent tryptophan residue via photoinduced electron transfer. Because of their well-known properties we chose carboxypeptidase A (CPA) as a model system. The self-quenching probes were used in homogeneous solution as well as on cross-linked PEG-coated surfaces in combination with single-molecule imaging techniques. However, even with standard fluorescence spectrometers we achieved sensitivity below the picomolar range.

Keywords: Exopeptidase-assay; Self-quenching substrates; Single-molecule imaging

1. Introduction

During the past years, the interest in fast and sensitive assays for proteolytic enzymes, i.e. enzymes that cleave peptide bonds, has increased considerably, due to their major role in many diseases such as cancer or viral infections. Meanwhile, many fluorescence-based assays have been developed for the detection of endopeptidases in solution using quenched enzyme substrates. Usually they are based on a fluorescence resonance energy transfer (FRET) [1, 2] system. On one side of the recognition sequence a donor dye and on the other side an acceptor dye is located, thus an efficient FRET occurs. Upon cleavage of the peptide substrate by the target enzyme the close

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proximity between the fluorophores gets lost. Instead of a FRET pair two identical fluorophores can be applied. Due to hydrophobic interactions in aqueous solution fluorophores form non- or only weakly fluorescent H-type dimers [3–6]. Both methods enable the direct monitoring of protease activity by measuring the increase in the fluorescence intensity.

Apart from the endopeptidases, the exopeptidases are also of great medical interest. For example the metallocarboxypeptidase thrombin activatable fibrinolysis inhibitor (TAFI) a proenzyme that is proteolytically activated by thrombin in a process enhanced dramatically by the cofactor thrombomodulin plays an important role during blood coagulation and fibrinolysis [7, 8], and in pathogenesis of thrombotic disorders in lung cancer patients [9] as well as interstitial lung diseases [10]. Another medical interesting exopeptidase, the angiotensin I-converting enzyme (ACE), a peptidyl-dipeptidase that is generally membrane-bound, is important in elevation of blood pressure and cardiovascular diseases, through formation of angiotensin II (vasoconstrictor) and destruction of bradykinin (vasodilator) [11]. Other exopeptidases such as carboxypeptidase N [12] or carboxypeptidase U [13], both metallocarboxypeptidases, and several cathepsins [14–16] play major roles in inflammation, fibrinolysis and cancer. Although the interest in assaying exopeptidases is rapidly increasing, to date no generally applicable fluorescence-based assay has been developed. Hence, the commercially available enzyme assays that usually apply doubly-labeled substrates are not appropriate for the detection of exopeptidases, because the enzymes are not able to digest dye-labeled amino acid residues. Thus no cleavage between the two labels occurs.

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This article presents a novel sensitive assay for exopeptidases based on self-quenching substrates containing only one fluorescence dye. Therefore, we applied the oxazine derivative MR121 that is efficiently quenched by a tryptophan residue. Furthermore, this dye can be excited by standard laser diodes emitting at 635 nm and is therefore often applied in single-molecule spectroscopy experiments. In the substrate a tryptophan residue is located adjacent to the dye-labeled lysine. Thus the relative quantum yield in aqueous solution decreases from 1 to 0.1–0.2 depending on the salt concentration. In the presence of the target enzyme the tryptophan is removed by digestion of the substrate resulting in a significant increase of the fluorescence intensity. We chose carboxypeptidase A as the model system because of its well-known properties, availability and uncomplicated handling. The new method is suitable for assays in homogeneous solution as well as on surfaces. In order to ensure the operativeness of the substrates and the enzymes on the surface we applied a special cross-linked PEG-coating that can also be used to immobilize the substrates. The fluorescence signal was read out by highly sensitive single-molecule imaging techniques.

2. Experimental

2.1 *Fluorescent dyes and conjugates*

All dyes used in this study were red-absorbing oxazine and cyanine derivatives, respectively. The oxazine derivative MR121 was provided courtesy of K.H. Drexhage (Universität-Gesamthochschule, Siegen). The dye was transferred into its N-hydroxysuccinimidyl ester by an equimolar amount of N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide

(NHS) in acetonitrile. The peptide substrates were labeled at the amino group (lysine residue or N-terminus) with the fluorescent dyes. Therefore, 20 μL of the activated dye (0.1 mM in acetonitrile) was mixed with an excess of peptides in 50 μL of carbonate buffer (0.1 M, pH 8.3). After a few hours the product was purified by HPLC (Hewlett-Packard, Böblingen, Germany) using a reversed-phase column (Knauer, Berlin, Germany). Separation was carried out in 0.1 M triethylammonium acetate, using a linear gradient from 0 to 75% acetonitrile in 20 min.

2.2 *Spectroscopy*

All absorption spectra were taken on a Cary 500 UV-vis-NIR spectrometer (Varian, Darmstadt, Germany) in standard cuvettes. Fluorescence measurements were performed on a Cary Eclipse fluorescence spectrometer (Varian, Darmstadt, Germany). In all measurements, concentrations were kept below 1 μM to avoid reabsorption and reemission effects. Relative fluorescence quantum yields, $\Phi_{f,rel}$, of the fluorescently labeled peptides were measured with respect to the fluorescence intensity of the free dye under otherwise similar conditions.

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Ensemble fluorescence lifetime measurements were taken at the emission maximum applying the time-correlated single-photon counting (TCSPC) technique with an IBH spectrometer (model 5000MC; Glasgow, UK). A pulsed laser diode emitting at 635 nm with a pulse length of ~200 ps (FWHM) or LEDs emitting at 495 nm or 590 nm with pulse length of ~1 ns (FWHM) with a repetition rate of 1 MHz were taken as excitation sources. With this set-up, instrument response functions (IRFs) of 220 ps (FWHM) for the laser diode and ~1 ns for the LEDs (FWHM) were measured. With this apparatus it was possible to measure fluorescence lifetimes down to 40 ps (for the pulsed laser diode), and ~200 ps (for the pulsed LED). Typically, 3000–5000 photon counts were collected in the maximum channel using 4096 channels. The decay parameters were determined by least-squares deconvolution, and their quality was judged by the reduced χ^2 values and the randomness of the weighted residuals. In the case that a monoexponential model was not adequate to describe the measured decay, a multiexponential model was used to fit the decay.

2.3 *Surface modification*

Four-arm star isothiocyanate modified polyethylene glycol was purchased from the PEG-shop (South Korea). For efficient cross-linking of the PEG via the isothiocyanate groups, cover slips were incubated in a PEG-solution (5 mg mL^{-1} in de-ionized water) for 2 h and rinsed twice with de-ionized water prior to use.

2.4 *Single-molecule imaging*

The experimental set-up for single-molecule experiments on PEG-coated cover slips consists essentially of a standard inverse fluorescence microscope. A pulsed laser diode with a wavelength of 635 nm, a repetition rate of 64 MHz, and a pulse length of <100 ps (PDL800B; Picoquant, Berlin, Germany) served as the excitation source. The elliptical shape of the laser beam profile was converted into a circular (Gaussian-like) profile by the use of two cylindrical lenses. The collimated laser beam passes an excitation filter (639DF9; Omega Optics, Brattleboro, VT), and was then

directed into an inverted microscope (Axiovert 100TV; Zeiss, Germany) via the backport. It was coupled into an oil-immersion objective (100×, NA = 1.4; Nikon, Japan) by a dichroic beam splitter (645DMLP; Omega Optics, Brattleboro, VT). The average laser power was adjusted to approximately 20 μW at the sample. The fluorescence signal was collected with the same objective and focused through the TV outlet of the microscope onto a 50-μm pinhole. Afterwards, the fluorescence light was spectrally separated by a nonpolarizing dichroic beam splitter (670DMLP; Omega Optics, Brattleboro, VT) and imaged onto the active area of an avalanche photodiode (AQR-14; EG&G, Canada). For the generation of fluorescence images, the microscope was equipped with a motion controller-driven x, y microscope stage (SCAN 100×100, MC2000; Märzhäuser, Wetzlar, Germany).

3. Results and discussion

Fluorescent dye molecules which are influenced by their environment can act as molecular probes that exhibit information about neighboring groups or changes in the polarity of the microenvironment. It is known for many years that several red-absorbing rhodamine and oxazine derivatives are efficiently quenched by the nucleic base guanosine [17–19]. This property has successfully been used in specific and highly-sensitive DNA-assays [20–22]. Furthermore, it has been shown that different amino acids influence the fluorescence properties of some fluoresceine and boradiazaindacene derivatives [23–25]. Indeed the fluorescence quenching by tyrosine as well as tryptopan is relatively weak because it is due to a mainly dynamic quenching mechanism. We found the red-adsorbing oxazine derivative to be efficiently quenched by the amino acid tryptophan, whereas the effects of all other amino acid residues are negligible [26]. The quenching efficiency and the quenching mechanism can be determined by Stern–Volmer analysis. Therefore, the fluorescence intensity F and the fluorescence lifetime τ of the dye are measured in the presence of an increasing concentration of the quencher molecule. The fluorescence lifetime is decreased only by a dynamic quenching mechanism that is due to collision, whereas the fluorescence intensity is decreased by dynamic as well as by static quenching caused by the formation of non-fluorescent ground state complexes. The quenching constants can be determined by:

$$F_0/F = 1 + K_s[Q] = 1 + k_{q,s}\tau_0[Q] \tag{1}$$

$$\tau_0/\tau = 1 + K_{dyn}[Q] = 1 + k_{q,dyn}\tau_0[Q] \tag{2}$$

where τ_0 and F_0 are the fluorescence lifetime and intensity in the absence of a quencher, τ and F are the fluorescence lifetime and intensity in the presence of the quencher Q with the concentration $[Q]$, and K_s and K_{dyn} denote the static and dynamic Stern–Volmer constants, respectively. The static constant K_s is equal to the association constant of the non-fluorescent dye-quencher complex, if the quenching mechanism is exclusively of static nature. In the case of mixed quenching mechanisms the fluorescence decrease can be described by:

$$F_0/F = (1 + K_{dyn}[Q])(1 + K_{ass}[Q]) \tag{3}$$

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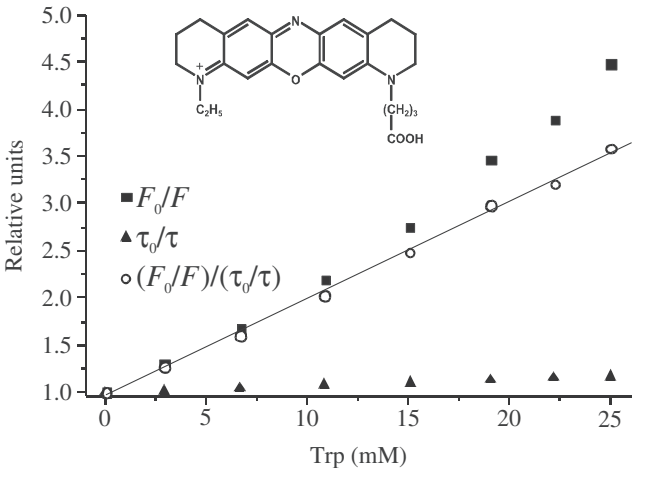

Figure 1. Fluorescence intensity (F_0/F) and lifetime (τ_0/τ) Stern–Volmer plots for the tryptophan quenching and the molecular structure for the used oxazine derivative MR121. Measurements were carried out in PBS (pH 7.4). The dynamic quenching constant K_{dyn} was calculated to $7.3\,M^{-1}$ and the association constant K_{ass} to $96\,M^{-1}$, respectively.

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The Stern–Volmer plot for the oxazine derivative with the amino acid tryptophan as the quencher molecule is shown in figure 1. The black curve as well as the cut-out illustrates the dependence of the fluorescence lifetime on the tryptophan concentration. Via the slope of these data a dynamic quenching constant of $K_{dyn} = 7.3\,M^{-1}$ was calculated. The red data points show the measured fluorescence dependence. Since we are obviously dealing with a mixed quenching mechanism the association constant can be calculated to $K_{ass} = 96\,M^{-1}$ via equation (3) by plotting $(F_0/F)/(\tau_0\tau)$ as illustrated by the green data points. These results demonstrate, in contrast to former investigated dyes, e.g. fluoresceine derivatives, that the static quenching mechanism dominates, whereas the dynamic mechanism can usually be neglected.

3.1 *Assaying exopeptidases in solution*

The general principal for assaying exopeptidases in solution is shown in figure 2. The fluorescent dye is coupled to one end of a peptide, whereas a tryptophan residue is located at the other end. Due to the close proximity between dye and tryptophan residue, the fluorescence is efficiently quenched via photoinduced electron transfer. In the presence of the target enzyme, the peptide bond is hydrolyzed and the contact formation between fluorescent dye and tryptophan residue consequently gets lost. This results in an increase in fluorescence intensity.

For assaying carboxypeptidase A (CPA), the red-absorbing oxazine derivative MR121 was covalently coupled to the amino residue of the lysine group of the dipeptide lysine-tryptophan. The dipeptide lysine-tryptophan was used because the best quenching efficiency is obtained with the dye directly adjacent to the tryptophan residue. The relative quantum yield decreases to a value of 0.1 in a buffer containing 0.025 M Tris/HCl, 0.5 M NaCl, 0.05 M LiCl. Lithium chloride was added to establish optimal cleavage conditions for CPA. (http://www.worthington-biochem.com/COA/default.html) Accordingly a ten-fold increase of the fluorescence intensity is observed after digestion.

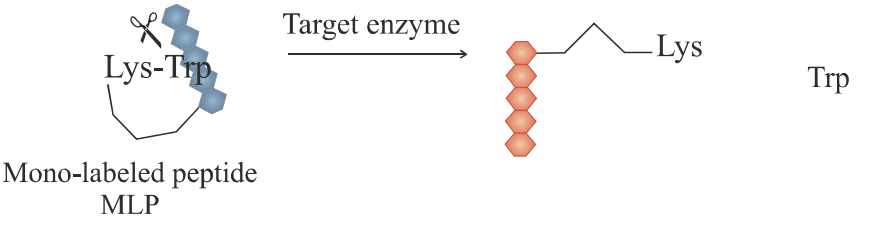

Figure 2. Principle of the new assay for the detection of exopeptidases in solution. If the target enzyme digests the peptide, the contact formation between the fluorescent dye and the tryptophan residue is annihilated and thus, the fluorophore is no longer quenched.

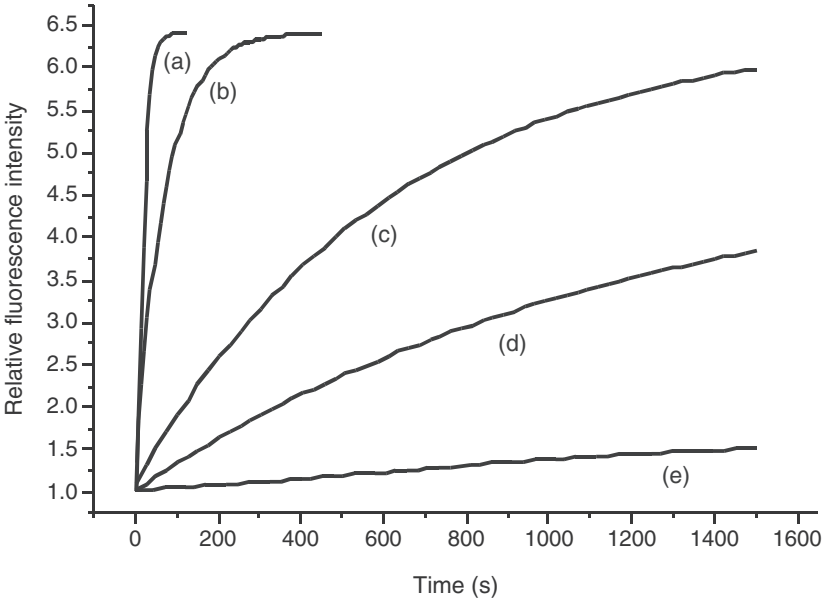

Figure 3. Relative fluorescence intensities *vs.* time for the MR121-labeled dipeptide lysine-tryptophan (10^{-7} M) after addition of different concentrations of carboxypeptidase A (CPA). (a) 10^{-6} M CPA, (b) 10^{-9} M CPA, (c) 10^{-11} M CPA, (d) 10^{-13} M CPA, and (e) 10^{-15} M CPA. Measurements were performed in pure water at 25°C (excitation wavelength: 640 nm; emission wavelength: 690 nm).

To ascertain the sensitivity and the detection limit of the assay, measurements with different CPA-concentrations were performed (figure 3). To avoid unspecific adsorption of CPA the glass walls of the cuvette were coated with PEG. Since salt-containing buffers enhance the adsorption tendency of the CPA the handling low concentrations is problematic. Therefore, all measurements were carried out in pure water although the fluorescence quenching is less efficient and the substrate shows a quantum yield of approximately 0.15, corresponding to a 6.5-fold increase after cleavage. The maximum increase is obtained in a few seconds after addition of 10^{-6} M CPA which corresponds to a ten-fold excess with respect to the dye-labeled substrate (figure 3a). Figure 3(d) shows that even a CPA concentration of 10^{-13} M, which correlates to one enzyme molecule per 10^6 substrate molecules, effects a 2.5-fold increase in fluorescence intensity within 10 min. To detect lower concentrations

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longer measurement times are required. However, our data demonstrate that a CPA concentration of 10^{-15} M can be handled. After 25 min a 1.5-fold increase in fluorescence intensity was observed.

3.2 *Adsorption effects on surfaces*

Adsorption effects of organic matter and biomolecules on surfaces constitute a major problem for all analytical techniques at the single-molecule level. The more hydrophobic the dye, peptide or enzyme is, the stronger are the interactions with the surface. The adsorbed molecules are in a dynamic equilibrium with the solvent molecules. If the molecules are very hydrophobic, the equilibrium is shifted strongly towards the adsorbed molecules, so that they virtually cannot dissociate from the surface. In concentrated solutions ($>10^{-8}$ M) the number of adsorbed molecules is negligible compared to those in solution. However, at the single-molecule level ($<10^{-9}$ M) the concentrations of the dissolved molecules can be strongly influenced by adsorption effects. The magnitude of the adsorption depends on many different factors (e.g. temperature, pH-value, salt-concentration of the solution, concentration of other substances in the solution) and is therefore difficult to predict.

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To investigate adsorption effects of analyte molecules on glass surfaces, we used scanning confocal fluorescence images of the oxazine dye MR121, MR121-lysine-tryptophan, and MR121-CPA on untreated cover slips (figure 4, left panels) and compared them to PEG-4-arm-coated cover slips (figure 4, right panels). The surfaces were covered with a highly-diluted solution of the respective molecule in bi-distilled water in which the concentration has to be in a range ($<10^{-12}$ M) to ensure that single molecules could be observed on the glass surface. However, an exact concentration is difficult to determine, because of adsorption at plastic vials and pipettes used for dilution. The scanning confocal fluorescence images of the surfaces were taken after 15 min. All pixels with an intensity below 10 kHz are colored black.

The left panels in figure 4 show many fluorescence spots, which are due to immobile dye molecules during the measurement period of 20 s (20 scan-lines). It is not possible to remove them even by washing the surface with bi-distilled water or ethanol. Beside these spots, many stripes were observed, which only existed during one scan-line. They originate from dye molecules that stayed immobile on the same place of the surface for no longer than 1 s, before they moved or photobleached. The PEG-coated cover slips (figure 4, right panels) show a strong reduction of the adsorption. Only a few spots could be observed which are due to coating defects. Consequently, PEG-4-arm-coatings are ideally suited to prevent adsorption effects of biomolecules on glass surfaces.

A closer look at the images of the untreated glass surfaces shows that the ratio between stripes, caused by dynamic adsorption of the molecules, and spots differ significantly from each other depending on the observed molecule. For the pure dye MR121, six times more stripes than spots could be counted, whereas for the dye-labeled dipeptide lysine-tryptophan the ratio declines to 4.5. The MR121-labeled carboxypeptidase A (CPA) exhibits a stripes to spots ratio of 2.5. The outcome of this is that CPA shows the strongest adsorption tendency of all investigated molecules. This might represent a major drawback associated with assaying of CPA and related enzymes at very low concentrations at the single molecule level.

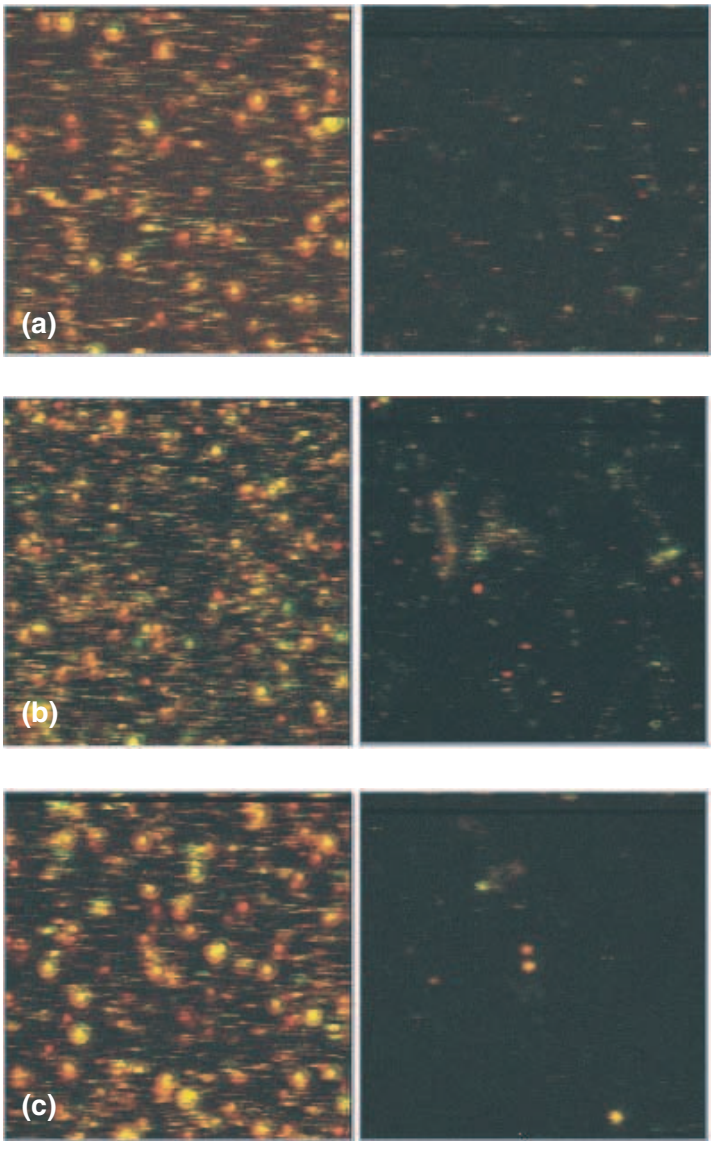

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Figure 4. Single-molecule fluorescence scanning confocal images ($20 \times 20\,\mu m$) of highly diluted ($< 10^{-12}$ M) solutions of (a) MR121, (b) MR121-lysine-tryptophan, and (c) MR121-CPA in water. The resolution was 20 (pixel)μm^{-1}, the integration time 1 ms $(pixel)^{-1}$. A pulsed diode laser (635 nm, 64 MHz, 10 μW) was focused on the upper glass surface of the cover slip. Scanning was performed from top left to bottom down. The left pictures show untreated cover slips, whereas the pictures on the right show PEG-4-arm-coated cover slips (intensity scale: 0–20 kHz).

3.3 *Assaying exopeptidases on surfaces*

For surface-based assays, the enzyme substrates have to be immobilized on cover slips. In a first step, the glass surfaces were coated with isothiocyanate-modified star-shaped polyethylenglycole (4-arm PEG). Therefore, the monomers were cross-linked via the hydrolysis of isothiocyanate groups to primary amines which react with further

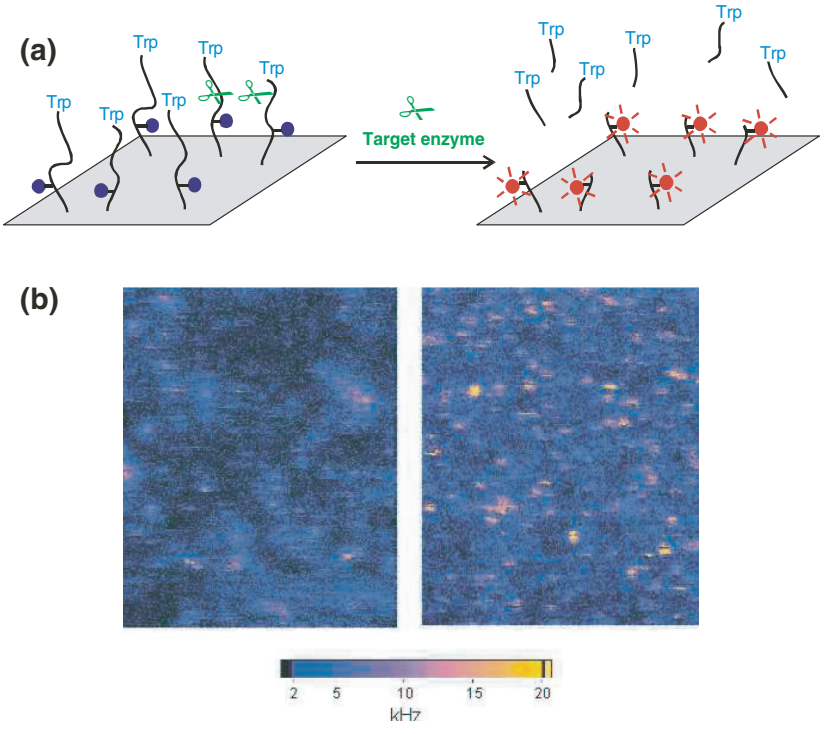

Figure 5. (a) Scheme for the detection of carboxypeptidase A on surfaces. A dye-labeled peptide (cysteine–glycine–glycine–lysine–tryptophan) was covalently linked to a PEG-coated surface using the SH-group of cystein. (b) 15 μm × 20 μm images of a substrate loaded surface before (left) and 15 min after addition of 10^{-10} M CPA (right). The excitation power was 5 μW at the sample.

isothiocyanate groups. After complete hydrolysis, some primary amines still remain as anchor points. By the dint of GMBS (N-(γ-maleimidobutyryloxy)succinimide ester), they are converted into maleimide on which thiol-containing enzyme substrates can be covalently linked. Figure 5(a) illustrates a general principal of the surface-based enzyme assays. Due to the close proximity of the dye and the tryptophan residue the substrates are efficiently quenched even after coupling to the PEG-coated surface, whereas the quenching is abolished by linking to uncoated glass surfaces. After addition of the target enzyme the peptide is digested, thus the tryptophan residue is removed and the fluorescence of the dye is released. It must be ensured that the enzyme does not cleave any peptide bond between the surface and the dye, otherwise the dye will be removed from the surface and no fluorescence can be imaged.

Figure 5(b) shows a 15 μm × 20 μm scanning fluorescence confocal image of a PEG-coated surface on which the dye-labeled enzyme substrate (cysteine–glycine–glycine–lysine(MR121)–tryptophan) is covalently linked and covered with the reaction buffer containing 0.025 M Tris/HCl, 0.5 M NaCl, 0.05 M LiCl, pH 7.5. The two glycine residues are incorporated in the substrate to act as a spacer between the surface and the recognition sequence of the target enzyme. The left image was taken before and the right 15 min after addition of 10^{-10} M CPA. In the left image only a few spots exhibit fluorescence intensities of more than 15 kHz, whereas most fluorescence signals are significantly below 10 kHz. The bright spots might be due to impurities

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or unquenched substrates. The quenching could be prevented by strong interactions of the fluorophores with the glass surface enabled by coating-defects.

Upon adding the carboxypeptidase A (CPA) at a concentration of approximately 10^{-10} M the peptide bond between the dye-labeled lysine and the tryptophan residue is cleaved. Thus the quencher is removed and the fluorescence of the dye increases. Hence, the CPA is not able to cleave peptide bonds on the N-terminal side of the dye-labeled lysine residue, the now fluorescent substrate remains covalently linked to the surface and can be easily detected. The image on the right side of figure 5(b) was taken 15 min after addition of the CPA and shows many spots with fluorescence intensities between 10 and 20 kHz which is equal to 10 to 20 photons per pixel. This corresponds to about a few thousand photons per detected dye. Counting only the spots with a maximum intensity above 10 kHz yields to approximately 10-fold more spots after addition of the CPA. The fragmentary spots are due to sudden photo-bleaching of the chromophore. The failure of high fluorescence signal during some pixels or even whole scanning lines is caused by long dark states of the dye that can last from some milliseconds to several seconds. Both phenomena, the abrupt photobleaching as well as the 'blinking' behavior are generally accepted as signatures of individual fluorophores.

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4. Conclusion

We have developed new self-quenching peptide substrates for highly-sensitive assaying of exopeptidases in homogeneous solution as well as on surfaces. The substrates are based on the efficient fluorescence quenching of the oxazine derivative MR121 especially by the amino acid tryptophan. In the presence of the exopeptidase, a tryptopane residue is removed and the fluorescence intensity increases. In principle, this assay system is also working for endopeptidases, if their recognition sequence is located between the dye and the tryptophan residue. In contrast to most fluorescence-based assay systems using double-labeled substrates, the new single-modified substrates exhibit considerable advantages. Besides less-complicated and less-expensive synthesis, incomplete labeling is unproblematic because unlabeled substrates do not fluoresce, whereas incomplete labeling of the commercially available substrates containing two dyes or rather a dye and a quencher leads to the simulation of cleaved substrates. Therefore, the background signal increases limiting the sensitivity, especially if single-molecule detection techniques are applied.

Furthermore, doubly-labeled substrates are usually not applicable for assaying exopeptidases because most of them are not able to digest labeled amino acid residues. However, we presented the first fluorescence based exopeptidase assay that applies a natural amino acid residue as part of the substrate itself as a fluorescence quencher. Moreover, the new assay is generally applicable because it works in homogeneous solution as well as on surfaces using standard fluorescence spectrometers or single-molecule detection techniques.

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