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Using two photon microscopy to quantify enzymatic reaction rates on polymer beads

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Two-photon fluorescence microscopy was introduced as a tool to assess enzyme accessibility and to quantify enzyme reactions rates on solid supports.

Enzyme catalysis on substrates that are linked to solid supports (solid phase biocatalysis) is becoming increasingly important as polymer supported synthesis[1] and high throughput screening methods[2] are developed. Despite successes both in synthesis and analysis, fundamental understanding of the kinetics and thermodynamics of such enzyme catalysed reactions is limited. It is increasingly clear that the fundamental rules for solid phase chemistry are different from those of solution phase chemistry,[3] and the same can be expected for solid phase biocatalysis. These rules need to be better understood in order to exploit the advantages of solid phase reactions in full. Hence, we recently initiated research efforts aimed at a better fundamental understanding of solid phase biocatalysis.

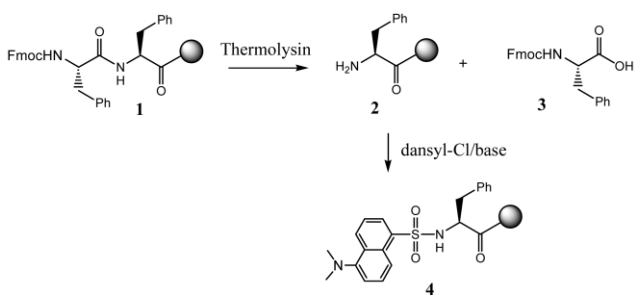
We have already obtained insight into the thermodynamics of amide hydrolysis reactions.[1b,e,f] In addition, we[1g] and others[1d,2] have gained a better understanding of the factors that govern accessibility of proteins to polymer beads. We have identified the combination of polymer $PEGA_{1900}$ and the proteolytic enzyme thermolysin as a very suitable model system where all reactive sites on the polymer appear to be accessible to the enzyme.[1b,d–f]

One important aspect of solid phase biocatalysis that has not been studied are the kinetics or reaction rates of these enzyme catalysed reactions. While it is well established that many enzymes are catalytically active within polymer beads,[1,2] it is not known *how* active they are when compared to aqueous solutions. Solid phase enzyme kinetics are expected to be significantly different from those of solution reactions for a number of reasons. First, the enzyme (large, thermolysin is 35 kDa) now has to diffuse towards the (small, typically 100 Da) substrate rather than *vice versa*. It is well known that the diffusion of small molecules into immobilised polymer particles can limit reaction rates,[1h,j] so limitation by enzyme diffusion could be very significant. Furthermore, reduced local diffusion rates will affect encounter frequencies, which might limit reaction rates. Second, the polymer bound substrate is inherently different from its solution analogue due to its linkage to the solid support. Third, the reaction takes place inside the polymer bead micro-environment, which is different from aqueous buffer. These three effects can be expected to lower enzymatic rates, and researchers often apply large amounts of enzyme in attempts to override these limitations. Insight into the *true* enzymatic reaction rate is therefore highly desirable for future applications where large amounts of enzymes are not always readily available.

To study enzymatic reaction rates systematically, a suitable analytical method is required. It is desirable to spatially and temporally analyse the distribution of chemical groups within the solid phase supports. Fluorescence microscopy is an obvious method for such analysis, since fluorescence methods are well developed in solution phase biocatalysis. However, conventional (confocal) fluorescence microscopy methods were found to be unreliable for analysis of high concentrations of fluorophores on polymer beads due to a variety of effects such as absorption/re-absorption of excitation and emission radiation, photo-bleaching and quenching.[4] We recently demonstrated that these effects are less important when using two photon microscopy (TPM).[5] In TPM, the sample is irradiated with a laser with a wavelength that is approximately twice that of the normal excitation wavelength of the fluorophore. As a result, excitation can only occur when two photons are absorbed simultaneously. Such two-photon events occur at a very high photon density that is reached only at the focal point of the laser beam. Hence, the fluorescence detected originates only in the part of the sample that is in focus. Away from the focal point there is essentially no absorption of the exciting beam due to the long wavelength used. TPM therefore avoids the artefacts that were described for confocal fluorescence microscopy.[4] We[5] and others[1j] recently demonstrated that TPM allowed for the spatial resolution of fluorophores within polymer beads. The emitted fluorescence could be linearly related to the fluorophore loading over the polymer bead size and loading ranges commonly used in solid phase chemistry.[5] Thus, we demonstrated that spatial quantification of fluorescent groups is possible by using TPM. Here, we report the first spatially and temporally quantified enzymatic reaction rate on a polymer support by TPM, using the enzyme/polymer combination thermolysin/$PEGA_{1900}$ as a model system.

The general set-up for measurement of the enzymatic reaction rate is outlined in Scheme 1. Upon hydrolysis of immobilised dipeptide **1** to give **2** and **3**, free amino groups are exposed only at those sites where enzymatic catalysis has occurred. The exposed amines (indicating the sites of enzyme catalysis) in **2** are chemically acylated with dansyl chloride to give **4**.

The resulting TPM images of single beads after reacting for different lengths of time with thermolysin (*i.e.* bearing different ratios of starting material **1** and product **4**) are shown in Fig. 1. Areas where peptides are hydrolysed enzymatically are light



Scheme 1 Thermolysin catalysed hydrolysis of solid supported Fmoc–Phe–Phe.

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after reaction with fluorescent dansyl chloride while unreacted areas remain dark.

These images (Fig. 1) reveal that the enzymatic reaction is initially limited by diffusion of enzyme into the resin bead, resulting in a light ring on the outside of the resin bead. In the course of time (after about 45 min) the ring expands until the full resin bead is penetrated by enzyme. The next step was to quantify the emitted fluorescence and convert it into biocatalytic reaction rates. By integrating the pixel intensity data of the TPM images quantitative values could be obtained. To determine the total amount of fluorophore present the pixel intensities were integrated and corrected for attenuation related to bead size as discussed before.[5] Fluorescence intensities that were obtained could be directly compared to the conversion data obtained by HPLC analysis of **3** and gave a good correlation (data not shown). This observation illustrated that TPM can be used for reliable quantification of chemical groups on PEGA beads.

The spatial resolution of our measurements has allowed us to estimate the time required for diffusion of thermolysin to the centre of the $PEGA_{1900}$ bead (Fig. 2) at around 45 min. In order to interpret this value, it can be compared to the expected diffusion time over the same distance through an aqueous solution using the diffusion equation for a homogeneous sphere. For the enzyme concentration at the centre of the bead to reach 95% of the concentration of the surrounding buffer, a time of $0.37\ L^2\ D^{-1}$ is then required. Here, L is the sphere radius and D is the effective diffusion coefficient inside the bead. As a first approximation we assume that the bead environment is water-like, which is reasonable because swollen $PEGA_{1900}$ beads consist of $>90\%$ water. The diffusion coefficients for proteins in the molecular weight range 20–150 kDa have values between 5 and 10×10^{-11} $m^2\ s^{-1}$.[6] In spherical beads of radius 100 μm, the time required for effective diffusion calculates to *ca.* 1 min. The observed enzyme diffusion in the $PEGA_{1900}$ beads is much slower (45 min) and it therefore seems likely that the PEG chains restrict and slow down the diffusion of enzyme molecules through the bead interior.

The limiting diffusion of the enzyme as described above is not expected to play a role at the edge of the beads. Here enzyme diffusion through the bulk buffer solution can be expected to rapidly bring the enzyme concentration up to the bulk concentration (0.5 mg mL^{-1} or 15 μM). The initial reaction rate could be estimated to be 0.13 mmol $(L\ min)^{-1}$.† We can then calculate the enzymatic reaction rate to be 0.26 μmol $(mg\ enz.\ min)^{-1}$. This value may be compared with 3.5 μmol $(mg\ enz.\ min)^{-1}$ for thermolysin-catalysed hydrolysis of a similar peptide (Z–Phe–Leu–NH_2 at 10 mM)[7] in aqueous solution.‡ Hence, the enzymatic rate on an immobilised substrate on the surface of $PEGA_{1900}$ was about an order of magnitude lower than that observed in free solution. Interestingly, an order of magnitude reduction would be expected if the observed biocatalytic rate on solid support was purely controlled by diffusion-limited formation of the enzyme/substrate complex. However, this simple explanation does not seem to be valid here§ and the precise reasons for the observed difference in reaction rate requires further study. We are currently investigating enzymatic catalysis on substrates that fluoresce upon enzymatic conversion and allow for real time analysis of reaction kinetics.

In summary, we have shown that TPM can be used for the spatially resolved quantitative measurement of enzyme kinetics on polymer supported substrates. When comparing the solid phase system to a solution reaction it was found that immobilisation of the enzyme substrate reduced the biocatalytic rate by about one order of magnitude. In $PEGA_{1900}$, the observed rate of reaction was found to be further limited by the diffusion of enzyme into the polymer, which was 1–2 orders of magnitude slower than diffusion rates in aqueous media. Overall we have provided a further step toward the understanding of solid phase biocatalysis and showed that TPM provides a useful analytical tool for analysis of the kinetics of this class of reactions.

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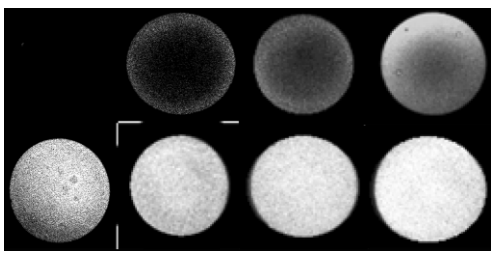

Fig. 1 Thermolysin catalysed hydrolysis of $PEGA_{1900}$ bound dipeptide **1** as examined by TPM. From left to right the images represent 5, 10, 20,45, 60, 90, 120 and 240 min.

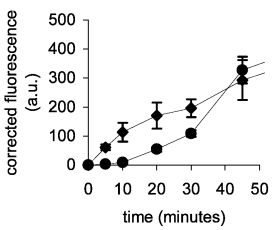

Fig. 2 Spatially resolved analysis of fluorescence intensities at the edge (●) and centre (◆) of $PEGA_{1900}$ beads bearing **1**. Initially the reaction is limited by diffusion of enzyme into bead, but after 45 min the enzyme had accessed all areas within the bead resulting in the same reaction rate throughout the bead.

Notes and references

† The initial rate of increase in fluorescence intensity at the surface is, per min, 1.3% of the maximal value for the fully dansylated bead. This is equivalent to 0.13 mmol $(L\ min)^{-1}$ based on an amino group loading of 0.1 mmol g^{-1} and the swollen bead volume of 10 mL g^{-1} (both taken as uniform).[5]

‡ It was not possible to measure rates accurately with Fmoc–Phe–Phe–NH_2, because of its very low aqueous solubility.

§ The biocatalytic rate constant for the reaction in terms of enzyme concentration is around $10^5\ M^{-1}\ s^{-1}$. This is 3 orders of magnitude smaller than the diffusion limit and hence formation of the enzyme/substrate complex does not appear to be rate limiting.

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