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Highly stable protein immobilization *via* maleimido-thiol chemistry to monitor enzymatic activity†

 Jonas Schartner,^a Jörn Güldenhaupt,^{ID a} Sarah Katharina Gaßmeyer,^b
 Katharina Rosga,^a Robert Kourist,^{ID ‡ b} Klaus Gerwert,^{ID a} and Carsten Kötting^{ID * a}

Immobilizing enzymes for biocatalysis offers many advantages, including easy separation of the enzyme from the product and repeated and continuous use. ATR-FTIR spectroscopy is a versatile tool to monitor immobilized enzymes and has been applied to many proteins. However, while the common and convenient immobilization *via* oligohistidine on mono-NTA layers is adequate for the measurement of difference spectra induced by ligand binding or photochemistry, it lacks the long term stability that is necessary for monitoring biocatalysis. Here, we report a new immobilization methodology based on maleimido-thiol chemistry. A 12-mercaptododecanoic acid NHS ester monolayer is reacted with 1-(2-aminoethyl)-maleimide to build a thiol reactive surface. Subsequently, NTA-C16-thiol is covalently attached and finally oligohistidine tagged enzymes were immobilized to this surface, which remained bound with a five times higher EC₅₀-value compared to typical mono-NTA layers. To demonstrate the high potential of the surface we analysed decarboxylation reactions catalyzed by arylmalonate decarboxylase. With ATR-FTIR both the enzyme and its substrate conversion can be monitored label free. Correct folding of the enzyme can be evaluated based on the amide band of the immobilized enzyme. In addition, the infrared absorption spectra of educt and product are monitored in real time. We show that hybrid hard-soft multivariate curve resolution improves separation of the product and educt spectra from other effects during the experiments, leading to clean kinetic traces and reaction rates for the catalytic process. Our approach can in principle be extended to any enzyme and is ideally suited for the development of biocatalysts.

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Introduction

The catalytic application of enzymes is an important and dynamic research field. Especially, the rational design for biotechnology approaches requires a well characterized enzyme.^{1,2} Many conventional assays are performed in solution and use absorbance or fluorescence spectroscopy for analysis of the efficiencies of the substrate conversions. Immobilization of proteins provides many advantages. In biocatalysis, these are improved handling, reusability and easy separation of the enzyme from the product.³ During enzyme characterization and optimization, ATR-FTIR spectroscopy can deliver infor-

mation concerning the orientation, the secondary structure, the activity (substrate conversion), and the interaction with small molecules or other proteins.^{4–11} We and other groups demonstrated the high potential of germanium ATR-crystals for protein attachment.^{10,12–16} Especially, the stability of the protein is crucial for a reliable investigation. Besides the covalent immobilization *via* NHS-ester/amine coupling,¹⁵ maleimido/thiol linkage¹⁷ or others,¹⁸ the immobilization *via* histidine-tag is still an excellent opportunity.^{19,20} This idea was refined by synthesizing derivatives of the NTA head group with up to four divalent cation chelating residues in solution and three residues for immobilization.^{21,22} Besides the complexation of Ni(II), recently the oxidation of Co(II) to Co(III) was employed to deliver highly stable and permanent immobilized proteins.^{23,24} The disadvantages of these methods are a time-consuming synthesis of the tris-NTA and in case of the cobalt, the usage of hydrogen peroxide which would damage most proteins. Therefore, we focused on the establishment of a methodology which is easily accessible due to commercially available compounds and which provides a platform for highly stable natively folded immobilized proteins. This was achieved

^aDepartment of Biophysics, Ruhr-Universität Bochum, Universitätsstrasse 150, 44801 Bochum, Germany. E-mail: carsten.koetting@rub.de

^bJunior Research Group for Microbial Biotechnology, Ruhr-Universität Bochum, Universitätsstrasse 150, 44801 Bochum, Germany

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‡Current address: TU Graz, Institut of Molecular Biotechnology, Petersgasse 14, 8010 Graz, Austria.

by combining different chemical surface modifications, which finally lead to a dense NTA surface *via* 1-(2-amino-ethyl)-pyrrole-2,5-dione (maleimido-amine) and -[N α ,N α -bis(carboxymethyl)-L-lysine]-16-mercapto-hexadecane-amide linkage (NTA-thiol). Each step of the reaction was monitored in real time with spectral resolution due to the usage of ATR-FTIR, which is a great advantage in contrast to common techniques like surface plasmon resonance or quartz crystal microbalance. The chemically modified ATR-germanium crystal was loaded with Ni(II) and this system was at first investigated with the small GTPase N-Ras_{1-180-His10} to have a gold standard for comparison with recently developed systems.^{10,12,13,25,26} As model enzyme we analysed the arylmalonate decarboxylase (AMDase), which performs the decarboxylation of several malonic acid derivatives, providing direct access to optically pure α -substituted carboxylic acids.²⁷ In order to obtain the pure spectra of both, the educts and the products, and the kinetics of the enzymatic reaction of the immobilized AMDase in addition, we utilized multivariate curve resolution alternating least squares (MCR-ALS) analysis. MCR-ALS is a powerful method for analysing spectroscopic data recorded from chemical reactions²⁸ and is capable of extracting pure spectra from the involved chemical components together with their respective concentration profiles. The term concentration profile can thereby represent reaction time, retention time (in chromatographic applications), pH range or any other variable which is linked to the analysed spectra series. This method allowed us to filter out artefact spectra and directly extract the pure educt and product spectra and the respective rate constants.

Experimental

Arylmalonate decarboxylase

Arylmalonate decarboxylase from *Bordetella bronchiseptica* was produced and purified as described.²⁹

ATR-FTIR

The detailed experimental set-up for the ATR-FTIR measurements was described recently.^{10,12,13}

Surface preparation

If not stated otherwise all chemicals were purchased by Sigma-Aldrich (Taufkirchen, Germany). In the first step, the germanium ATR-crystal was functionalized with 12-mercaptododecanoic acid NHS-ester outside the spectrometer as published previously.¹³ Before each reaction step, a new reference spectrum in the desired solvent was recorded. The thiolation was done in 2-propanol, therefore a 3 min water rinsing step was required at the beginning, before equilibrating the surface with the reaction buffer 50 mM NaPi (pH 6.5). In this buffer a solution 30 mM of 1-(2-amino-ethyl)-pyrrole-2,5-dione hydrochloride (maleimido-amine) was flushed over the functionalized surface for 120 min. Subsequently, the surface was washed to remove unreacted molecules and a 2.5 mM solution of (pH 6.5) N-[N α ,N α -bis(carboxymethyl)-L-lysine]-16-mercapto-

hexadecanamide in 50 mM NaPi was applied overnight. In case of the control experiment 3-mercapto-1-propanol (10 mM in reaction buffer) was employed as described above. After the coupling of the maleimide with the desired thiol and a washing step, the surface was equilibrated with binding buffer (20 mM Hepes pH 7.4, 150 mM NaCl). To enable binding of histidine-tagged proteins the surface was loaded with 30 mM NiCl₂ (binding buffer) for 10 min. After rinsing the surface with binding buffer, a 3 μ M solution of casein was applied to suppress unspecific protein binding.¹¹ Subsequently, unbound proteins were removed by washing and the germanium ATR-crystal was equilibrated for at least 15 min with protein binding buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 0.1 mM GDP). Finally, the desired protein (3.8 μ M for AMDase; 1 μ M for N-Ras) was injected into the system and circulated for 45 min. After the successful immobilization, the surface was washed to remove unbound proteins. The substrate conversion by AMDase was directly monitored by adding 1 mM naproxen malonic acid or flurbiprofen malonic acid to the immobilized protein. The activity of the Ras protein was measured as described elsewhere.¹²

MCR-ALS

Before performing the MCR-ALS analysis the ATR spectra were baseline corrected using a linear baseline with the mean of the spectral regions 2800–2780 cm⁻¹ and 1860–1850 cm⁻¹ as supporting points. Afterwards, the spectra were cropped from 1900 to 1150 cm⁻¹ and the MCR-ALS analysis was started. The decomposition of the measured data $D(m \times n)$ with m spectra and n points on the spectral axis by use of a bilinear model into a number of p component spectra $S^T(p \times n)$ and their corresponding concentration profiles $C(m \times p)$ results in a residual matrix $E(m \times n)$ which ideally contains only noise:

$$D = CS^T + E$$

No information about reaction chemistry or kinetics is needed to perform an MCR-ALS analysis, but applying constraints on the analysis helps to reduce the rotational ambiguity of the results. Besides classical constraints, such as non-negativity and closure we used the “hard-soft” kinetic modelling approach (HS-MCR) which set as shape constraint on the concentration profile that is derived from a reaction scheme.^{30–32} The number of components was set to four in each experiment; one educt, one product and two artefact carrying components were used. The initial estimation of the concentration profiles of these four components was obtained by normalizing the difference between the absorbance kinetics at 1363 and 1338 cm⁻¹ (each average of a 5 cm⁻¹ region) for the educts, the complementary of this for products and two random number vectors of between 0 and 1×10^{-5} for the artefacts. MCR-ALS constraints can be used to minimize the rotational ambiguity of possible solutions. We applied non-negativity and a closure (= 1) constraint on educt and product concentration profiles; both using the *fnnls* algorithm. Additionally, we applied a shape constraint on the concen-

tration profiles (mixed “hard-soft” modelling) computed from the kinetic model of the first order reaction $A \rightarrow B$. The model was applied to the first two components and was initialized with rate constants of 0.001 s^{-1} whereas components 3 and 4 were optimized with no kinetic constraints. The spectra pre-processing and the MCR-ALS analysis using the freely available MCR-ALS Toolbox 2.0³³ (<http://www.mcrals.info>) were performed using MATLAB R2016b (Mathworks, Natick, MA, USA).

Results and discussion

Formation of a maleimido-terminated monolayer

We investigated the generation of a maleimido-terminated surface and the adjacent reaction with molecules bearing a terminal thiol-group. At first, a polished germanium surface was activated with hydrofluoric acid (HF), followed by the incubation with 12-mercaptododecanoic acid NHS ester for 24 h, as described previously.^{13,34} The modified germanium surface was subsequently analysed in the FTIR spectrometer. By adding a 30 mM solution of 1-(2-aminoethyl) maleimide hydrochloride (maleimido-amine) in sodium phosphate buffer (50 mM, pH 6.5) the NHS-esters were substituted, and the maleimido-group was covalently attached (Fig. 1 and 2). The maleimido-amine is highly water soluble and a small molecule compared to the bulky $N\alpha,N\alpha$ -bis(carboxymethyl)-L-lysine (NTA-amine), which is usually used.^{10,35} The reaction is almost complete after 2 h as indicated by the kinetics shown in Fig. 2B, which illustrates the disappearance of the NHS-esters by the negative band at 1730 cm^{-1} and the formation of the maleimido group with the positive band at 1562 cm^{-1} . This band corresponds to the amide II vibration of the formed peptide bond, which is also indicated by the amide I band at 1630 cm^{-1} . The intensity of the amide I is diminished due to the displacement of water molecules from the probe volume by the attached molecule, resulting in a negative absorbance

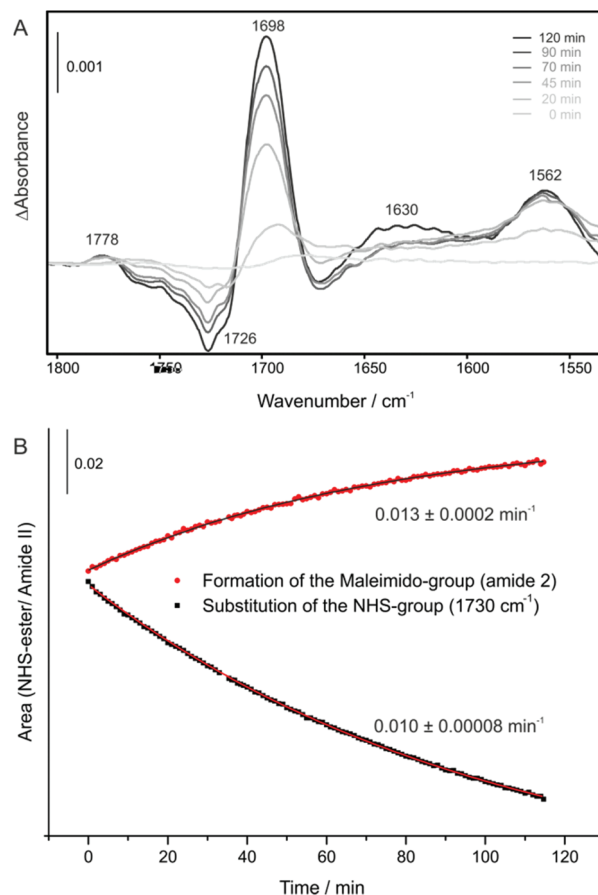


Fig. 2 (A) ATR-FTIR spectra of the reaction of 1-(2-aminoethyl) maleimide hydrochloride with the NHS-ester modified germanium surface. The positive bands at 1778 and 1698 cm^{-1} correspond to the maleimido-carbonyls, whereas the negative band at 1730 cm^{-1} is caused by the substituted NHS-ester. The formed peptide bond is characterized by the amide I (1630 cm^{-1}) and the amide II (1562 cm^{-1}). (B) Kinetics of the maleimido-amine reaction of the NHS-thiol terminated surface. The red kinetics (black fit) shows the growth of the formed amide II linkage, whereas the black kinetics (red fit) represents the substituted NHS-moiety.

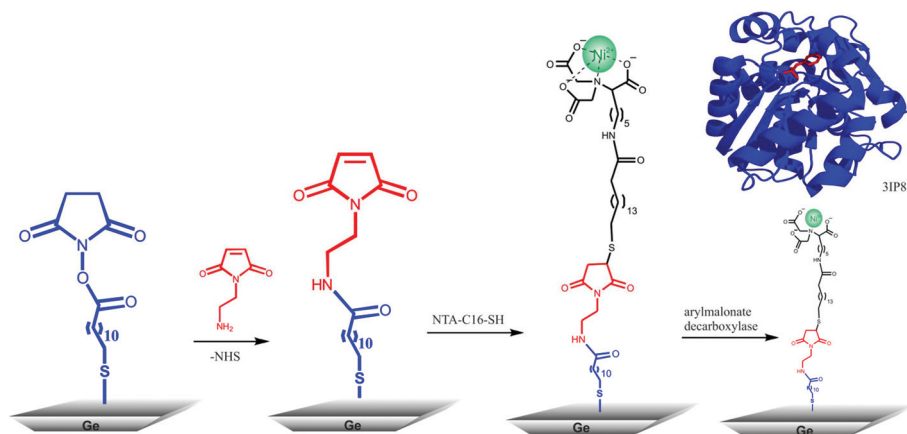


Fig. 1 Scheme of the performed surface chemistry to generate a maleimido terminated surface as a universal acceptor for thiol-carrying molecules. In the first step, an NHS-thiol terminated monolayer (blue) is prepared as described in the literature.¹³ Subsequently, the NHS-ester is reacted with 1-(2-Aminoethyl)maleimide (red). The maleimido-group was reacted with NTA-C₁₆-thiol, yielding an oligohistidine-tag capturing sensor surface. Finally, the target enzyme (here AMDase) can be easily attached to the surface.³²

in the amide I region. The successful reaction is further characterized by the positive bands at 1778 and 1698 cm^{-1} from the carbonyl groups of the maleimido-ring (Fig. 2A). The approach is simple, fast, and convenient because it can be performed in an aqueous environment, all components are commercially available, and no toxic organic solvents are required after assembling of the cuvette. Other promising approaches that also used maleimido-thiol linkage for protein immobilization required prior organic synthesis and a more complex surface chemistry.¹⁷ Due to the application of the thiol-chemistry, many tailored groups are commercially available and allow for stable modification of germanium.¹³

Reaction of the maleimido-group with thiols

To investigate the reactivity of the maleimido-group we employed ATR-FTIR difference spectroscopy. In phosphate buffer 10 mM of 3-mercaptopropanol was dissolved and the maleimido-terminated surface was incubated with this thiol overnight. After washing the surface to remove unbound molecules the resulting average spectrum of the bound 3-mercaptopropanol was obtained, which shows two negative bands at 1772 and 1700 cm^{-1} (Fig. S1A†). These bands correspond to the carbonyls in the maleimido-ring, which appear due to the different bond orders after addition at the double bond of the maleimido-group (Fig. S1A†). The positive band at 1645 cm^{-1} can be assigned to the OH-bending or a carbonyl vibration of the product (Fig. S1A†). Please note that the water HOH bending vibration is at the same position and might interfere. The corresponding kinetics of the changes in the carbonyls of the maleimido-group (1700 cm^{-1}) and of the bound hydroxyl-function (1645 cm^{-1}) are shown in Fig. S1B.† The successful binding of 3-mercaptopropanol demonstrates the reactivity of the maleimido group, which can be seen as a universal linker to tether thiol-carrying groups to the germanium surface. Due to the universality of the approach and common usage of histidine tags, immobilization on terminated surfaces Ni-NTA was the favourable concept pursued here.

Assembly of Ni-NTA via maleimido-linkage

To further analyse the reactivity of the surface we employed *N*-[N α ,N α -bis(carboxymethyl)-L-lysine]-16-mercaptohexadecanamide (NTA-C16-SH) to generate the desired Ni-NTA surface. In contrast to previous reports using NHS-ester and amino-functionalized NTA the competing hydrolysis reaction was excluded due to the Michael addition of thiol-group and the double bond of the maleimido moiety.^{10,12,13,35} A general reaction scheme is presented in Fig. 1. The usage of Ni-NTA is a very recommendable approach since proteins bearing a histidine-tag are easily accessible.²¹ Upon the addition of 2.5 mM NTA-C16-SH (50 mM NaPi, pH 6.5) several bands were observed in the ATR-FTIR spectrum. The vibrational bands at 2919 and 2850 cm^{-1} can be assigned to asymmetric and symmetric stretching mode of the CH_2 -groups of the elongated hydrocarbon chain (Fig. 3A). Further bands were detected in the region 1700–1778 cm^{-1} , these most likely correspond to the maleimido-carbonyls. The carboxylic acids are character-

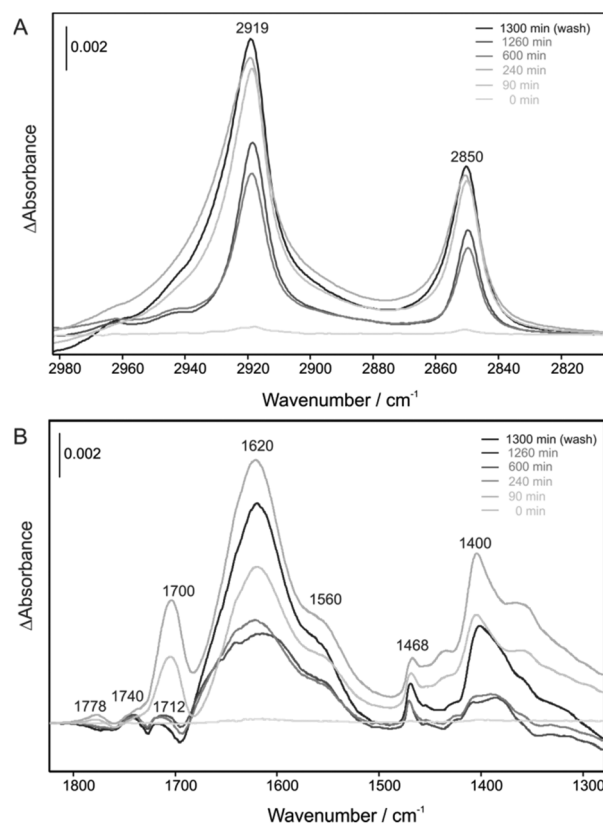


Fig. 3 (A) Reaction of NTA-C16-SH with the maleimido-terminated surface. The asymmetric stretching vibration at 2919 cm^{-1} and the symmetric stretching vibration at 2850 cm^{-1} were shown over time. (B) Characteristic bands of the carboxylic acids from the NTA moiety are shown, for example, the asymmetric at 1620 cm^{-1} and the symmetric stretching vibration at 1400 cm^{-1} . The additional bands show that a part of the acids is protonated and also further combinational vibrations.³⁵

ized by the asymmetric stretching vibration at 1620 cm^{-1} and the symmetric stretching vibration at 1400 cm^{-1} (Fig. 3B). The covalent attachment of the NTA-group was further analysed by the addition of 30 mM nickel chloride to the system. The resulting difference spectrum shows three signals in the CH-region at 2962, 2916 and 2650 cm^{-1} that indicate a structural change in the NTA-molecule (Fig. S2A†). Furthermore, three negative bands at 1710 cm^{-1} , 1640 cm^{-1} , and 1390 cm^{-1} represent the previous state of carboxylic acids, showing probably a small amount of deprotonation (Fig. S2B†). Positive bands at 1583 and 1410 cm^{-1} are shifted and can be assigned to the asymmetric and symmetric stretching vibration of the carboxylic acids (Fig. S2B†).¹² The high intensity compared to previous reports of Ni-coordination spectrum is probably caused by a higher surface concentration of the NTA groups. Such intense signals were observed on dextran-brushes with a higher number of reactive residues.¹²

Protein immobilization on Ni-NTA

To investigate the Ni-NTA surface N-Ras1-180 with decahistidine-tag was flushed over the prepared germanium crystal. As

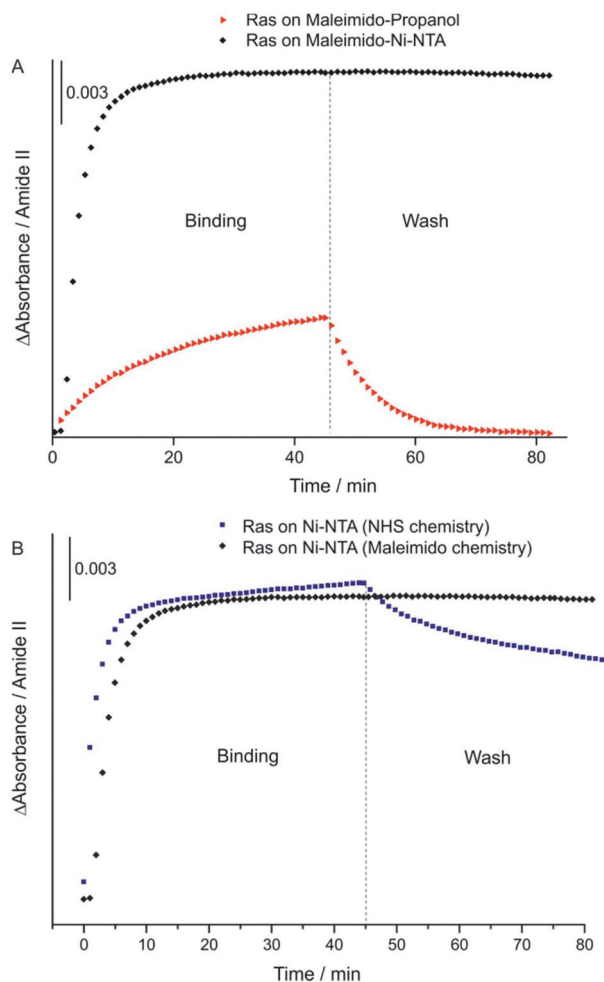


Fig. 4 (A) Immobilization kinetics of N-Ras with decahistidine-tag on maleimido-Ni-NTA (black) in comparison with a control surface maleimido-propanol (red). The black kinetics shows a fast and extremely stable binding of N-Ras. In contrast to this, the red kinetics shows much less binding and nearly all proteins are detached by washing with buffer. (B) Comparison of the immobilization with typical NTA surfaces (blue).³⁶

shown in Fig. 4 (black squares) the Ras protein binds within 15 min and remains extremely stable after washing the surface with buffer. The amount of immobilized proteins corresponding to an amide II absorption of 16 mOD is in agreement with a dense protein monolayer.¹³ In comparison to our recent work, the stability is strongly increased (Fig. 4B).¹³ Usually, significant amounts of protein bound to a mono-Ni-NTA surface are washed off within 45 min (Fig. 4B). With the maleimido based approach, the stability was strongly increased during washing with buffer as shown by the kinetics in Fig. 4A/B. The amount of protein that remains immobilized after 90 min of washing is 98.4%, in comparison to the previous surfaces that

showed 71.6% immobilized after 90 min of washing.³⁶ The high stability of the protein-immobilization was further analysed by determining the EC_{50} -value with different imidazole concentrations, which cause an elution of the protein from the surface (Fig. S3†).

The obtained value of 68 ± 7 mM imidazole is five times higher than the values obtained for other surface immobilizations based on mono-NTA (Table 1).^{13,22} This finding suggests that our new approach yields a higher Ni-NTA surface concentration compared to the literature. As discussed above the maleimido-thiol reaction is very specific and does not compete with a hydrolysis reaction and thus enables the formation of very dense Ni-NTA monolayers as also indicated by the strong absorbance signals as shown in Fig. 3A and B. The NTA absorbance spectra are over ten times higher compared to our recent study, indicating a higher surface concentration is causing the high stability.¹³ The obtained EC_{50} -value is comparable to values as described for tris-Ni-NTA by Piehler and coworkers.^{21,22} The usage of tris-Ni-NTA requires a complex organic synthesis, whereas the approach described here is easily accessible and the compounds are commercially available.

In a control experiment, the Ras protein was flushed over a maleimido modified surface that was reacted with 3-mercaptopropanol. Small amounts of Ras protein bind to this surface, but after washing the surface with buffer all proteins were rinsed off (Fig. 4A, red triangle). This emphasizes the specific immobilization ($\geq 99\%$) *via* Ni-NTA. The long term stability was also investigated by washing the immobilized protein for six hours with in total 360 mL buffer (1 mL min^{-1} , 1 mL total volume in the system), which resulted in only a small decrease (8%) of the protein signal (Fig. S4†). A time frame of several hours is sufficient for analysing the protein and the great advantage of a highly stable immobilized protein is that the interaction with small molecules or ligands is not disturbed by negative protein dissociation bands. This was proven by the usage of beryllium fluoride, a small molecule that mimics the GTP state ("ON") in the Ras protein by interacting with bound GDP ("OFF"), which results in high quality difference spectra of the protein (Fig. S5†). Due to the high stability, also ligands or potential drugs for the Ras protein or other target proteins could be employed and analysed in real-time.

In the first instance, it seems surprising that the new methodology results in such a significant improvement compared to former reports, but several reasons contribute: the attachment of the NTA groups *via* the maleimido-thiol chemistry does not compete with a hydrolysis reaction as in the case of the reaction of an NHS ester with an amine. Further, the linker consists of two long hydrophobic carbohydrate chains separated by the hydrophilic region with an

Table 1 Comparison of the EC_{50} -values for the displacement of decahistidine tagged proteins from different NTA surfaces by Imidazole

Surface	Mono NTA (Schartner <i>et al.</i>) ¹³	Mono NTA (Lata <i>et al.</i>) ²²	Tris NTA (Lata <i>et al.</i>) ²²	Mono NTA (this work)
EC_{50}	13.3 mM	13.8 mM	61.0 mM	68 mM

amide bond and the reacted maleimido group. This leads to an improved separation from the germanium surface and to a more flexible cushion like surface that can adapt better to the enzyme and its oligohistidine tag.

Analysis of decarboxylases

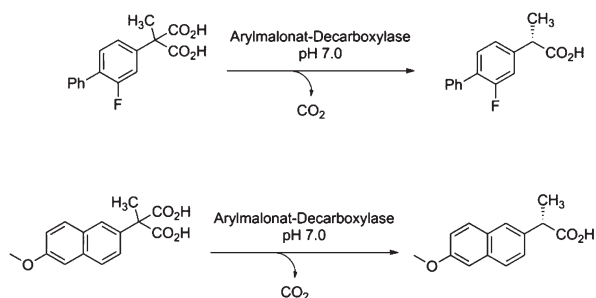
While the stability of the former surfaces was sufficient for obtaining difference spectra of light induced or ligand induced reactions from fast processes, they were not suitable for following enzymatic reactions over hours. With the method developed here, also slow reactions and low affinity protein reaction are accessible. The observable is not the influence of the ligand on the enzyme but the secondary structure of the protein and the conversion of the ligand in solution. The analysis of enzymatic reaction requires a stable immobilization of the target protein. Therefore, we employed the developed approach to monitor the enzymatic decarboxylation. As candidate enzyme the arylmalonate-decarboxylase (AMDase)^{27,29} was immobilized and the reactions were investigated by ATR-FTIR. The AMDase catalyses the decarboxylation of arylmalonic acids resulting in an optically pure α -arylpropionic acid (Scheme 1).

The protein binds within 30 min and remains stable on the surface after washing with buffer (Fig. S6†). The activity of the immobilized enzyme was tested with different substrates. The time series of spectra were measured against a buffer background in a circulating flow through system and therefore represents a mixture of the educt and product spectra in varying intensities according to the concentrations of the substances in the circulating volume at the spectra recording time. The spectra time series except those early spectra showing the process of filling of the cuvette with educt solution were analysed using HS-MCR to extract the pure educt and product spectra and their corresponding concentration profiles as shown in Fig. 5. As the intensity of the recorded difference spectra is very low compared to the overall absorbance of the sample obtaining a mixture of signal and artefact spectra is a common issue in thin-film ATR-FTIR spectroscopy. Artefact spectra could arise from surface instabilities, educt or product surface adsorption, temperature effects or other setup related spectral influences. These artefact processes were separated

from the signals of interest by HS-MCR because of their reaction-uncorrelated concentration profiles (Fig. S7†). The extracted difference spectra obtained by MCR analysis, thus providing a clear spectrum of the educt (black) and the product (grey) for each substrate as shown in Fig. 5. The ATR-FTIR difference spectrum revealed a band pattern in the region between 1700 and 1200 cm^{-1} . The bands in between 1700 and 1500 cm^{-1} are probably caused by the asymmetric stretching vibration of the carboxylates and the aromatic ring system of the substrates. The bands at 1393–1330 cm^{-1} correspond to the symmetric stretching of the acid (Fig. 5). The main differences in the educt/product IR-spectra were the disappearance of the 1408 and 1339 cm^{-1} bands, which result from the removal of the carboxylic acid and the appearing bands at 1655, 1624, 1393 and 1364 cm^{-1} probably correspond to changes in the aryl ring system and the remaining acid residue (Fig. 5). The obtained reaction rate for the conversion of flurbiprofen malonic acid to flurbiprofen was $1.7 \times 10^{-3} \text{ s}^{-1}$. The conversion of naproxen malonic acid into naproxen is faster with a rate of $4.7 \times 10^{-3} \text{ s}^{-1}$. Similar rates are obtained by an exponential fit of the absorbance difference of a product (1339 cm^{-1}) and an educt (1364 cm^{-1}) band (Fig. S8†). The 2.8-fold higher activity of the (*R*)-selective wildtype enzyme in the synthesis of naproxen compared to flurbiprofen agrees well with the decarboxylation with purified enzyme in solution, where naproxen malonate was converted with 90 U mg^{-1} and flurbiprofen with 40 U mg^{-1} .²⁹ In conclusion, the analysis of AMDase by ATR-FTIR provides detailed information about the educts/products of the biochemical reaction and can in principle be used as universal tool for the analysis of enzymatic reactions. The method has been used for four other proteins. Only one of these was not active on this surface but was also inactive on all other types of NTA surfaces we prepared. We assume that certain proteins are incompatible with the high charge density of a dense NTA surface.

Enzymes on surfaces

The analysis of enzymes by immobilization is a widely used approach. Protein immobilization is achieved by direct adsorption, which is an unspecific process highly depending on the intrinsic stability of the protein.^{37,38} Besides adsorption the covalent immobilization is a common approach, often performed by amine NHS-ester coupling.^{15,39} Furthermore, the usage of polymers caving the proteins is a promising concept,^{12,40} especially in biocatalysis applications.^{41,42} In addition, the immobilization *via* His-tag is still a common and reliable system for investigating proteins on surfaces or also for protein purification.^{10,23,43} The advantages of the presented assay, a surface providing highly stable immobilized enzymes combined with ATR-FTIR techniques, is the direct information about the biochemical reaction and the enzyme itself without requiring any label. The revealed IR-spectrum can be seen as a biochemical fingerprint of the reaction and can help to identify unknown products of the enzymatic reaction. Since the enzyme is embedded in flow through system reactants or



Scheme 1 Decarboxylation of flurbiprofen malonic acid into flurbiprofen (above) and naproxen malonic acid into naproxen (below) catalysed by AMDase.

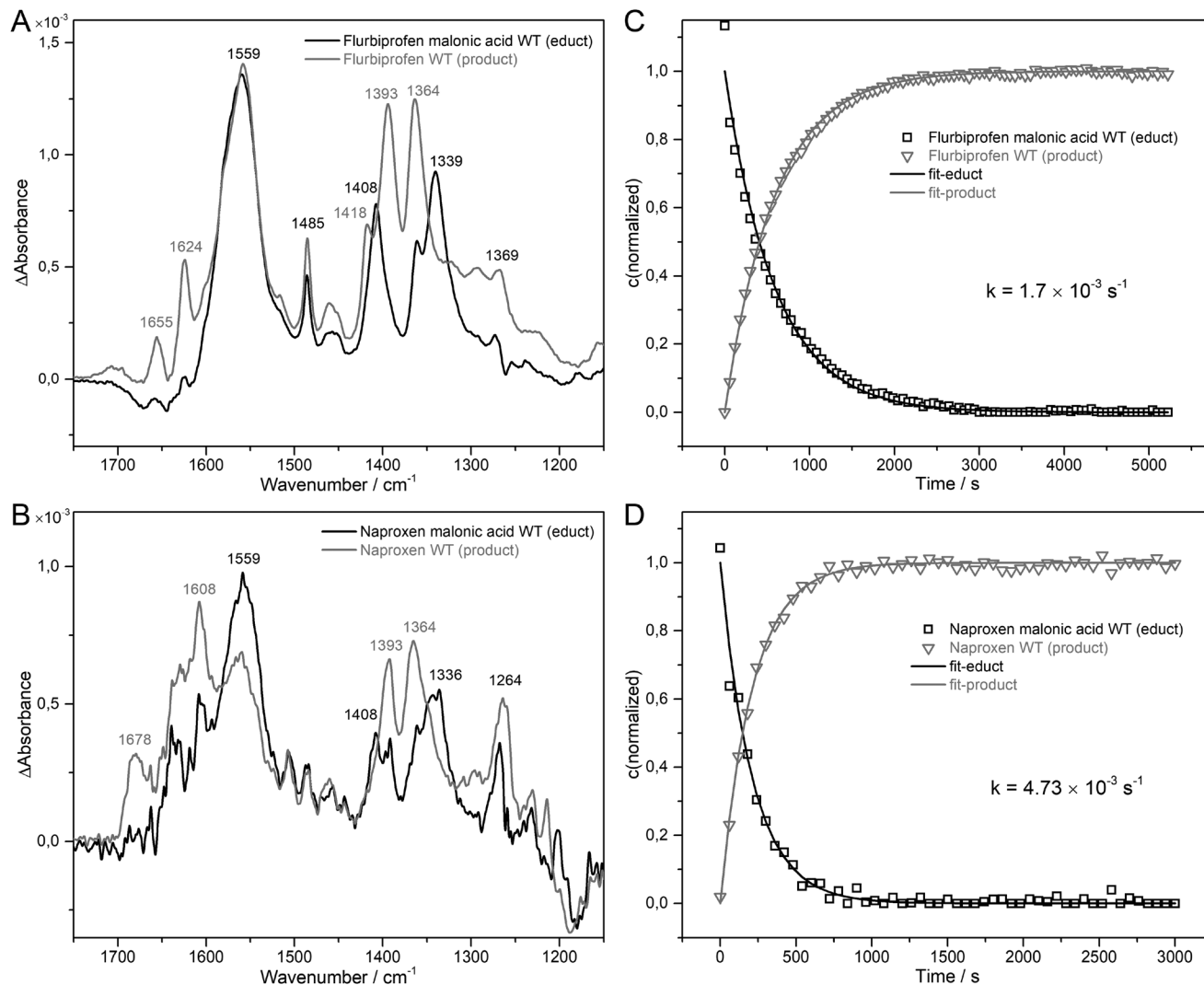


Fig. 5 Pure educt and product spectra of the AMDase catalysed decarboxylation reactions as extracted from HS-MCR analysis of the time series of ATR-FTIR spectra. The resulting spectra from AMDase WT with flurbiprofen malonic acid (A) and naproxen malonic acid (B) are and the corresponding concentration profiles are shown.

different conditions are easily applied and the information is directly obtained providing real time kinetics.

Comparison with other recent techniques

Protein immobilization is an emerging field. Antibodies are immobilized to create biosensors⁴⁴ or even diagnostic tools.^{11,45} Enzymes are also immobilized in biocatalysis applications.^{46,47} Recently, the use of cobalt(III) as a mediator for NTA showed the formation of an irreversible complex between NTA and protein.^{48,49} To obtain such a complex hydrogen peroxide is required. In our hands, this was not compatible with immobilized Ras and we even observed a damage of the germanium surface.

The properties as an internal reflection element in the mid IR of germanium are superior to other materials. We recently developed three approaches for protein immobilization on germanium employing silane-chemistry,¹⁰ thiol-chemistry¹³ and

finally dextran-brushes for highly packed protein surfaces.¹² So far, surface plasmon resonance,^{50,51} surface acoustic waves⁵² or quartz crystal microbalance^{53,54} are often employed for protein immobilization, but they all lack the spectral resolution. Without the spectral information, the chemical functionalization is performed blind and without a control of the progress. Furthermore, analysis of substrates especially the functional groups is not possible. Nevertheless, the advantages are that these systems only need a small amount of sample and are commercially available. In general more effort is needed to make germanium more easily accessible for protein immobilization.

In conclusion, we have shown that maleimido chemistry provides advantages in the assembly of surfaces for protein immobilization that enables new applications. The newly developed immobilization technique will be also interesting for other surfaces like gold or silicon because highly stable immobilization of proteins is crucial for any analysis on any surface.

Conflicts of interest

There are no conflicts to declare.

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