Polydimethylacrylamide surface functionalization for the optimization of enzymatic assays in technical and diagnostic applications

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Summary

Enzymes are biochemical reaction catalysts that are of crucial importance for everyday life. Beside playing an essential role in accelerating and regulating biochemical reactions in living organisms, enzymes contribute significantly to both technical and medical applications. The ability to regulate the rate and specificity of reactions gives enzymes their importance. In technical applications, enzymes have already led to significant changes as they offer a sustainable and environmentally friendly alternative to conventional chemical processes. For healthcare, enzymes are valuable diagnostic and therapeutic targets as they are associated with a wide range of diseases.

The objective of this work was to address current limitations and drawbacks of enzymatic applications, while also optimizing their applicability and expanding their scope. The first aim was to develop a method to enhance existing enzymatic processes, thus providing an essential tool on the way to greener and more sustainable chemistry. In the second part of the project, this method was to be adapted for the use in a diagnostic assay to detect bacterial enzymes. In order to achieve these goals, different strategies involving polydimethylacrylamide surface functionalization were implemented for enzymatic applications in technical and biomedical contexts.

In order to enhance enzymatic assays for more sustainable reactions, different polymer surfaces were functionalized with a polydimethylacrylamide hydrogel in a simple one-step method. PDMA was crosslinked as a copolymer with methylacryloylbenzo-phenone by UV-induced C,H-insertion reactions and thus immobilized on the surface. The surface functionalization aimed to increase the hydrophilicity of the polymer surfaces, leading to less non-specific adsorption of enzymes. Various enzymatic assays were used to test the effect of the surface functionalization on enzyme performances. The integration of PDMA led to decreased non-specific adsorption of the enzymes to the surfaces, resulting in enhanced enzymatic performance in all assays. The study revealed significant increases in enzymatic substrate conversion, and both initial and maximum velocities.

In course of this research, the one-step UV-crosslinking process for PDMA surface functionalization was further modified by adding β-lactam antibiotics and β-lactamase inhibitors. The incorporation of these molecules aimed to provide additional functio-

nality to the PDMA-functionalized surfaces, with the intention of optimizing β-lactamase assays and potentially improving diagnostic applications as well as antibiotic susceptibility testing for bacterial infections. Representatives of different classes of β-lactam antibiotics and β-lactamase inhibitors were successfully immobilized on the surface and the interaction with various β-lactamases was tested in a chromogenic β-lactamase assay. Results proved that sufficient amounts of antibiotics and inhibitors were immobilized using this method and that they are not losing their accessibility for β-lactamase bindings. The assays comprising various antibiotics and inhibitors revealed that the developed surface functionalization enabled successful susceptibility testing of β-lactamases from different classes. Antibiotic susceptibility testing was also proved possible for β-lactamases isolated from bacterial cultures, which mimicked clinical samples. During this study it was further accomplished to transfer the method of antibiotic immobilization to 3D printed microfluidic devices, paving the way for the development of point-of-care tests.

Zusammenfassung

Enzyme sind biochemische Reaktionskatalysatoren, die für das tägliche Leben von entscheidender Bedeutung sind. Neben ihrer essentiellen Rolle bei der Beschleunigung und Regulierung biochemischer Reaktionen in lebenden Organismen, leisten Enzyme einen wichtigen Beitrag zu technischen und medizinischen Anwendungen. Enzyme sind aufgrund ihrer Fähigkeit die Geschwindigkeit und Spezifität von Reaktionen zu regulieren, von großer Bedeutung. In technischen Anwendungen haben sie bereits zu bedeutenden Veränderungen geführt, da sie eine nachhaltige und umweltfreundliche Alternative zu herkömmlichen chemischen Verfahren darstellen. Im Gesundheitswesen sind Enzyme wertvolle diagnostische und therapeutische Ziele, da sie mit einer Vielzahl von Krankheiten in Verbindung gebracht werden.

Ziel dieser Arbeit war es, den derzeitigen Limitationen und Nachteilen enzymatischer Anwendungen entgegenzuwirken und gleichzeitig deren Anwendbarkeit zu optimieren und deren Anwendungsbereich zu erweitern. Das erste Ziel bestand darin, eine Methode zur Verbesserung bestehender enzymatischer Prozesse zu entwickeln. Dadurch soll ein wesentliches Instrument auf dem Weg zu einer grüneren und nachhaltigeren Chemie bereitgestellt werden. Im zweiten Teil des Projekts sollte diese Methode für den Einsatz in einem diagnostischen Assay zur Detektion bakterieller Enzyme angepasst werden. Um diese Ziele zu erreichen, wurden verschiedene Strategien mit Polydimethylacrylamid-Oberflächenfunktionalisierungen in enzymatischen Anwendungen in einem technischen und biomedizinischen Kontext umgesetzt.

Um enzymatische Assays für nachhaltigere enzymatische Reaktionen zu verbessern, wurden verschiedene Polymeroberflächen in einem einfachen einstufigen Verfahren mit dem Hydrogel Polydimethylacrylamid funktionalisiert. PDMA wurde durch UV-induzierte C,H-Insertionsreaktionen als Copolymer mit Methylacryloylbenzophenon vernetzt und auf den Oberflächen immobilisiert. Die Oberflächenfunktionalisierung zielt darauf ab, die Hydrophilizität der Oberflächen zu erhöhen, was zu einer Verringerung der unspezifischen Adsorption von Enzymen führt. Die Auswirkungen der Oberflächenfunktionalisierung auf die Enzymleistung wurden anhand verschiedener enzymatischer Assays getestet. Die Integration von PDMA führte in allen Assays zu einer geringeren unspezifischen Adsorption der Enzyme an den Polymeroberflächen. Dadurch verbesserte sich die enzymatische Leistung in allen Assays. Die Studie ergab

eine signifikante Steigerung der enzymatischen Substratumwandlung, sowie der Anfangsgeschwindigkeit und der maximalen Geschwindigkeit.

Im Rahmen dieser Forschungsarbeit wurde der einstufige UV-Vernetzungsprozess für die PDMA-Oberflächenfunktionalisierung durch die Zugabe von β-Laktam-Antibiotika und β-Laktamase-Inhibitoren weiter modifiziert. Der Einbau dieser Moleküle sollte den PDMA-funktionalisierten Oberflächen zusätzliche Funktionalität verleihen, um β-Laktamase-Assays zu optimieren und potenziell diagnostische Anwendungen sowie Antibiotika-Empfindlichkeitstests für bakterielle Infektionen zu verbessern. Vertreter verschiedener Klassen von β-Laktam-Antibiotika und β-Laktamase-Inhibitoren wurden erfolgreich auf der Oberfläche immobilisiert und die Wechselwirkung mit verschiedenen β-Laktamasen in einem chromogenen Assay getestet. Die Ergebnisse beweisen, dass mit dieser Methode ausreichende Mengen an Antibiotika und Inhibitoren immobilisiert wurden, ohne ihre Bindungsfähigkeit an β-Laktamasen zu verlieren. Die Assays mit verschiedenen Antibiotika und Inhibitoren zeigten, dass die entwickelte Oberflächenfunktionalisierung eine erfolgreiche Empfindlichkeitsprüfung von β-Laktamasen aus verschiedenen Klassen ermöglicht. Diese war auch für β-Laktamasen möglich, die aus Bakterienkulturen isoliert wurden, die klinischen Proben imitieren sollten. Im Rahmen dieser Studie wurde zudem die Methode der Antibiotika-Immobilisierung auf 3D-gedruckte mikrofluidische Systeme übertragen, was den Weg für die Entwicklung von Point-of-Care-Tests ebnen könnte.

List of publications

Publication Project I

S. Rentschler, M. Borgolte, R. Csuk, S. Laufer, H.-P. Deigner, "Toward more Sustainable Enzyme Reactions: Enhancing Kinetics by Polydimethylacrylamide Implementation," *Green Chemistry* **2024**, *26*, 1653-1659

Publication Project II

S. Rentschler, M. Borgolte, A. Filbert, S. Laufer, H.-P. Deigner, "Highly efficient β-lactamase assay applying Poly-Dimethylacrylamide- based surface functionalization with β-lactam antibiotics and β-lactamase inhibitors," *Lab on a Chip* **2023**, *23*, 5120-5130

Project related review article

S. Rentschler, L. Kaiser, H.-P. Deigner, "Emerging Options for the Diagnosis of Bacterial Infections and the Characterization of Antimicrobial Resistance," *International Journal of Molecular Sciences* **2021**, 22, 456

Further publications

W. Schary, F. Paskali, S. Rentschler, C. Ruppert, G. E. Wagner, I. Steinmetz, H.-P. Deigner, M. Kohl, "Open-Source, Adaptable, All-in-One Smartphone-Based System for Quantitative Analysis of Point-of-Care Diagnostics," *Diagnostics* **2022**, 12, 589

A. A. Khan, S. Khan, S. Khan, S. Rentschler, S. Laufer, H.-P. Deigner, "Biosynthesis of iron oxide magnetic nanoparticles using clinically isolated *Pseudomonas aeruginosa," Scientific Reports* **2021**, 11, 20503

M. Borgolte, O. Riester, T. Kacerova, S. Rentschler, M. S. Schmidt, S. Jacksch, M. Egert, S. Laufer, R. Csuk, H.-P. Deigner, "Methacryloyl-GlcNAc Derivatives Copolymerized with Dimethacrylamide as a Novel Antibacterial and Biocompatible Coating," *Pharmaceutics* **2021**, 13(10), 1647

A. A. Khan, K.N. Manzoor, A. Sultan, M. Saeed, M. Rafique, S. Noushad, A. Talib, S. Rentschler, H.-P. Deigner, "Pulling the Brakes on Fast and Furious Multiple Drug-Resistant (MDR) Bacteria," *International Journal of Molecular Sciences* **2021**, 22, 859

M. Mahmoud, C. Ruppert, S. Rentschler, S. Laufer, H.-P. Deigner, "Combining aptamers and antibodies: lateral flow quantification for thrombin and interleukin-6 with smartphone readout," *Sensors and Actuators: B. Chemical* **2021**, 333, 129246

Author contributions

Contribution to Publication I

Simone Rentschler:	general concept, experimental work and data acquisition, data processing and interpretation, primary writing of the manuscript, proofreading and editing of the manuscript
Max Borgolte:	investigation (synthesis of PDMA), methodology
René Csuk:	scientific support, supervision, proofreading
Stefan Laufer:	scientific support, supervision, proofreading
Hans-Peter Deigner:	conceptualization and initiation of project, funding acquisition, supervision, review and editing of the manuscript, proofreading

Contribution to Publication II

Simone Rentschler:	general concept, experimental work and data generation, data processing and interpretation, writing of the manuscript and supporting information
Max Borgolte:	investigation (synthesis of PDMA), methodology
Alexander Filbert:	investigation (SEM image acquisition)
Stefan Laufer:	scientific support, supervision, proofreading
Hans-Peter Deigner:	conceptualization and initiation of project, funding acquisition, supervision, review and editing of the manuscript, proofreading

Abbreviations

AMR	antimicrobial resistance
AST	antibiotic susceptibility testing
BLI	β-lactamase inhibitor
BP	benzophenone
DBO	diazabicyclooctanone
ESBL	extended-spectrum β-lactamase
MBL	metallo-β-lactamase
MBP	methacryloylbenzophenone
PBPs	penicillin-binding proteins
PE	polyethylene
PDMA	poly(N,N-dimethylacrylamide)
pNPP	p-nitrophenyl phosphate
POC	point-of-care
PP	polypropylene
PS	polystyrene
SBL	serine-β-lactamase
TMB	3,3’,5,5’-tetramethylbenzidine

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1 Introduction

Enzymes are vital catalysts in biochemical reactions, and their specificity and ability to regulate reaction rates makes them indispensable tools in both technical and medical applications. They offer economic friendly alternatives to conventional chemical processes and serve as diagnostic and therapeutic targets in healthcare. This dissertation aimed to improve enzymatic applications by developing methods to enhance enzymatic processes and adapt them for diagnostic assays. To achieve this aim, strategies involving polydimethylacrylamide surface functionalization were employed. This chapter will introduce important aspects on surface functionalization as well as basics on enzymatic assays in technical and medical applications.

1.1 Polydimethylacrylamide surface functionalization

The following chapters provide an overview of the fundamental aspects of surface chemistry, the benefits of surface functionalization, and strategies for surface functionalization.

1.1.1 Surface chemistry

Surfaces play a crucial role in numerous chemical and biological processes across natural and industrial settings, primarily owing to their high chemical reactivity [1, 2]. A surface forms an interface with its surroundings, where various surface-related phenomena take place. The characteristics of the surface significantly affect these phenomena, directly impacting its interaction with biological components [3, 4]. Proteins rapidly adsorb from the surrounding solution onto the surface through diffusion processes as they approach an interface [5]. At the interface, proteins experience a partial loss of conformational and hydration energy [2]. Consequently, the interaction with the surface can trigger conformational and orientational rearrangements in the proteins [6]. The initial attachment of proteins and subsequent conformational changes are governed by the physicochemical properties of both the protein and the surface. These properties encompass electrostatic and hydrophobic interaction, isoelectric points, protein-protein interactions, ionic strength and temperature, as well as surface charge, topology, morphology, and roughness [7]. For instance, the overall hydrophobicity of the protein significantly influences the stability

of its structure, leading to varying degrees of structural rearrangements in the adsorbed molecule. Depending on the extent of structural rearrangement, this affects the activity of the protein to a greater or lesser degree [8]. The structural rearrangement in the protein molecule is also impacted by the hydrophobicity of the surface and is more prominently induced at hydrophobic surfaces. Furthermore, an increase in surface hydrophobicity corresponds to an augmentation in adsorption affinity [9, 10].

1.1.2 Surface functionalization

The phenomenon of protein adsorption on solid surfaces significantly impacts the performance of numerous materials and processes, making it imperative to exert control over this process. Surface functionalization emerges as a valuable tool for regulating surface properties and governing subsequent interactions [11, 12]. Strategies for surface functionalization encompass a variety of techniques, including physical methods such as the use of temperature, laser irradiation, plasma treatment, and polishing. Chemical methods involve UV irradiation and the formation of surface layers, either covalently bonded or physisorbed [13]. Additionally, biological modifications entail the introduction of biological entities such as peptides, proteins, and carbohydrates onto the surface [14]. In general, various materials can undergo modification, such as inorganic materials, ceramides, and their composites as well as synthetic organic materials, including polymers [15]. Synthetic non-degradable polymers are in widespread use in diverse fields due to their positive properties like low costs, specific mechanical properties such as excellent rigidity (polypropylene (PP)), high stability under environmental conditions or a high resistance to chemicals (polyethylene (PE) or polytetrafluoroethylene (Teflon^{TM})) [16, 17]. Polymers such as polystyrene (PS), PP, and PE, frequently employed as support materials in enzymatic applications, can be modified through processes like oxidation, reduction, hydrolysis, plasma and UV irradiation as well as wet chemical, and ionized gas treatments [16, 18].

1.1.3 Photochemistry of benzophenone

Functionalization of polymer surfaces can also be achieved through photochemical methods employing photoreactive reagents such as diazirines, aryl azides, nitrobenzils, cyclic disulfides, and benzophenones (BP). BP and its derivatives stand out

due to several advantageous characteristics that make them a widely preferred choice. In comparison to other photoreactive reagents, BP offers benefits like high stability in ambient light, reversible activation, and low reactivity with water [19]. BP is an aromatic ketone comprised of two benzene rings and a carbonyl carbon and serves dual roles as a photosensitizer and photoinitiator. BP can absorb light energy, transferring it to another molecule, and it can cleave to form initiating species [20, 21]. Irradiation with UV light at a wavelength of 280 to 400 nm first excites the BP molecule to an upper singlet state S2. Subsequent relaxation via an internal conversion process yields a diradical BP in a singlet state (S1). The excited S1 state rapidly undergoes intersystem crossing, resulting in an excited triplet state (T1) [22]. In the T1 state, the carbonyl oxygen is electron deficient and acts similarly as an electrophilic alkoxy radical. It can react with C-H bonds, forming relatively stable carbon radicals by hydrogen abstraction [21, 23]. The carbon radical, generated through this hydrogen abstraction, reacts with the BP ketyl radical to form a photo-crosslinked adduct.

The hydrogen abstraction reaction allows for the functionalization of substrates, encompassing basic reactions steps depicted schematically in Figure 1. During the process of triplet state formation, the excess energy of the BP singlet state is transferred to C-H groups on a substrate surface, resulting in the formation of excited C-H groups on the surface. The excited C-H groups and the triplet states of BP react by hydrogen abstraction, forming radicals on the substrate surface [22]. The surface radicals formed react with free radicals of BP, resulting in its immobilization on the substrate surface [19].

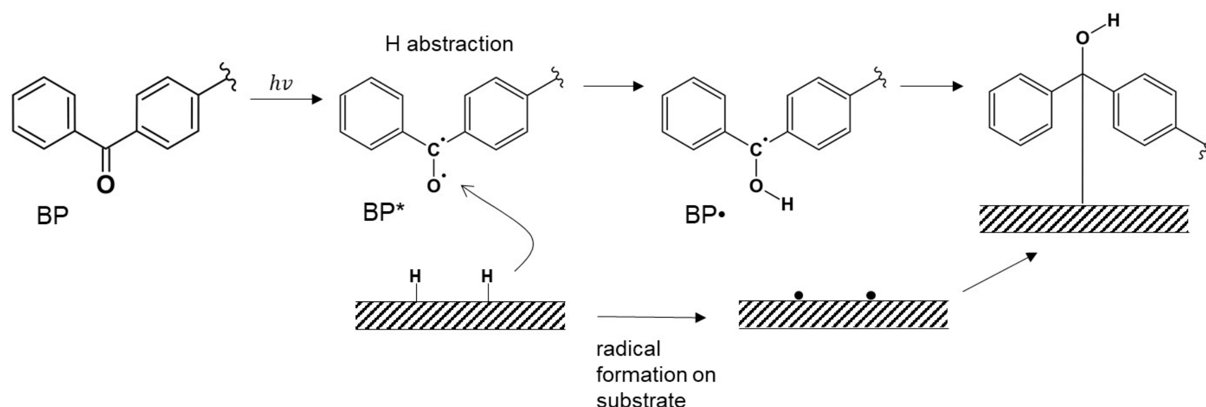


Figure 1 *Schematic picture of the basic reaction steps in surface functionalization with benzophenone (BP) as initiator. Image created with Biorender.com.*

1.1.4 Polydimethylacrylamide

Poly(N,N-dimethylacrylamide) (PDMA) is a highly valuable biocompatible polymer possessing favorable characteristics, including good antibacterial and antiviral properties [24]. PDMA is a water-soluble synthetic linear polymer derived from N,N-dimethylacrylamide monomers, undergoing a transition to water insolubility upon cross-linking. In its cross-linked form, PDMA has the ability to absorb and retain water, resulting in substantial swelling, defining it as a hydrogel [25, 26]. A surface functionalized with PDMA exhibits a protein-repellent nature, resulting in excellent non-fouling properties. The protein-repellent nature is attributed to steric hindrance, facilitated by the creation of networks with small mesh sizes. Proteins larger than the mesh size encounter impediments preventing their penetration into the network [27]. Moreover, the repulsive force generated by the balance between the entropic elasticity of the polymer chains and osmotic pressure prevents protein adsorption on the surface [24].

Based on its favorable characteristics, PDMA finds numerous applications, both in non-medical and medical domains. In non-medical applications, PDMA contributes to the paper industry, synthetic fiber industry, and printing. In the medical field, PDMA plays a pivotal role in applications such as grafting on contact lenses, drug delivery systems, and, owing to its non-fouling properties, engineering surfaces for vascular grafts, catheters, dialyzers, and blood containers [28, 29].

1.1.5 Photoimmobilization of molecules for surface functionalization

Surface functionalization with PDMA (Figure 2) can be realized by copolymerizing PDMA with benzophenone as polydimethylacrylamide-co-methacryloylbenzophenone (PDMA-MBP, **1**). As previously mentioned, UV excitation of BP initiates the generation of highly reactive benzyl radicals (**1***). These radicals can then insert into C-H bonds of a surface, such as polymeric substrates like PS (**3**), leading to the formation of a covalent bond between the surface and PDMA-MBP (**4**). Simultaneously, the radicals can also insert into the C-H bonds of molecules (**2**), forming covalent bonds with them. Consequently, the PDMA-coated substrate surface can be additionally functionalized with various C-H-containing molecules [30, 31]. Molecules such as DNA and proteins, including antibodies and glycoproteins, have been successfully immobilized on hydrogel surfaces through this method, leading to the generation of biofunctionalized

surfaces suitable for diverse applications [32 - 37]. Due to the nature of the C-H insertion reaction, the processes of PDMA-crosslinking, surface attachment and the incorporation of molecules into the forming network occur simultaneously [31].

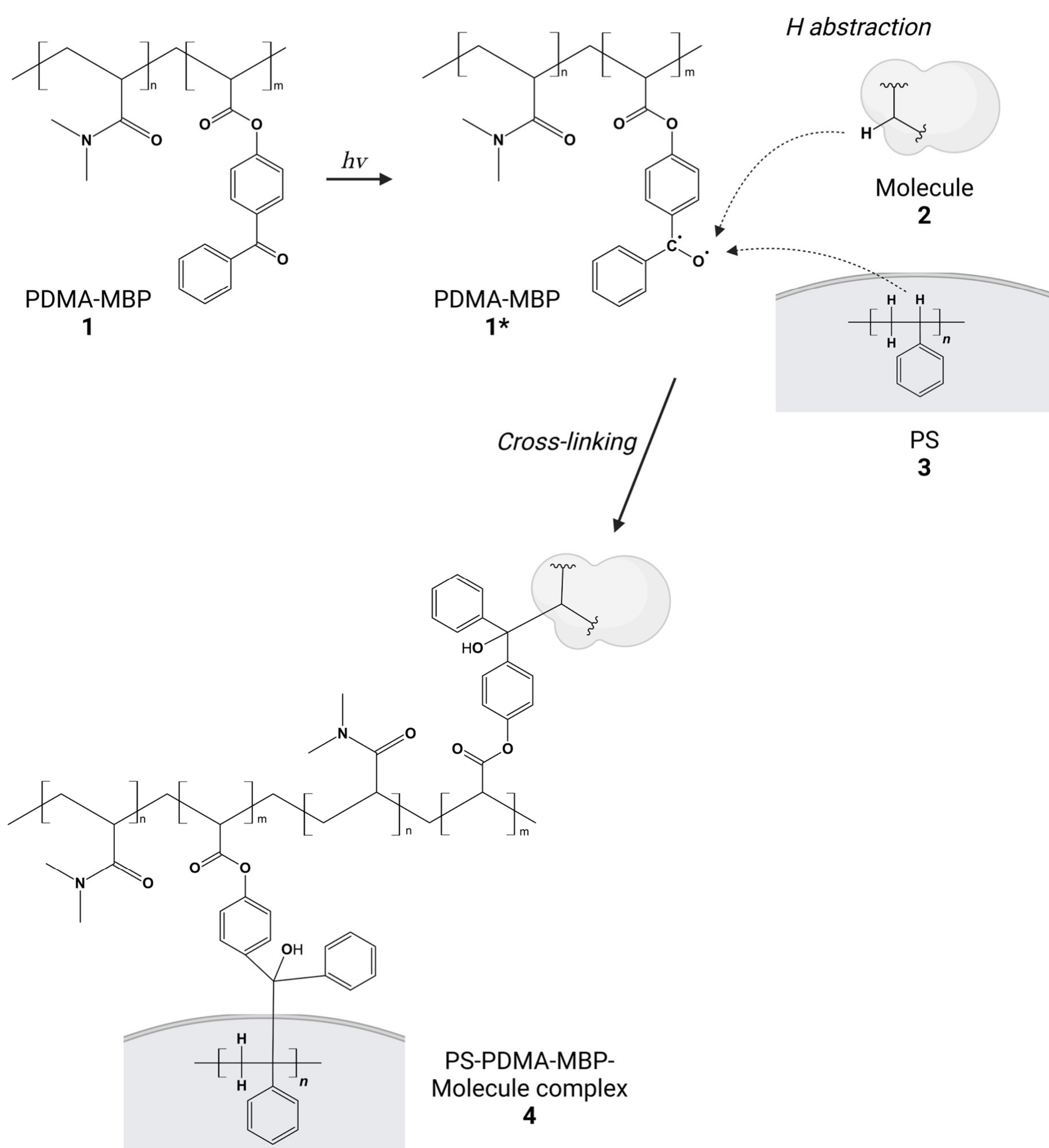


Figure 2 *Schematic illustration of the essential steps involved in functionalizing a polystyrene (PS) surface (**3**) with a target molecule (**2**), utilizing a copolymer comprising poly(N,N-dimethylacrylamide) (PDMA) and methacryloyl-benzophenone (MBP) (**1**). Crosslinking to the surface is initiated by benzyl radicals derived from MBP upon UV excitation. These radicals facilitate crosslinking by inserting into C,H-bonds of both the PS-surface and the target molecule through hydrogen abstraction. Image created with Biorender.com.*

1.2 Enzymes and their application for sustainable reactions

Sustainable and Green Chemistry has become increasingly important in recent decades. It is intended to contribute to the protection and preservation of people and the environment. Enzymes and their applications are major components in this effort. Consequently, the optimization of enzymatic applications is of great research interest.

This chapter outlines the principles of Green Chemistry, highlighting the advantages of using enzymes in technical applications and their distinctive characteristics.

1.2.1 Green Chemistry

In the 1990s, as concerns over chemical pollution and the depletion of natural resources intensified, the field of Green Chemistry, or Sustainable Chemistry, emerged as a dedicated research area within chemistry and chemical engineering. The fundamental objective of Green Chemistry is to optimize chemical processes and products by minimizing or eliminating the use and generation of substances hazardous to humans, animals, plants, and the environment [38 - 41]. The main principles include: First, the design of processes aiming to convert the maximum amount of feedstock into the desired product. Second, the utilization of renewable raw materials and energy sources. Third, the incorporation of safe and environmentally friendly substances, especially solvents, wherever feasible. Fourth, the creation of energy-efficient processes and fifth, the prevention of waste generation, which is considered to be the most effective approach to waste management [39, 40].

With its overreaching goal of achieving sustainability at the molecular level, the concept of Green Chemistry has exerted a significant impact not only in research laboratories, but also in industrial practices, educational initiatives, environmental efforts, and public awareness. Twelve principles of Green Chemistry serve as comprehensive guidelines, encompassing all aspects of the process life cycle. These principles are applied to the selection of raw material, the efficiency and safety of transformations, and the toxicity and biodegradability of products and reagents. One of these aspects is the use of catalysts [38, 42].

Catalysts enhance the rate of a chemical reaction, facilitating the efficient transformation of reactants into the desired product within a shorter timeframe. The catalyst itself remains unaltered and is not consumed during the reaction. This property

allows catalysts to enhance reaction efficiency by reducing the required input energy, minimizing the need for stoichiometric amounts of reagents, and increasing product selectivity. Consequently, catalysts contribute to decreased energy and feedstock consumption and generate less waste [43, 44]. Beyond mere efficiency, catalysis enables the realization for otherwise unfavorable reactions [38].

Common catalysts include inorganic solids like metals and metal salts, sulfides, oxides, metal salts of organic acids, organometallic compounds, and transition-metal complexes (e.g., Ni, Fe, $Mn_2(CO)_{10}$, $Cr(CO)_6$) along with organic materials such as hydroperoxide and ion exchangers [44 - 46].

As an important alternative to these chemical catalysts, biocatalysts, like enzymes and microorganisms, offer attractive benefits. The application of biocatalysts in chemical processes allows milder reaction conditions (temperature, pressure, pH), rendering the process more energy-efficient, cost-effective, and safer. Moreover, bioanalyzed processes, compared to conventional chemical methods, can avoid environmentally unfriendly reactants, involve fewer reaction steps, and result in fewer side products, simplifying purification processes [47 - 49].

1.2.2 Enzyme characteristics and mechanisms of enzyme action

Enzymes are biological catalytic molecules that selectively accelerate biochemical reactions by orders of magnitude, enabling the timescales necessary for proper cell function [50]. They contribute significantly to biological regulation and metabolism by converting macromolecular substrates and small molecules into products [51]. Most enzymes possess a remarkable specificity, typically catalyzing the conversion of either one specific type or a range of similar substrates. This substrate specificity arises from the shape and charge of the enzyme’s active site, a small specific region where substrate binding occurs [52].

The interaction between a substrate (***S***) and an enzyme (***E***) results in the formation of an enzyme-substrate complex (***ES***). This complex transforms through a transition state (***ES****) into an enzyme-product complex (***EP***), ultimately yielding the final product (***P***). The reaction mechanism can be summarized as:

$$E + S \leftrightarrow ES \leftrightarrow ES* \leftrightarrow EP \leftrightarrow E + P$$

This represents the simplest case of a reaction involving one substrate and one product. However, most enzymes catalyze more complex reactions involving two substrates and two products, or, occasionally even three or four substrates or products [53, 54].

Kinetic studies are performed to understand the specificity and physiological function of enzymes, providing insights into the mechanism, mode of regulation, reaction rate and kinetic parameters [54, 55].

The catalytic activity of enzymes is often described by a constant, k_{cat}, denoted as the turnover rate, representing the number of substrate molecules converted to product by a single enzyme molecule per unit time [52, 53, 56]. The observed change in concentration over the time interval during which this conversion occurs is referred to as the reaction rate [57].

Starting with a solution solely comprising substrate ***S***, the conversion of ***S*** to product ***P*** exhibits an initially nearly constant rate for a brief period after the onset of the reaction. From this linear range, the initial reaction rate (v_0), commonly used to characterize the enzyme activity, can be determined [58]. During this steady-state phase, the reaction is minimally affected by any phenomenon, influencing reaction rates or rate constants. Subsequently, in the course of enzyme catalysis, these constants can exhibit significant variations [59 - 61]. As the conversion progresses, the concentration of ***S*** diminishes, and ***P*** begins to accumulate, resulting in a gradual deceleration of the reaction rate. Upon reaching the point of equilibrium concentration, ***S*** and ***P*** are converted reciprocally at rates maintaining this equilibrium [54]. The time course of an enzyme catalyzed reaction displaying the product concentration versus time of reaction is shown in Figure 3A.

The simplest case in enzyme kinetics, applied to enzyme-catalyzed reactions involving one substrate and one product, is denoted Michaelis-Menten kinetics [62]. Michaelis-Menten kinetics is rooted in the law of mass action, which asserts that the rate of a chemical reaction is directly proportional to the product of the activities or concentrations of the reactants [54]. This law is derived from the assumptions of free diffusion and thermodynamically driven random collisions, influencing the rate of substrate transformation [63]. At low substrate concentrations, enzyme active sites are predominantly available for catalyzing substrate conversion upon binding. Increasing substrate concentration correlates with a heightened rate of enzyme-substrate

complex formation, given the increased frequency of random collisions at higher substrate and/or enzyme concentrations. The reaction rate v_0 escalates accordingly. Consequently, at high substrate concentrations, all enzyme active sites become occupied, leading to enzyme saturation. At this point, the maximum velocity (V_{max}) is achieved, and the reaction rate ceases to increase further, even with additional increase in substrate concentration. The substrate concentration at which the reaction rate reaches half of the maximum velocity is denoted by K_m [64, 65].

The formula of this kinetic model is given by the Michaelis-Menten equation:

$$\vartheta = \frac{VS}{(K_m + S)}.$$

A visualization of the Michaelis-Menten equation is shown in Figure 3B, displaying the initial velocity versus substrate concentration with the associated kinetic parameters V_{max} and K_m.

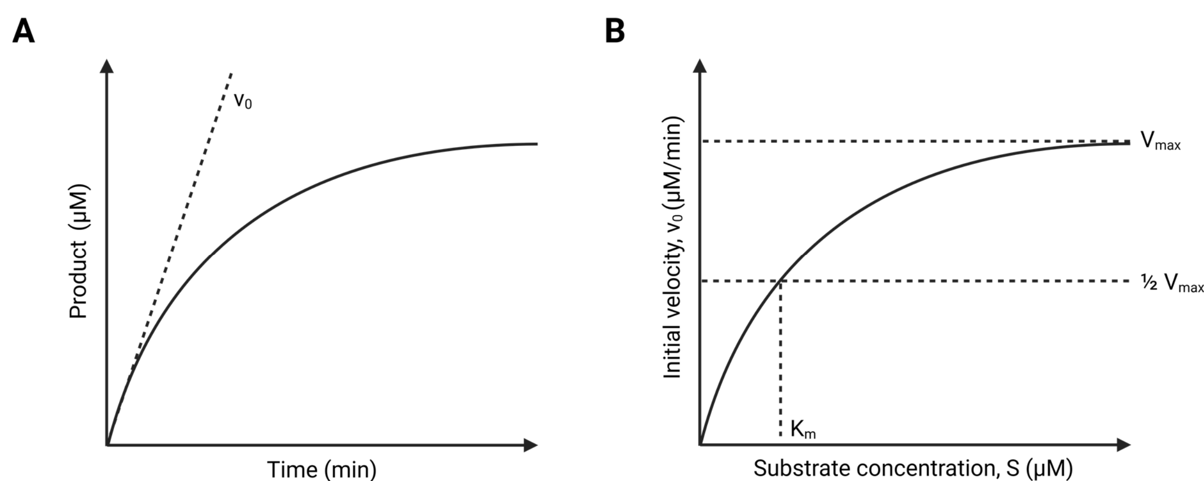


Figure 3 *Plots of an enzyme catalyzed reaction and a kinetic model. (A) Time course of an enzyme reaction: displayed is the product concentration versus time of reaction. The reaction rate v_0 is the slope in the linear range of substrate conversion. (B) Visualization of the Michaelis-Menten equation: Plot of initial velocity v_0 versus substrate concentration with the associated kinetic parameters V_{max} and K_m (described in the text). Image created with Biorender.com based on* [66] *and* [54].

The velocity of the reaction can be determined by monitoring either the disappearance of ***S***, or the formation of product ***P***. In the case of enzymatic reactions, this can be achieved through photometric determination of a (colored) substrate or product [67, 68].

1.2.3 Enzymes in industrial applications

Enzymes offer substantial value in a wide range of industrial products and processes, owing to their remarkable catalytic properties. Their utilization not only enhances reaction specificity and product purity but also mitigates the environmental impact of industrial operations. Derived from renewable sources and characterized by biodegradability, enzymes often circumvent the necessity of harsh conditions, such as high temperatures, extreme pH environments, and organic solvents [69, 70]. The range of applications for enzymes extends from the chemical industry, agriculture and energy industries to food processing and medicine [52, 69, 71, 72]. Noteworthy examples include their roles in detergents, textile and paper industries, organic synthesis, and biofuels production. Moreover, enzymes play major roles in food processing, including bakery, cheese manufacturing, wine making, and brewing [73].

In food industry, enzymes contribute not only in improving the production efficiency but also in elevating the quality of food components, such as nutritive value, flavor, texture, color, and appearance [74]. One notable application involves the utilization of lipases and triacylglycerol acylhydrolases in fat modification processes. Unlike chemical modification techniques, these enzymes catalyze the hydrolysis of oils and fats under milder conditions, thereby avoiding the introduction of impurities [75]. Furthermore, the beverage industry has implemented an enzyme-assisted beer production process, resulting in significant environmental benefits. This approach has led to a 7 % reduction in water consumption, a 14 % decrease in raw material usage, and a remarkable 78 % reduction in natural gas consumption and significantly reduced the adverse impact on the environment [76]. Enzymes also offer environmentally friendly alternatives in textile fiber processing, replacing conventional chemical processes. For instance, amylases are employed in desizing processes, while laccases and cellulases find application in denim finishing. Additionally, enzymes can be incorporated into detergent formulations, with proteases being particularly effective [77].

The applications of enzymes in medicine are numerous and, like in the industrial sector, are expanding fast. For example, fibrinolytic enzymes are beneficial in thrombosis therapy by aiding in the dissolution of blood clots and proteolytic enzymes assist in the removal of dead skin and burns [78]. Moreover, enzymes are valuable in organic synthesis and in the pharmaceutical industry, particularly in the production of enantiopure compounds. In the production of simvastatin, a cholesterol-lowering drug,

for example, traditional chemical routes necessitate extensive protection and deprotection steps to overcome problems related to regioselectivity. These routes also generate large amounts of solid waste, especially during the deprotection step. Using an acyltransferase that naturally catalyzes regiospecific eliminates these steps [72].

However, not all enzymatic-assisted processes, despite their theoretical feasibility or success on small scale, transition into industrial processes. Reasons include that engineering suitable biocatalysts need long lead times, enzymes and potentially necessary cofactors are expensive, and that the implementation of new processes often require new investments into a factory. The importance of economic success, time constraints, and the imperative to meet industry standards and expectations remain key drivers in industrial sectors, preventing some enzymatic processes from being implemented [79, 80]. Further advances in enzyme and reaction technology may enable the establishment and introduction of enzymatic synthesis as environmentally friendly, sustainable, and cost-effective methods in the industrial sectors [80].

1.3 Role of enzymes in antibiotic resistance and diagnostic applications

Enzymatic assays are significant tools in the medical field, given the indispensable roles enzymes play in human physiology and their involvement in multiple diseases. Antibiotic resistance, which represents a major challenge for the healthcare system, can be caused by bacterial enzymes, the β-lactamases. Accordingly, the development and improvement of diagnostic assays for the detection of β-lactamases is of immense importance in combating this threat. The forthcoming chapters provide an overview of the fundamental aspects of antibiotic resistance, the chemical basis of β-lactam antibiotics and β-lactamases, as well as their diagnostics.

1.3.1 Antibiotic resistance

Throughout the course of history, humanity has faced the challenge of combating infectious diseases, a struggle that has profoundly shaped the trajectory of both society and medical science. Among the milestones in this ongoing battle, the introduction of antibiotics is considered one of the greatest achievements in the annals of medical history. This breakthrough has not only revolutionized the treatment and management of infectious diseases, but has also played a pivotal role on reducing morbidity and

mortality rates, contributing to a substantial extension of human lifespan [81, 82]. However, the widespread use of antibiotics, coupled with their persistence in the environment has inadvertently given rise to a pressing concern - the global dissemination of antimicrobial resistance (AMR) [83]. AMR has now emerged as a major healthcare challenge, poised to potentially result in up to 10 million deaths and a cumulative economic deficit of 100 trillion USD in increased healthcare costs and lost productivity by the year 2050 [84]. In response to this looming crisis, it is imperative to implement strategic measures. Control programs, enhanced hygiene practices, and the development of improved antimicrobial agents are crucial components of a multifaceted approach aimed at curbing the spread of AMR [85].

1.3.2 β-lactam-antibiotics

β-lactam antibiotics constitute one of the most extensively utilized classes of antimicrobials, due to their notable efficacy, specificity, and the availability of numerous derivatives with high tolerability. Since the introduction of benzylpenicillin in the 1940s, the continuous discovery of β-lactam antibiotics has yielded compounds with improved properties, including increased potency, enhanced pharmacokinetics, expanded spectrum of activity, and heightened safety profiles, all designed to address the emergence of antibiotic resistance [86 - 89].

The mechanism of action of β-lactam antibiotics involves the interference with bacterial cell wall assembly through covalently binding to and inactivating enzymes called penicillin-binding proteins (PBPs). This disrupts the peptidoglycan biogenesis, resulting in compromised cell integrity and ultimately bacterial cell lysis [90]. However, akin to all antibiotics, bacteria have evolved mechanisms to circumvent the action of β-lactam antibiotics. Resistance to benzylpenicillin was identified almost simultaneously with its discovery. The challenge of resistance to β-lactam antibiotics is of paramount concern for healthcare workers worldwide, spanning both hospital and community settings [87, 91]. Four mechanisms have been identified to contribute to this resistance: modification or reduced production of outer membrane porins resulting in decreased antibiotic access, alteration of PBPs, production of efflux pumps (actively transporting antibiotics out of bacterial cells), and cleavage of antibiotics by β-lactamases, enzymes produced by bacteria themselves [87, 92].

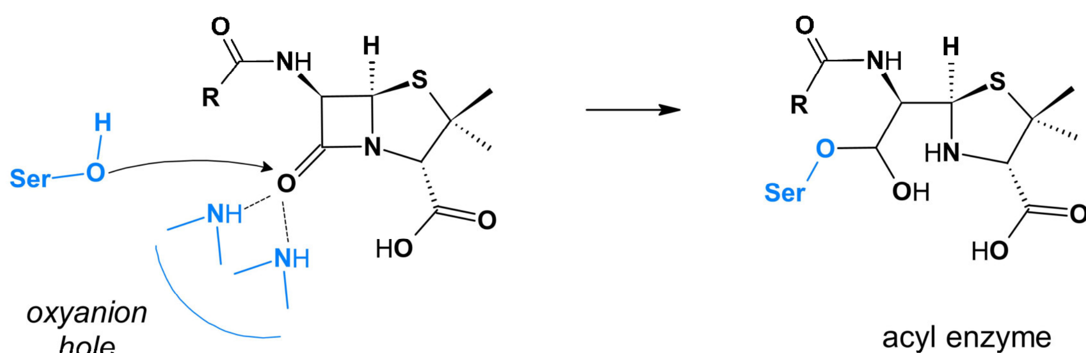
The emergence of resistance to the first β-lactam antibiotics spurred researchers to develop new subclasses within this class of antibiotics. Strategies such as modifying the chemical structure and incorporating bulky side groups have been employed to create effective antibiotics [93, 94]. The various β-lactam antibiotics introduced can be classified into subclasses, including penicillin derivatives, cephalosporins, monobactams, and carbapenems. All these subclasses share a structural similarity, a four-membered β-lactam ring, crucial to their bactericidal activity [87, 95, 96]. This ring forms a stable covalent acyl-enzyme complex with the PBPs by acylating serine and cysteine nucleophiles in the active site of the PBPs [97]. The classification into subclasses is based on the chemical nature of the ring fused to the β-lactam pharmacophore unit, resulting in a diverse array of bicyclic ring structures. Penicillins incorporate an attached thiazolidine ring, cephalosporins include a six-membered sulfur-containing ring (dihydrothiazine ring), and carbapenems possess a cyclopentene ring. In contrast, monobactams exist as isolated β-lactam rings without attached ring structures [95, 98]. Within each subclass, a variety of antibiotics were generated by modifying the ring structures at specific sites with different substituents. The physicochemical attributes associated with the core structure and the substituents determine the biological activities of the β-lactam antibiotics and influence their interaction with target proteins and resistance mechanisms [97, 98].

1.3.3 β-lactamases

The primary resistance mechanism to β-lactam antibiotics is the production of one or more β-lactamases, primarily synthesized by Gram-negative bacteria. These enzymes catalyze the irreversible hydrolysis of the amide bond of the β-lactam ring, yielding biologically inactive products [86, 88, 99]. The evolution of β-lactamases has been rapid, resulting in a staggering increase in their number and diversity. Presently, over 8000 [100] β-lactamases have been identified, exhibiting significant variation in phenotypic, genetic, and biochemical characteristics. The classification of β-lactamases is based on diverse criteria, including amino acid sequence, molecular mass, molecular structure, isoelectric point, substrate profiles, inhibition profiles, and their encodement (plasmid or chromosomally mediated) [88, 101]. They are categorized into four main Ambler classes (A-D), with classes A, C, and D representing serine-β-lactamases (SBLs), and class B encompassing zinc-dependent metallo-β-

lactamases (MBLs). SBLs hydrolyze antibiotics through the formation of a hydrolytically labile acyl-enzyme intermediate involving an active-site serine, while MBLs employ at least one active-site zinc catalyzing reactions without a covalent intermediate (Figure 4) [86, 102].

Serine-β-lactamase



H_2O

Metallo-β-lactamase

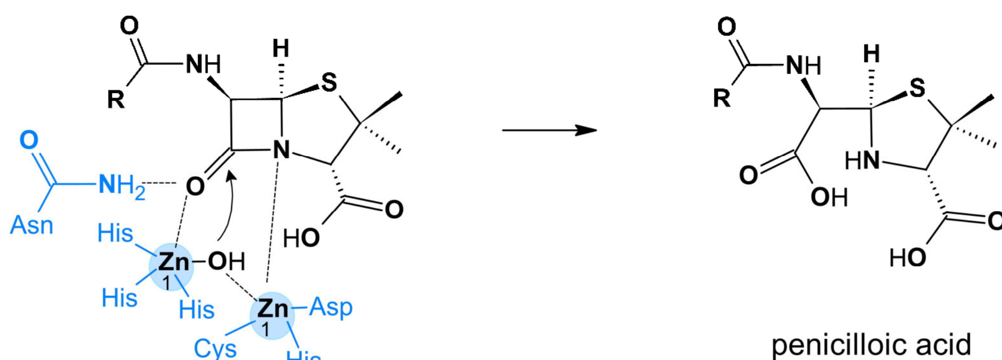


***Figure 4** Reaction mechanisms of penicillin hydrolysis by serine-β-lactamases (SBLs) and metallo-β-lactamases (MBLs). SBLs rely on an active site serine residue as a nucleophile and form an acyl enzyme intermediate, followed by subsequent hydrolytic deacylation. MBLs employ a zinc-bound water (or hydroxide) as active-site nucleophile and hydrolyze penicillin without the formation of a covalently bound acyl enzyme intermediate. Image created with ChemSketch, based on* [103].

The β-lactamases of the different Ambler classes vary in their substrate specificity, which can be relatively narrow, covering only one antibiotic class, or broader, covering an extended spectrum of several antibiotic classes. Class A β-lactamases, primarily known as penicillinases, rather hydrolyze penicillins than cephalosporines. However, several variants within this class may exhibit significant broad-spectrum activity against cephalosporins and carbapenems. Class B β-lactamases (metallo-β-lactamases) generally exhibit a broad-spectrum substrate specificity, encompassing all β-lactams

except monobactams (e.g., aztreonam). Class C β-lactamases (cephalosporinases) show a substrate preference for cephalosporins, while class D β-lactamases (oxacillinases) predominantly target oxacillin and related penicillins [104].

All four classes are widely distributed across multiple species of clinically significant bacterial pathogens and environmental bacteria, including Enterobacteriaceae such as *Escherichia coli* and *Klebsiella pneumoniae*, and non-fermenting species such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Notable enzyme families include TEM, CTX-M, KPC and SHV (class A), NDM and VIM (class B), CMY and ADC (class C), and the oxacillinase (OXA) of class D [105].

Of particular concern are enzymes capable of targeting expanded-spectrum β-lactams, including AmpC (class C cephalosporinases), extended-spectrum β-lactamases (ESBL) (classes A and D), and carbapenemases that hydrolyze most β-lactams, including carbapenems (classes A, B, and D) [106]. These enzymes are typically associated with resistance to multiple antibiotics, leaving limited therapeutic options [107].

1.3.4 β-lactamase inhibitors

The challenge posed by β-lactamases is driving researchers to develop novel β-lactam antibiotics designed to evade enzymatic inactivation by β-lactamases. An alternative strategy is the introduction of β-lactamase inhibitors (BLIs), which share structural similarities with penicillin and also contain the amide bond of the β-lactam group, albeit with a modified side chain. The unique structural features of BLIs enable them to render β-lactamases inactive upon binding, thereby preventing these enzymes from hydrolyzing the β-lactam antibiotic [87, 97].

Following the discovery of clavulanic acid as a β-lactamase inhibitor, the exploration of mechanistically related compounds revealed sulfone-substituted β-lactams, like sulbactam and tazobactam [108, 109]. These function as “suicide inhibitors” by forming irreversible, covalent adducts with the catalytic site in the enzyme, inactivating it permanently. However, these three inhibitors are effective primarily against most β-lactamases of class A SBLs. They do not provide protection against β-lactamases from the other subclasses (class B MBLs, classes C and D SBLs) [105, 108, 110]. In response to this limitation, new classes of non-β-lactam β-lactamase inhibitors have

been developed to broaden inhibitor activity to a wider range of β-lactamase targets. One such class is the diazabicyclooctanones (DBOs), including avibactam and relebactam, sharing the same bicyclic DBO molecular scaffold. The difference between avibactam and relebactam is in the side chains, where relebactam has an additional piperidine ring [111]. The DBO structure, mimicking a β-lactam, distinguishes them from earlier β-lactam inhibitors, allowing evasion of known resistance mechanisms [110]. DBOs covalently form carbamyl ester to the active-site serine of β-lactamases, leading to their inhibition [111]. This binding is reversible and once deacetylated, the recyclization of the 5-membered urea ring allows the regeneration of the active inhibitor [112]. The spectrum of activity of DBOs includes most class A and class C β-lactamases and several class D β-lactamases [110].

Other non-β-lactam BLIs, such as vaborbactam, are boronic acid compounds that are effective against SBLs (class A, C, and D) [108].

However, these inhibitors exclusively target SBLs, leaving Ambler class B MBLs unaffected, as no covalent intermediate is formed during MBL catalysis. [111]. The mechanism of action of currently marketed BLIs is not applicable to MBLs. Additionally, the evolving nature of bacteria has led to an increasing number of inhibitor-resistant bacterial strains, challenging the efficiency of presently available BLIs [112].

1.3.5 Diagnostic assays and resistance screenings for β-lactamases

The accurate identification of the pathogen causing an infection is imperative for successful treatment, as not all antibiotics are effective against all bacteria. However, the identification of bacterial pathogens can be an intricate and time-consuming process, leading to the common practice of frequent and indiscriminate antibiotic prescription. This has consequently fostered the ongoing emergence of pathogens that are increasingly resistant to antibiotics, posing a significant global health threat. Of particular concern are resistances to β-lactam antibiotics, primarily caused by pathogens producing antibiotic inactivating β-lactamases [113].

Fast and accurate detection of bacterial susceptibility to antibiotics, including the detection of β-lactamases, holds paramount importance for both epidemiological and clinical purposes. It is essential for guiding appropriate antibiotic therapy decisions,

improving patient outcomes and preventing the emergence of multidrug-resistant bacteria [114, 115].

Traditional methods for detecting bacterial infections and conducting antibiotic susceptibility testing (AST) rely on cultivating pathogens in cell culture, followed by biochemical methods aimed at identifying strains and species of microorganisms [116]. Bacterial cultivation methods, while cost-effective and typically yielding specific diagnoses, suffer from extended turnover times, lack sensitivity, and are susceptible to errors related to collection conditions and specific growth media requirements [117, 118]. Additionally, these methods have limitations, particularly in reliably identifying and characterizing β-lactamase producers, as resistant strains often harbor multiple ESBL and/or AmpC genes from different resistance gene families [119].

Molecular methods such as polymerase chain reaction (PCR)-based diagnostics and DNA sequencing offer faster and often more cost-effective alternatives. Another advantage lies in their capability to not only detect pathogens, but also identify antimicrobial resistance genes carried by microorganisms, including β-lactamase resistance genes [120 - 122]. Nevertheless, limitations and potential pitfalls of screening for specific genes and resistance determinants include false-positive results (amplification of silent genes or pseudogenes), mutations in primer binding sites, and the inability to address new and as-yet-uncharacterized resistance mechanisms. This complexity makes it challenging to detect highly variable and rapidly evolving resistance mechanisms, as is the case with β-lactamases [123, 124]. Furthermore, these methods only confirm the presence of a specific resistance gene and do not indicate the level of its expression. Bacteria with the resistance gene present but not expressed, or expressed at low levels, may still be susceptible to the specific antibiotic [125].

An alternative strategy focuses on the direct detection of β-lactamases through immunological methods, utilizing monoclonal antibodies targeted against specific β-lactamases [126, 127]. Although highly sensitive and specific [128, 129], these tests are limited to known β-lactamase variants and may fail to detect emerging enzyme variants [125]. Moreover, these tests cannot assess the hydrolytic activity of β-lactamases against different antibiotics.

Colorimetric tests can not only monitor the production of β-lactamases but also assess their hydrolytic activity. These tests rely on chromogenic substrates that change color

upon hydrolysis by the β-lactamases [130]. This color change can be detected either visually or quantitatively using a spectrophotometer. Chromogenic substrates, such as nitrocefin and CENTA, both containing a cephalosporin backbone, are used for the detection of β-lactamases [131, 132]. Chromogenic methods are fast, convenient, and highly sensitive to most β-lactamases across all classes [99, 132].

This type of test affirms the presence of active β-lactamases within a sample, but does not facilitate the identification of specific β-lactamase variants [99]. However, this limitation is inconsequential, as such information does not necessarily contribute to the selection of antibiotics suitable for therapeutic applications. To determine effective β-lactam antibiotics and β-lactamase inhibitors, they can be integrated into the colorimetric β-lactamase assays. If an antibiotic is effective, it binds to the β-lactamases and deactivates them, preventing the conversion of the chromogenic substrate and avoiding a color change. In cases where an antibiotic is ineffective, either due to a lack of binding to the β-lactamases or because the β-lactamases hydrolyze it, a color change occurs as the β-lactamases convert the chromogenic substrate. The basic principle of this colorimetric β-lactamase assay is schematically provided in Figure 5.

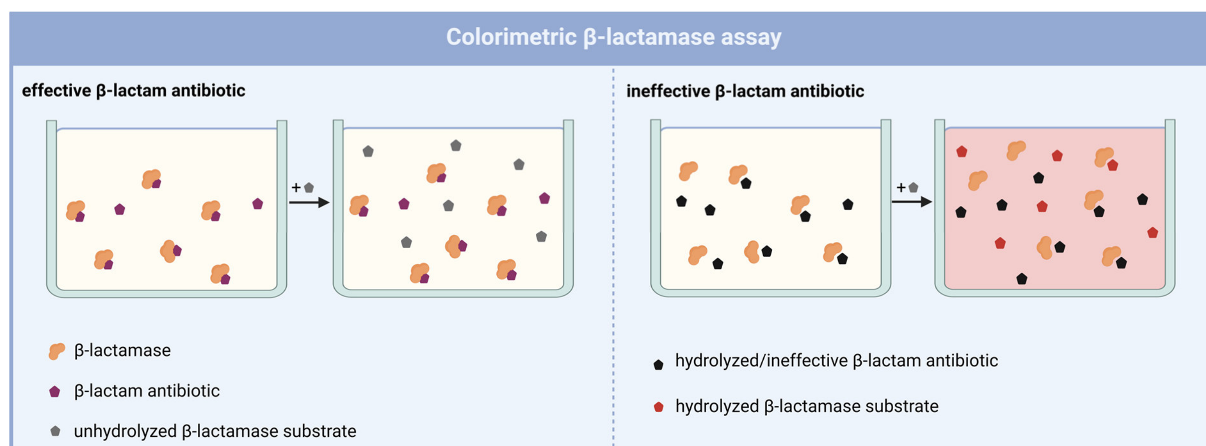


Figure 5 *Scheme of a colorimetric β-lactamase assay for antibiotic susceptibility testing. Effective β-lactam antibiotics bind to β-lactamases, preventing the conversion of the β-lactamase substrate. If a β-lactam antibiotic cannot bind to a β-lactamase or is hydrolyzed by the β-lactamase, the β-lactamase is still active and can hydrolyze the chromogenic β-lactamase. This results in a detectable color change that indicate the ineffectiveness of the β-lactam antibiotic. Image created with Biorender.com.*

However, there are limiting factors affecting the sensitivity of these chromogenic tests. If an enzyme is expressed at a low level, the amount in the test sample might not be sufficient for a color change or may only occur after a prolonged incubation time. Moreover, the hydrolytic activity of an enzyme relies on the extraction protocol and the pharmacokinetic properties of the enzyme/substrate pair. Sensitivity also depends on the characteristics of the indicator and the method of measurement, with photometric measurement being more sensitive than visual observation [125]. It is for these reasons that the development of optimized enzyme assays, particularly in the field of antibiotic resistance testing, is of high relevance.

2 Objectives

Enzymes serve as fundamental catalysts in biology, essential for accelerating and regulating biochemical reactions crucial for life. Their significance lies in controlling reaction rates and specificity. Beyond vital cellular functions, enzymes find applications in various technical fields, feed industry, and food processing, prompting greener and more sustainable practices. In medicine, enzymes are important for diagnostics and therapies targeting enzyme-related diseases.

The objective of this research was to address the drawbacks and limitations associated with enzymatic applications, aiming to optimize and broaden their applicability and scope. The work comprises two central projects, which are described in detail in this chapter. **Project I** focuses on enhancing enzyme applications to promote more sustainable processes, while **Project II** aims to adapt the approach developed in **Project I** to optimize an enzyme assay in the biomedical field. To achieve these goals, various strategies involving polydimethylacrylamide (PDMA) surface functionalization were investigated.

2.1 Project I: Kinetic enhancement

The application of enzymes has already led to transformative changes across various industries, serving as sustainable and environmentally friendly alternatives to conventional chemical processes. Enzymes facilitate reactions under mild conditions, enabling reduced energy consumption and minimized waste production. These attributes have paved the way for advancements in the industrial sector towards more sustainable and Green Chemistry practice. With the global community increasingly embracing sustainability, optimizing enzymatic applications provides an opportunity to expand the positive impact of enzymes.

The objective of **Project I** was to enhance enzymatic assays for more sustainable enzyme reactions by improving enzyme kinetics. It is known that the reaction rate of enzymatic reactions can be increased by elevating substrate and/or enzyme concentrations. However, this is not in line with the principles of Green Chemistry, which aims to minimize resource consumption. This research sought an alternative method for enhancing reaction rates. Proteins, including enzymes, are known to non-

specifically adsorb to surrounding surfaces. This effect thus occurs in all enzymatic applications comprising enzymes in solution. Modifying these surfaces to reduce non-specific enzyme adsorption is desirable. With fewer enzymes adsorbing, more remain in solution, increasing chances of random enzyme-substrate-collisions and thus the reaction rate. With this increased reaction rate either substrate, enzyme or time can be saved.

To achieve this objective, various polymeric surfaces (PS, PP and PC) were functionalized, and the impact on protein adsorption and enzyme performance was tested using various enzymatic assays. The goal of surface functionalization was to increase the hydrophilicity of the polymer surfaces, thereby reducing non-specific protein adsorption, as more hydrophilic surfaces adsorb fewer proteins. PDMA hydrogel was chosen for functionalization due to its higher hydrophilicity compared to the applied polymeric surfaces and its ease of coating in a one-step process. As a copolymer with methacryloylbenzophenone (MBP), PDMA can be crosslinked by UV-induced C,H-insertion reactions and immobilized on the surface.

Model enzymes used in this study included TEM-1 β-lactamase (hydrolyzing nitrocefin substrate), horseradish peroxidase (oxidizing 3,3',5,5'-tetramethylbenzidine (TMB) substrate), and alcohol dehydrogenase (hydrolyzing p-nitrophenyl phosphate (pNPP) substrate). The enzymatic substrate conversion and kinetic parameters were the main objectives for investigating the effect of PDMA functionalization in these enzymatic assays.

2.2 Project II: Diagnostic β-lactamase assay

The emergence of β-lactamases, enzymes produced by bacterial pathogens, has significantly contributed to the rise and spread of antibiotic resistance, posing a serious threat to global health. Current methods for β-lactamase detection and for AST are often time-consuming, technically demanding, or insufficient in sensitivity and specificity.

Project II aimed to optimize β-lactamase assays and potentially improve diagnostic applications as well as AST in bacterial infections by utilizing the beneficial effects of PDMA surface functionalization on enzyme activities. For this purpose the method of PDMA surfaces functionalization via UV-crosslinking through C,H-insertion reactions

was applied and investigated for its applicability to immobilize β-lactam antibiotics and β-lactamase inhibitors. The workflow of **Project II** is in Figure 7.

β-lactamases are diverse enzymes with different molecular structures and functional characteristics enabling them to hydrolyze β-lactam antibiotics of different classes and generations as well as β-lactamase inhibitors. In this project, the immobilization of ampicillin (penam), cefoperazone (cephalosporine), meropenem (carbapenem), and aztreonam (monobactam) as representatives of different classes of β-lactam antibiotics was targeted. Additionally, the β-lactamase inhibitors tazobactam and sulbactam as well as avibactam and relebactam as non-β-lactam β-lactamase inhibitors were studied for their ability to be immobilized via PDMA-crosslinking. The surfaces functionalized with antibiotics were then used in β-lactamase assays, applying the chromogenic β-lactamase substrate nitrocefin to detect interactions of the immobilized antibiotics with β-lactamases. The aim was to verify whether a sufficient amount of antibiotics had been immobilized and was accessible for binding of the β-lactamases or if steric hindrance might prevent binding. Inhibition assays were conducted using β-lactamases from all Ambler classes to determine potential applicability over a wide range of the highly diverse β-lactamase population.

Considering that the functionalized surfaces are intended for use in healthcare diagnostics, another aim of the project was to ensure the applicability of the surfaces to clinical samples. Therefore, the experiments were also performed with β-lactamases isolated from clinically relevant bacterial strains.

In anticipation of the development of a potential point-of-care (POC) diagnostic assay, an additional objective was the investigation of the transferability of the method from the initially used 96-well plates to microfluidic devices. For this purpose, microfluidic devices were 3D-printed using a polymeric filament, and antibiotics were immobilized in the microfluidic system via the UV-induced C,H-insertion reaction.

3 Summary of results

As part of this research work, stable surface coatings with PDMA on different polymeric substrates were obtained using one-step benzophenone crosslinker chemistry. The functionalized surfaces were then employed in diverse enzymatic assays.

Project I demonstrated that the implementation of PDMA resulted in reduced non-specific adsorption of model enzymes TEM-1 β-lactamase, HRP, and ALP across all examined polymeric substrates. Subsequently, it was observed that the substrate conversion of enzyme solutions was significantly higher following the incubation on functionalized surfaces compared to their non-functionalized counterparts. This can be attributed to the decreased non-specific enzyme adsorption to the PDMA-functionalized surfaces, allowing more enzymes to remain in solution. Moreover, the incorporation of PDMA resulted in a considerable increase in substrate conversion rates by the enzymes. Notably, HRP exhibited up to 40 % higher substrate conversion, while TEM-1 and ALP demonstrated even greater improvements. The increase of substrate conversion by TEM partially exceeded 100 %, for ALP the increase ranged up to 200 %.

Further investigation revealed that the extent of substrate conversion increasement was dependent on the quantity of PDMA immobilized on the surfaces. Higher amounts of PDMA correlated with higher substrate conversions. At first, substrate conversion initially exhibited a linear increase with rising amount of PDMA, reaching saturation at a certain amount of PDMA. Moreover, the kinetic analysis revealed an enhancing effect of PDMA-functionalization on enzyme catalytic performances. Both initial velocities and maximum velocities were notably elevated across all enzymes tested. Maximum velocities were increased by 90 % for TEM-1, by about 45 % for HRP, and by at least 160 % for ALP.

In course of this research work, β-lactam antibiotics and β-lactamase inhibitors were added to the one-step UV-crosslinking process. Comprehensive surface characterization utilizing Fourier-transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) confirmed proper crosslinking of the PDMA and the successful immobilization of various β-lactam antibiotics and β-lactamase inhibitors of different classes. Subsequent β-lactamase assays conducted on these functionalized surfaces, using nitrocefin as the enzymatic substrate, demonstrated that the immobilized

antibiotics and inhibitors were accessible for enzyme binding, resulting in a decrease of substrate conversion. This decrease was contingent upon the quantity of antibiotic or inhibitor immobilized, with higher amounts leading to higher degrees of enzyme inhibition.

The results of assays performed with β-lactamases of different classes, comprising various antibiotics and inhibitors, demonstrated that the developed surface functionalization allowed for successful susceptibility testing. These tests reliably determined the susceptibility and resistance profiles of the different β-lactamases to the tested antibiotics and inhibitors. In course of the project, the feasibility of conducting AST on β-lactamases isolated from bacterial cultures, mimicking clinical samples, was explored. The assays again succeeded in differentiating between antibiotic-sensitive and antibiotic-resistant β-lactamases isolated from clinically relevant bacterial strains. Furthermore, this project achieved the successful transfer of the method for immobilizing antibiotics via PDMA from conventional well plates to microfluidic devices. PDMA microfluidic devices were produced using a 3D printer and functionalized with β-lactam antibiotics and β-lactamase inhibitors. These functionalized devices succeeded in assaying β-lactamase AST.

All experiments conducted in **Project II** consistently revealed that substrate conversion by resistant β-lactamases was higher and faster on functionalized surfaces compared to non-functionalized surfaces. These results align seamlessly with the findings made in **Project I.**

4 Project I: Kinetic enhancement

Publication Project I

Toward more sustainable enzyme reactions: enhancing kinetics by polydimethylacrylamide implementation

Simone Rentschler, Max Borgolte, René Csuk, Stefan Laufer and Hans-Peter Deigner

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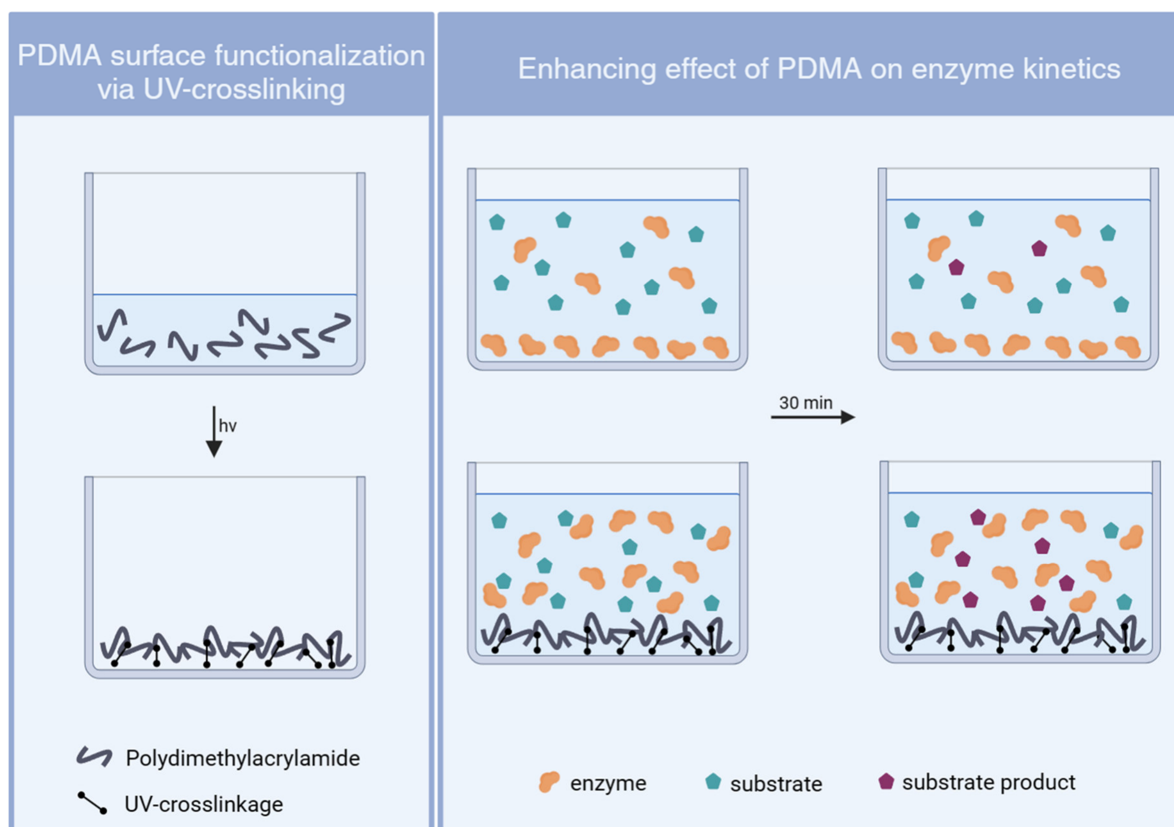

Figure 6 *Graphical Abstract Publication Project I: PDMA surface functionalization via UV-crosslinking is leading to increased substrate turnover and initial velocities, allowing more sustainable enzymatic reactions* [133].

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Toward more sustainable enzyme reactions: enhancing kinetics by polydimethylacrylamide implementation†

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Enzymes, with their ability to accelerate reactions under mild conditions, have become invaluable tools in a wide range of applications and have revolutionized various industrial sectors. As industry strives for sustainability, optimizing enzymatic applications is a key factor in unlocking the full potential of enzymes. Considering that surfaces have a major impact on the behaviour of biological components, modifying surfaces in reaction systems enable the regulation of reaction conditions and can yield profound improvements. In this article, we investigate the effect of a one-step surface functionalization with polydimethylacrylamide on enzymatic reactions, aiming to enhance enzyme performance and prompt sustainability. Functionalized wells were used to assay substrate turnover and kinetic measurements of various enzymes and substrates. We observed increased substrate turnover and initial velocities for all enzymes and substrates. Our results show that the incorporation of PDMA in a reaction system can save up to two-thirds of the substrate and therefore hold significant implications for enzymology and biotechnological applications. Optimized enzymatic reactions can reduce resource consumption, waste production, time, and costs. In diagnostics, PDMA-enhanced assays might lead to earlier and more accurate disease detection, improving patient outcomes. Furthermore, as we were able to demonstrate the effect of PDMA surface functionalization for enzymes of different types, it can be assumed that the positive properties can also be used for other enzymes, assays and diverse industrial applications.

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Introduction

Enzymes stand as indispensable tools across diverse applications, owing to their remarkable efficiency, specificity, and pivotal role in facilitating biochemical reactions.[1] Enzymatic applications have revolutionized various sectors by offering sustainable and environmentally friendly alternatives to conventional chemical processes. Their ability to accelerate reactions under mild conditions, to reduce energy consumption, and to minimize waste production, has paved the way for advancements in pharmaceuticals, agriculture, food production, biofuels and more.[2,3] In the field of medicine, enzymes are vital in diagnostic tests, enabling rapid and sensitive detection and monitoring of diseases, as well as for therapeutic interventions, offering tailored and specific treatments for various diseases.[4,5] As the global community increasingly embraces sustainable practices, the optimization of enzymatic applications emerges as a way to further harness the potential of enzymes.

However, despite immense potential, the full exploitation of enzymatic applications often encounters challenges related to enzyme stability, activity, substrate specificity, and unfavourable reaction conditions. Enzymes exhibit sensitivity to fluctuations under environmental conditions, variations in substrate concentrations, and the presence of inhibitors. To address these limitations, researchers are driven to explore innovative approaches to enhance the efficiency and effectiveness of enzymatic reactions. Optimized enzymatic applications can lead to more efficient bioprocessing, reducing resource consumption, waste generation, and overall costs, prompting sustainability.[6]

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Surface functionalization presents a promising avenue to optimize enzymatic applications as surface properties govern the behaviour and overall performance of materials. Robust surface functionalization offers control over the efficiency, sensitivity, and stability of a system.[7] By modifying surfaces with specific chemical groups or coatings, subsequent surface interactions and responses can be controlled, unlocking a wide array of additional functionalities.[8] Generally, almost any material such as synthetic organics, inorganics, ceramics, and their composites can be modified by various methods including chemical conjugation, polymerization, mineralization and physical adsorption. For instance, in enzymatic applications, synthetic non-degradable polymers often serve as support materials and can be modified using methods like plasma treatment, aminolysis, and hydrolysis.[9–11] However, these methods often require time-consuming synthesis and multi-step processes to incorporate suitable chemical groups onto polymeric surfaces.[12] Therefore, achieving simple and cost-effective surface functionalization methods that yield robust surfaces is highly desirable for optimizing enzymatic applications.

This research paper explores a one-step surface functionalization technique applied to polystyrene, polypropylene and polycarbonate microtiter plate wells using polydimethylacrylamide (PDMA) copolymerized with methacrylol benzophenone (MBP). The process involves photoactive UV-crosslinking of the benzophenone moiety. Our focus was to investigate the impact of PDMA implementation in enzymatic assays, with special attention to its influence on the catalysis and kinetic behavior of enzymes, aiming for potential enhancements in sensitivities and performances. The functionalized wells were used to conduct enzymatic assays, measuring substrate turnover and kinetic parameters for various enzymes and substrates. Notably, we observed increased substrate turnover and initial velocities across all enzymes studied. This research provides valuable insights to the field of enzymology, offering a tool for the development of more efficient enzymatic processes in industry, medicine and biotechnology. Furthermore, the implementation of PDMA in enzymatic assays could have significant implications for the optimization of enzymatic assays by enhancing assay performance, sensitivity and limits of detection. This will enable, among other things, earlier and more accurate disease diagnosis, increased efficiencies, and promoted sustainability.

Experimental

Chemicals and materials

PDMA-MBP was synthesized and characterized as previously described.[13] 96- and 48- polystyrene (PS) well plates as well as 96- polypropylene (PP) well plates were purchased from Greiner AG (Kremsmünster, Austria). 96-Polycarbonate (PC) well plates were fabricated with an Ultimaker 3 (Ultimaker, Netherlands) FDM 3D printer with a 0.4 mm nozzle head using a transparent 2.85 mm PC polymer filament (filament-world.com, Germany). TEM-1 β-lactamase was purchased from Prospec-Tany Technogene Ltd (Ness-Ziona, Israel) and a nitrocefin chromogenic substrate from ApexBio Technology LLC (Houston, USA). A horseradish peroxidase (HRP)-conjugate was purchased from PeproTech ES Limited (London, UK) and 3,3′,5,5′-tetramethylbenzidine (TMB) from Thermo Fisher Scientific Inc. (Waltham, USA). 4-Nitrophenyl phosphate disodium salt hexahydrate (pNPP) and alkaline phosphatase (ALP) were acquired from Thermo Fisher Scientific (New Jersey, USA).

Solutions

The solutions used in this study included a 0.01 M phosphate-buffered saline (PBS, pH 7.4) and a 1 M Tris–HCl buffer (pH 8.0). TEM-1 β-lactamase was diluted in PBS to a concentration of 3 nM. The β-lactamase substrate nitrocefin was dissolved in DMSO to a concentration of 8 mM and further diluted in PBS to a final substrate concentration of 2 mM. The HRP-conjugate was diluted 1 : 20 000 in PBS and the TMB substrate was used undiluted. ALP was diluted to a concentration of 10^{-5} U μl^{-1} in Tris–HCL and the ALP substrate pNPP was dissolved in Tris–HCL to a concentration of 2 mM.

Preparation of polymer coatings

PDMA was diluted to a concentration of 10 mg mL^{-1} in an 80% ethanol mixture in H_2O. From the mixture, 40 μL was pipetted into each well of a 96-well plate (PS, PP, and PC), and 80 μl into each well of a 48-well plate (PS). The plates were allowed to dry at room temperature for at least 4 h and cross-linked with 3 J cm^{-2} UV light at 365 nm, followed by 3× washing with 250 μL of PBS. Well plates for control measurements were equally treated, using 40 μL (96-well plate) and 80 μL (48-well plate) of 80% ethanol mixture in H_2O without PDMA.

Varying amounts of PDMA were used to investigate the effect of PDMA on enzymatic substrate turnover. PDMA was diluted to concentrations of 10, 8.75, 7.5, 6.25, 5, 3.75, 2.5 and 1.25 mg mL^{-1} in an 80% ethanol mixture in H_2O. Further preparation of the plates was carried out as described above.

FTIR data of the prepared polymer coatings were recorded on a Spotlight 400 FT-IR microscope (PerkinElmer, Waltham, USA) in the range of 4000–400 cm^{-1} using the transmittance mode of operation. The spectra were acquired and processed using the associated Spectrum 10™ software.

Determination of protein adsorption onto PDMA and different polymer wells

70 μl of each enzyme solution (TEM-1 (3 nM), HRP and ALP (10^{-5} U μl^{-1})) were added to separate PS-, PP- and PC-wells functionalized with PDMA as well as to non-functionalized wells. The plates were incubated for 30 min at room temperature. After the incubation, the enzyme solutions were transferred to fresh wells that were not functionalized and mixed with the specific substrates as follows: 65 μl of pre-incubated TEM-1 β-lactamase was mixed with 35 μl of nitrocefin dilution

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(1 mM), 6 μl of pre-incubated HRP was mixed with 144 μl of TMB, and 50 μl of pre-incubated ALP was mixed with 50 μl of pNPP dilution (2 mM). The substrate product formation was monitored with a TECAN Infinite 200 PRO as an absorbance increase at 405 nm (pNPP), 492 nm (nitrocefin) and 650 nm (TMB).

Determination of activities and kinetic studies of enzymes in PDMA treated wells

65 μl of TEM-1 (3 nM) were added to each well of a prepared PS, PP and PC 96-well plate and mixed with 35 μL nitrocefin dilution for final substrate concentrations ranging from 0 to 700 μM. 15 μl of HRP dilution was added to each well of a prepared PS 48-well plate and mixed with different volumes of the TMB substrate ranging from 175 μL to 325 μL. The volume in each well was adjusted with PBS to a final volume of 350 μL. 50 μl of ALP (10^{-5} U μl^{-1}) were added to each well of a prepared PS 96-well plate and mixed with 50 μL of pNPP dilution for final substrate concentrations ranging from 0 to 1 mM. The substrate product formation was monitored with a TECAN Infinite 200 PRO at 405 nm (pNPP), 492 nm (nitrocefin) and 650 nm (TMB) at 10–15 second intervals over a defined period of time. The initial reaction rates were determined from the linear range of substrate turnover at different substrate concentrations. Substrate turnover analysis was performed after a 60-minute incubation period. The amounts of substrates converted by the enzymes were determined from the measured absorbance values and the corresponding extinction coefficients of 18 000 M^{-1} cm^{-1} (pNPP), 17 400 M^{-1} cm^{-1} (nitrocefin) and 39 000 M^{-1} cm^{-1} (TMB).

Results

Microtiter plates made of different polymers (PS, PP, and PC) were functionalized with PDMA in a one-step process to investigate the effect of surface functionalization on enzymatic activities. FTIR analyses were performed to demonstrate successful functionalization. The corresponding spectra and microscopic images are provided in the ESI (Fig. S1 and S2†).

Adsorption of enzymes to PDMA and different polymer wells

First, the effect of PDMA surface functionalization on nonspecific protein adsorption was investigated. Enzyme solutions were pre-incubated in PDMA-functionalized wells made of PS, PP and PC, as well as in non-functionalized wells. Following pre-incubation, enzyme activity was assessed by measuring the formation of the substrate products. The corresponding diagrams are presented in Fig. 1, revealing remarkable differences in substrate product formation among enzymes pre-incubated in wells that were PDMA-functionalized and those that were not. Thereby, the substrate turnover was lower for all enzymes (TEM-1 in Fig. 1A, HRP in Fig. 1B, and ALP in Fig. 1C) after incubation in non-functionalized wells. This effect was observed across all tested polymer types, including PS, PP and PC.

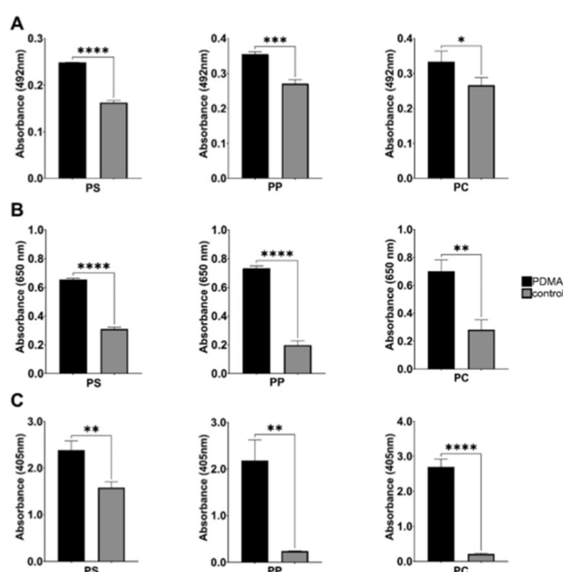

Fig. 1 Adsorption of enzymes onto different polymers. Plots of enzyme activity assays displaying the substrate turnover of (A) TEM-1, (B) HRP and (C) ALP after 30 min pre-incubation in PS, PP and PC wells functionalized with PDMA against non-functionalized wells (control). Each data point represents the mean ± SD of three different experiments.

Effect of PDMA on enzymatic substrate turnover

Starting with the determination of substrate turnover, the effect of PDMA-functionalization on the activities of TEM-1, HRP and ALP was tested. The substrate turnover of different amounts of substrates was measured spectrophotometrically after 60 minutes of incubation. Fig. 2 displays the amount of substrate that the enzymes turned over. For all enzymes, higher amounts of substrates were converted in the wells functionalized with PDMA than in the unmodified control wells. Differences in the amounts of substrate converted are observed at all substrate concentrations, with greater differences at higher concentrations, except for the oxidation of TMB, where no difference in substrate turnover was observed at low substrate concentrations (Fig. 2B). However, for higher substrate concentrations of 30% (for 100 μM) and 40% (for 120 μM) more substrate was converted. The hydrolysis of nitrocefin (Fig. 2A) revealed more significant results with an increase in substrate conversion of 40–50% at lower concentrations (<100 μM) and of 80–>100% at higher concentrations. For example, with 250 μM nitrocefin being used, only 100 μM was converted in the unmodified wells, whereas 225 μM was converted in the PDMA functionalized wells. The enhancement of substrate conversion was found to be even more significant for the hydrolysis of pNPP. As shown in Fig. 2C, the increase is already up to 80% for lower concentrations (125 μM and below) and 100–200% for higher concentrations (>200 μM). For a substrate concentration of 1 mM, 50 μM was turned over in unmodified and 150 μM in PDMA-functionalized wells.

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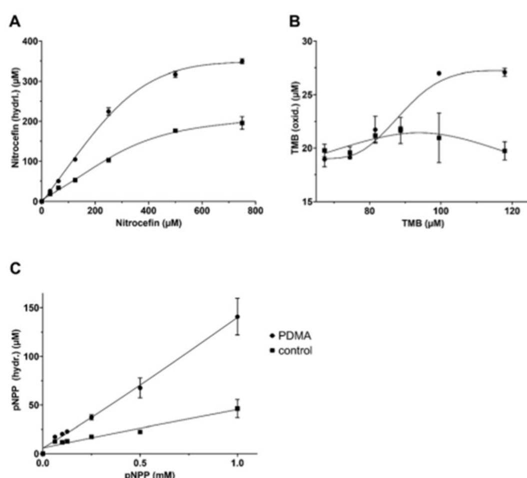

Fig. 2 Effect of PDMA on enzymatic substrate turnover. Plots of enzyme activity assays displaying substrate turnover after 60 minutes on PDMA hydrogel coated wells performed with (A) TEM-1 β-lactamase hydrolyzing varied concentrations of the nitrocefin substrate (492 nm), (B) HRP oxidizing varied concentrations of the TMB substrate (650 nm) and (C) ALP hydrolyzing varied concentrations of the pNPP substrate (405 nm). Each data point represents the mean ± SD of three different experiments. Detailed tables of the values presented are provided in the ESI (Tables S1–S3†).

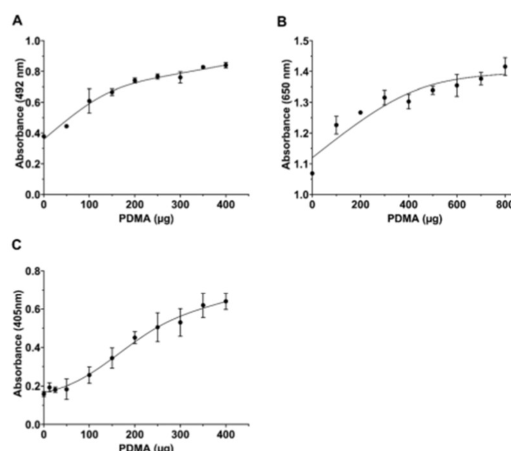

Fig. 3 Effect of PDMA amount on enzyme activity. Plots of enzyme activity assays displaying substrate turnover after 60 minutes in wells coated with different amounts of PDMA: (A) TEM-1 β-lactamase hydrolyzing the nitrocefin substrate (492 nm), (B) HRP oxidizing the TMB substrate (650 nm) and (C) ALP hydrolyzing the pNPP substrate (405 nm). Each data point represents the mean ± SD of three different experiments. Detailed tables of the values presented are provided in the ESI (Tables S4–S6†).

Effect of PDMA amount on enzymatic activity

For more detailed investigation of the effect of PDMA and to gain evidence for the possible underlying mechanisms, different amounts of PDMA were immobilized in the wells. As shown in Fig. 3, an effect of PDMA amount on substrate turnover can be detected. After 60 minutes of incubation, the enzymes in wells with higher amounts of PDMA converted more substrate. The absorption and thus the substrate turnover initially increased linearly with increasing amounts of PDMA and tended to saturate at a certain amount. This amount is around 250 µg in the case of TEM-1 β-lactamase (Fig. 3A), 400 µg for HRP (Fig. 3B) and above 350 µg for ALP (Fig. 3C).

Effect of PDMA on enzyme kinetics

The effect of PDMA-functionalization on enzyme catalytic performances was assessed by substrate turnover kinetic analyses. The results are displayed in the form of the initial velocities in Fig. 4, using Michaelis–Menten plots. The curves reveal that both higher initial velocities and maximal velocities are achieved with PDMA-functionalization. In Fig. 4A, the maximal velocity of TEM-1 β-lactamase was increased by 90% from 1.75 µM min^{-1} to 3.31 µM min^{-1} by implementing PDMA in the reaction system. For HRP, an elevation of about 45% from 0.67 µM min^{-1} to 0.96 µM min^{-1} could be achieved (Fig. 4B). Similarly, a significant increase in maximal velocity can be observed for ALP in Fig. 4C, although this cannot be determined exactly as both curves have not saturated completely. Nevertheless, an increase of about 160% or even more can be estimated (from ~0.15 µM min^{-1} to ~0.4 µM min^{-1}).

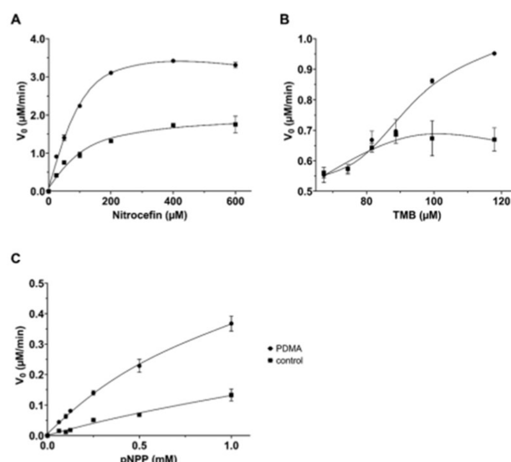

Fig. 4 Effect of PDMA on enzyme kinetics. Michaelis–Menten plots of (A) nitrocefin hydrolysis by TEM-1 β-lactamase, (B) TMB oxidation by HRP and (C) pNPP hydrolysis by ALP in PDMA hydrogel coated wells (circle) and in untreated control wells (squares). Each data point represents the mean ± SD of three different experiments. Detailed tables of the values presented are provided in the ESI (Tables S7–S9†).

Effect of PDMA-functionalization of different polymers

In order to investigate the effect of PDMA-functionalization on different polymers, not only PS microtiter plates but also PP

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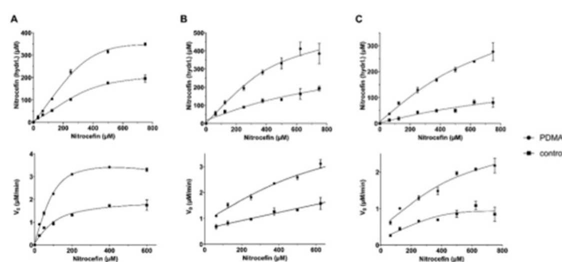

Fig. 5 Effect of PDMA-functionalization of different polymers on TEM-1 β-lactamase activity. Plots of TEM-1 activity assays displaying substrate turnover after 60 minutes (top) and Michaelis–Menten plots of nitrocefin hydrolysis by TEM-1 (bottom) in microtiter plate wells made of (A) PS, (B) PP and (C) PC functionalized with PDMA and those not functionalized (control). Each data point represents the mean ± SD of three different experiments. Detailed tables of the values presented are provided in the ESI (Tables S1, S7, S10–S13†).

and PC plates were functionalized with PDMA. For this purpose, TEM-1 β-lactamase was used as the model enzyme, and the turnover of the nitrocefin substrate, along with the determination of initial velocities, was analyzed on these plates. The results displayed in Fig. 5 indicate that PP and PC show similar effects to PS, when functionalized with PDMA. The substrate turnover in PDMA-functionalized PP wells (Fig. 5B (top)) and PC wells (Fig. 5C (top)) was significantly higher for all substrate concentrations compared to the non-functionalized wells. The same applies for the initial velocities and maximal velocities, which were also significantly higher in PP wells (Fig. 5B (bottom)) and PC wells (Fig. 5C (bottom)) with PDMA-functionalization.

Discussion

Surfaces play an important role in biology and medicine, as most biological reactions occur at surfaces and interfaces. The surface properties of a material are directly related to the interactions between biological components and the surface, influencing their performance.[14] The functionalization of surfaces and thus the modification of surface properties can be used to improve this performance. Therefore, we investigated the impact of simple surface functionalization on the catalytic behavior of enzymes. Effects on both temporal substrate turnover and the initial reaction rate were evident.

It is known that enzyme concentration directly impacts catalysis rates, leading to increased reaction rates with higher enzyme concentrations due to a higher likelihood of enzyme–substrate collisions. Similarly, substrate concentration plays a critical role as enzyme activity typically follows a saturation curve, where the initial increase in substrate concentration leads to a proportionally higher catalysis rate until the enzyme becomes saturated. Differences in the catalytic behaviour of the enzymes can thus be attributed to both enzyme and substrate concentrations. As the same concentration of enzyme or substrate was used in each of the experiments, the obtained results can be related to the PDMA surface functionalization.

Proteins adsorb rapidly from the surrounding liquid phase to a surface. Thereby, the surface properties have an influence on the amount and the conformation of the adsorbed proteins.[14] Depending on the material of the surface, proteins exhibit different affinities, influencing both the likelihood of adsorption and the strength of adherence. The interaction between the protein and surface occurs through intramolecular bonds, such as hydrophobic interactions, hydrogen bond formation and charge transfer.[15] Hydrophobic polymers, such as PS used as a support material in this study, adsorb greater amounts of proteins compared to less hydrophobic surfaces.[16,17] It has been demonstrated that PDMA surface functionalization results in a more hydrophilic surface with strong swelling properties in aqueous media and high resistance to protein adsorption.[18] This can be attributed to the strong binding of water molecules to the material surface groups, preventing proteins from displacing the surface-bound layer of water molecules.[19] The experiments performed here confirmed that PDMA surface functionalization reduces non-specific protein adsorption on PS as well as PP and PC surfaces. After pre-incubation of different enzymes in PDMA-functionalized wells, higher substrate product formation was observed compared to non-functionalized wells. The substrate product formation is directly related to the enzyme concentration, as more enzymes can convert higher amounts of substrate at the same time. As the initial concentrations were identical, it can be concluded that there was a decrease in the enzyme concentration in the non-functionalized wells caused by non-specific adsorption of the enzymes to the polymer wells. Therefore, when implementing PDMA to enzymatic reaction systems, it is expected that enzymes adsorb rapidly to the unmodified hydrophobic polymer surfaces, whereas there is significantly less adsorption of the enzymes to the PDMA-functionalized polymer surfaces. Here, the enzymes remain in solution in close proximity to the substrate, increasing the probability of enzyme–substrate collision, observed as increased reaction rates. In addition, proteins adsorbing through hydrophobic interactions to nonpolar surfaces like PS can cause significant conformational changes and even denaturation.[14,20] Thus, due to the conformational changes upon adsorption, a protein with a usual specific activity in solution may lose its activity when adsorbed onto a surface.[21] In the experiments on enzymatic substrate turnover, we showed that after the same time (after 60 min), higher absorption values were measured with PDMA-functionalization, resulting in more substrate conversion than without PDMA-functionalization. Based on the results of the experiments investigating different amounts of PDMA, evidence was provided that the observed effect is related to PDMA-functionalization. Applying a small amount of PDMA functionalizes only a small area of the well surface, and the effects occur only at a narrow area, resulting in no major difference in substrate turnover compared to unmodified wells. With higher amounts of PDMA, a larger surface area is functionalized, resulting in a greater effect. Above a

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certain amount of PDMA, as soon as the entire well surface is functionalized, the effect cannot be further enhanced, and the curves reach saturation, as shown in Fig. 3.

In addition to increased substrate turnover on PDMA-functionalized surfaces, we were able to demonstrate increased initial velocities and maximal velocities. The maximal velocities are reached when all enzymes form an enzyme–substrate complex. With more enzymes present, more enzyme–substrate complexes can form, allowing a higher maximal velocity. The maximal velocities are thus likewise dependent on the amount of enzymes. The determination of the maximal velocities is based on the initial velocities, which are determined in the initial linear phase of substrate turnover. The effects caused by the PDMA result in faster assembly of the enzyme–substrate complexes and thus higher initial velocities as well as higher maximal velocities. In turn, this means that with PDMA-functionalization, more enzymes are present in complexes earlier, respectively, at a smaller substrate concentration, than in the case without PDMA. Hence, without PDMA, more substrate is required to achieve the same reaction rate. Implementing PDMA in processes applying enzymes can result in reduced substrate usage and thus lower costs and decreased environmental impact.

In summary, the method presented here offers several possibilities for optimizing enzymatic applications. Up to two-thirds less substrate can be used to produce the same amount of converted substrate or product in the same reaction time. This allows the amount of substrate to be reduced, saving resources and causing less environmental impact. Considering biotechnological applications, it is conceivable that the use of polymeric beads in, for example, reactors for enzymatic reactions will bring further increase in effectiveness. We demonstrated that the effect of surface functionalization depends on the amount of PDMA and correspondingly the size of the functionalized surface. The implementation of polymeric beads in a reaction system will increase the surface area that can be functionalized and therefore the amount of PDMA in the system. In addition, our results demonstrate that the substrate turnover can be detected earlier by using PDMA surface functionalization. Assuming a fixed amount of substrate in an enzymatic assay, substrate turnover can be detected earlier by using PDMA, as the reaction is faster. Therefore, the results can be obtained more quickly.

Moreover, the positive effect of surface functionalization with PDMA has further potential applications in the field of diagnostic assays based on enzymatic reactions. Enzyme assays perform well when the enzyme is abundant and highly active; however, real-world applications often involve different conditions. Most often, the issue is identifying dilute solutions and therefore weak enzymatic activity in a reaction medium containing a complex matrix.[22] This is particularly relevant as enzymatic assays are used for the routine monitoring of a large number of enzymes that serve as clinical markers. In these assays, enzyme profiles are determined in serum, urine, *etc.*, and their levels are often associated with disease conditions.[23] Enhancing enzyme assays in terms of sensitivity and detection limit can revolutionize diagnostics by enabling earlier and more accurate detection, potentially leading to improved treatment outcomes and patient care.

Conclusion

We demonstrated a method for enhancing enzyme performance by implementing the use of polydimethylacrylamide in enzymatic reaction systems. Simple one-step surface functionalization *via* UV-crosslinking was applied to form stable PDMA coatings in polystyrene, polypropylene and polycarbonate microtiter plate wells. We observed increased substrate turnover and initial velocities for various enzymes and substrates in the functionalized wells. These effects are attributed to improved enzyme–substrate interactions facilitated by the hydrophilic nature of PDMA-functionalized surfaces, which prevent protein adsorption.

Overall, this work outlines an advantageous approach to optimizing enzymatic applications for more sustainable and efficient bioprocessing by reducing resource consumption, waste generation and overall costs. Our results demonstrate that incorporating PDMA in a reaction system can save up to two-thirds of the substrate. As we demonstrated the positive effect of PDMA surface functionalization for polymers and enzymes of different types, it can be assumed that these properties can also be beneficial for other polymers, enzymes, assays and diverse industrial applications.

Author contributions

Conceptualization: H.-P. Deigner; data curation: S. Rentschler; funding acquisition: H.-P. Deigner; investigation: S. Rentschler and M. Borgolte; methodology: S. Rentschler and M. Borgolte; resources: H.-P. Deigner, S. Laufer, and R. Csuk; supervision: H.-P. Deigner, S. Laufer, and R. Csuk; visualization: S. Rentschler; writing – original draft: S. Rentschler; writing – review & editing: H.-P. Deigner, S. Laufer, and R. Csuk.

Conflicts of interest

There are no conflicts to declare.

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5 Project II: Diagnostic β-lactamase assay

Publication Project II

Highly efficient β-lactamase assay applying polydimethylacrylamide-based surface functionalization with β-lactam antibiotics and β-lactamase inhibitors

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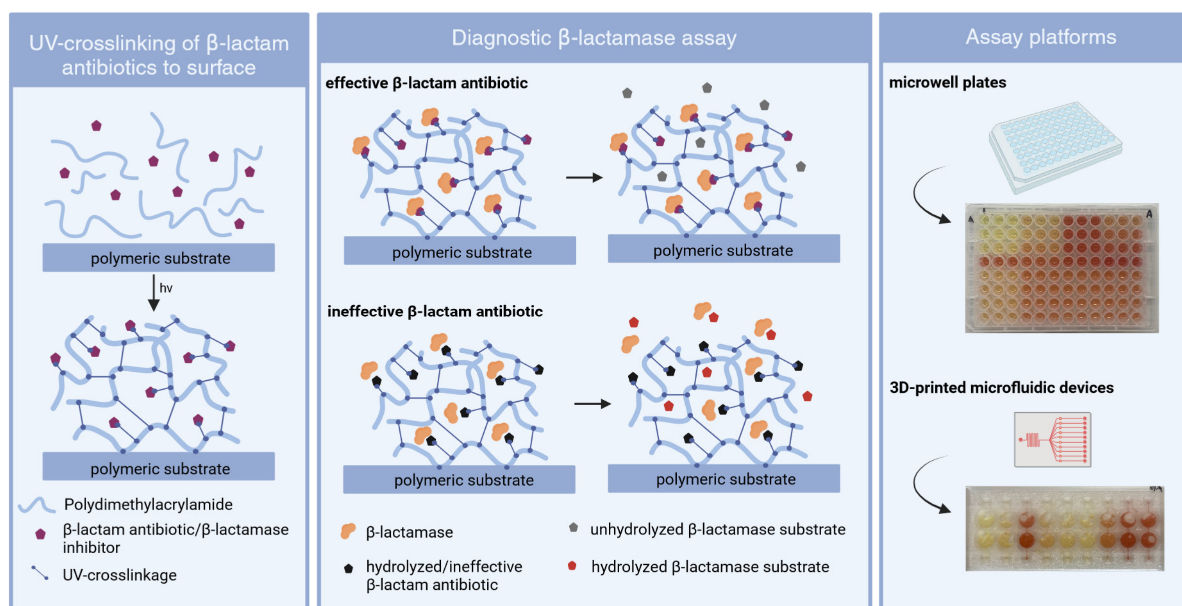

Figure 7 *Graphical Abstract Publication Project II: Immobilization of antibiotics to surfaces via PDMA by UV-crosslinking, providing a highly efficient diagnostic β-lactamase assay. The method is transferable to different substrate materials and devices as well as antibiotics and chemical substances.* [134]

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Highly efficient β-lactamase assay applying poly-dimethylacrylamide-based surface functionalization with β-lactam antibiotics and β-lactamase inhibitors†

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In recent decades, the rise of β-lactamases has substantially led to the emergence and wide spread of antibiotic resistance posing a serious global health threat. There is growing need for the development of rapid, cost-effective and user-friendly diagnostic assays for the accurate detection of β-lactamases to optimize patient outcomes and prevent the spread of multidrug-resistances. In this article, we present a poly-dimethylacrylamide (PDMA)-based surface functionalization to immobilize β-lactam antibiotics and β-lactamase inhibitors of different subclasses. Immobilization was induced *via* UV-crosslinking through C, H-insertion reactions. The functional coatings were successfully applied in a highly efficient assay for the determination of recombinant β-lactamases as well as β-lactamases isolated from clinically relevant bacterial strains. Thus, this method describes an innovative approach with several significant benefits for diagnostic applications: the creation of specific detection platforms tailored for β-lactamase activity, the development of high-throughput diagnostic assays and benefits regarding stability and shelf-life. Furthermore, this method is highly adaptable to other surfaces, antibiotics, and analytes, offering far-reaching implications for various biomedical, environmental, and antimicrobial applications.

Introduction

The emergence and spread of antibiotic resistance among bacterial pathogens represent an urgent global health concern. The limited efficacy of conventional methods of infection control, such as antibiotics and disinfectants, coupled with the alarming rise in multidrug-resistant strains, necessitates the development of innovative strategies to combat this growing health crisis.[1,2] Among the diverse range of antibiotics, β-lactam antibiotics have been widely employed for their efficacy against various bacterial infections.[3] However, the emergence and dissemination of β-lactamases, capable of hydrolyzing these antibiotics, pose a significant challenge to their therapeutic effectiveness. The extensive spread of β-lactamases in bacteria has rendered many β-lactam antibiotics, including penicillins, cephalosporins, and carbapenems, increasingly ineffective, limiting treatment options for infectious diseases and exacerbating morbidity and mortality rates.[4,5]

Accurate and timely determination of bacterial susceptibility to antibiotics, as well as the detection of β-lactamases, is crucial for guiding appropriate antibiotic therapy decisions to optimize patient outcomes and preventing the spread of multidrug-resistant bacteria.[6] In recent years, various diagnostic methods have been explored for antibiotic susceptibility testing (AST) and for the identification of β-lactamases, including phenotypic tests, genotypic assays, and biochemical techniques. However, many of these methods are time-consuming, technically demanding, or lack the required sensitivity and specificity.[7,8] Therefore, there is growing need for the development of rapid, cost-effective, and user-friendly diagnostic assays.

The functionalization of surfaces with antibiotics holds tremendous potential to revolutionize diagnostic applications in bacterial infections. By immobilizing antibiotics on

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different kinds of surfaces, such as polymeric materials, biosensors, and medical devices, it becomes possible to detect and monitor the presence of specific bacterial pathogens and particularly β-lactamases.[9]

In this context, poly-dimethylacrylamide (PDMA), a versatile and highly tuneable polymer, can be seen as a promising candidate for surface modification due to its unique attributes and remarkable adaptability. It is a synthetic polymer derived from dimethylacrylamide monomers, which exhibits excellent film forming capabilities, high optical quality and transparency as well as low toxicity and thus biocompatibility.[10–12] PDMA possesses a highly cross-linked structure, resulting in a robust and durable surface coating that can withstand diverse environmental conditions.[13] Furthermore, its chemical composition enables facile modification through the incorporation of functional groups, allowing for tailored and specific interactions with various substrates and surfaces.[14]

To immobilize the β-lactam antibiotics, methacryloyl benzophenone (MBP) is incorporated into the PDMA polymer. MBP acts as a photoactive crosslinking agent, enabling the immobilization of the antibiotics on the solid surface. The immobilization process occurs through a C,H-insertion reaction, which occurs when the benzophenone moiety absorbs UV light generating highly reactive benzoyl radicals. These radicals can insert into the C–H bonds of a surface to be modified, *e.g.* polymeric substrates, forming covalent bonds between the surface and the PDMA–MBP as well as into C–H bonds of the β-lactam antibiotics, forming covalent bonds between the antibiotic and the PDMA–MBP.[15,16]

Through this photoactive UV-crosslinking process, the β-lactam antibiotics become immobilized onto the surface, becoming covalently attached to the PDMA copolymer. This immobilization confers several significant benefits for diagnostic applications, as the creation of specific detection platforms tailored for β-lactamase activity, the possibility for the development of high-throughput diagnostic assays and benefits regarding stability and shelf-life of diagnostic assays.

Here we describe a method using poly-dimethylacrylamide copolymerized with methacryloyl benzophenone to immobilize different β-lactam antibiotics and β-lactamase inhibitors on solid surface. Surface anchoring was accomplished *via* photoactive UV-crosslinking of the benzophenone moiety on polypropylene surfaces.

We comprehensively explored the potential of surface functionalization with crosslinked β-lactam antibiotics as diagnostic tool for the detection of recombinant β-lactamases as well as β-lactamases isolated from clinically relevant bacterial strains. Besides the detection of β-lactamases it was also possible to determine their activity and resistance profile *versus* various β-lactam antibiotics and β-lactamase inhibitors tested.

This research aims to contribute to the development of innovative approaches for combating bacterial infections and determining strain-dependant antibiotic susceptibility by exploring the potential of surface functionalization with antibiotics *via* crosslinking. The study significantly contributes to the design and fabrication of advanced biomaterials with enhanced antimicrobial properties, thus addressing the urgent need for effective infection control in various healthcare and biomedical applications.

Experimental

Chemicals, materials and solution

PDMA–MBP was synthesized and characterized as previously described.[17] β-lactam antibiotics meropenem trihydrate, cefoperazone sodium and ampicillin sodium were purchased from Sigma-Aldrich Inc. (St. Louis, United States) and aztreonam from Thermo Fisher Scientific (New Jersey, United States). β-lactamase inhibitor tazobactam was purchased from Sigma-Aldrich, Inc. (St. Louis, United States), avibactam sodium was acquired from Combi-Blocks Inc. (San Diego, United States), relebactam and sulbactam from Career Henan Chemical Co. (Zhengzhou City, China). β-lactam antibiotics and β-lactamase inhibitors were dissolved in 0.01 M phosphate-buffered saline (PBS, pH 7.4) to a concentration of 5 mg ml^{-1}. Varying concentrations of β-lactam antibiotics/inhibitors were used to investigate their enzyme inhibiting effect. Antibiotics/inhibitors were diluted to concentrations of 2.5, 1.25 and 0.625 mg mL^{-1} in an 80% ethanol mixture in H_2O either containing PDMA or not (for controls).

LB broth (Lennox) as well as all cultivation media components were purchased from Sigma-Aldrich, Inc. (St. Louis, United States). Further cultivation media used were nutrient agar (tryptone 5.0 g, meat extract 1.0 g, yeast extract 2.0 g, sodium chloride 5.0 g, distilled water 1 L) and trypticase soy yeast extract medium (trypticase soy broth 30.0 g, yeast extract 3.0 g, distilled water 1 L).

Nitrocefin chromogenic substrate was purchased from ApexBio Technology LLC (Houston, USA). The substrate nitrocefin was dissolved in DMSO to a concentration of 8 mM and further diluted in PBS to a final substrate concentration of 0.5 mM. 96-well polypropylene plates were purchased from Greiner AG (Kremsmünster, Austria).

Fabrication of 3D printed microfluidic devices

CAD model of a microfluidic device was created in Autodesk Fusion 360 (Autodesk, USA) and sliced with the open source slicer software Cura (Version 4.6.1, Ultimaker, Netherlands) to adjust the parameters to the polymer used for 3D printing. Microfluidic devices were fabricated with an Ultimaker 3 (Ultimaker, Netherlands) FDM 3D printer with a 0.4 mm nozzle head using transparent 2.85 mm polymethylmethacrylate (PMMA) polymer filament (**https://filamentworld.com**, Germany).

Preparation of polymer coatings

PDMA was diluted to a concentration of 3 mg mL^{-1} in an 80% ethanol mixture in H_2O. From the mixture, 40 μL was

pipetted into each well of a 96-well polypropylene plate. The plates were allowed to dry at room temperature under the fume hood for at least 4 h and crosslinked with 2 J cm^{-2} UV light at 365 nm. 20 μl of the prepared β-lactam antibiotics or β-lactamase inhibitors was added into the dedicated wells. The plates were again allowed to dry at room temperature under the fume hood for at least 2 h and crosslinked with 1 J cm^{-2} UV light at 365 nm, followed by 3× washing with 250 μL of PBS. Well plates for reference measurements (to determine non-specific binding of antibiotics and inhibitors) were equally treated, using 40 μL of 80% ethanol mixture in H_2O respectively 20 μl of antibiotic dilution/solution in 80% ethanol mixture H_2O without PDMA.

Samples for FTIR and SEM characterization were prepared by carefully pipetting and distributing 40 μl of the polymer solution on a polystyrene surface to obtain a fully coated surface. The surfaces were let dry in air for at least 4 h, followed by crosslinking with 3 J cm^{-2} at 365 nm. Washing 3× with ddH_2O and 3× with ethanol, followed by drying in an N_2 stream yielded the final coating, which was used directly for IR spectroscopy. Samples for SEM characterization were further dried in vacuum and thereafter coated with an approximately 5 nm thick Au/Pd layer (SC7620 sputter coater, Quorum, Laughton, UK).

Microfluidic devices were prepared by adding 28 μl of PDMA dilution (3 mg ml^{-1} in H_2O) to each of the lower chambers. The chips were incubated at room temperature for 4 h and crosslinked with 2 J cm^{-2} UV light at 365 nm. 20 μl of the prepared β-lactam antibiotics or β-lactamase inhibitors was added into the dedicated lower chamber of the microfluidic device. The chips were again incubated at room temperature for 2 h and crosslinked with 1 J cm^{-2} UV light at 365 nm. The chambers were washed 3× with 100 μl of PBS from the lower chamber side upwards and the chambers dried with an N_2 stream.

FTIR and SEM surface characterization

IR data were recorded on a Spotlight 400 FT-IR microscope (PerkinElmer, Waltham, USA) in the range of 4000–400 cm^{-1} using the transmittance mode of operation. The spectra were acquired and processed using the associated Spectrum 10™ software. Scanning electron microscope (SEM) images were obtained with a FIB-SEM Crossbeam 550L (Carl Zeiss AG, Oberkochen, Germany) at 1–10 kV.

Recombinant β-lactamases

Recombinant *E. coli* TEM-1 β-lactamase (Lot: 1121PLACTB) was purchased from Prospec-Tany Technogene Ltd. (Ness-Ziona, Israel), recombinant *A. baumannii* β-lactamase OXA-23 (Lot: 202677) and recombinant *K. pneumoniae* β-lactamase IMP-4 (Lot: 202676) from LifeSpan BioSciences Inc. (Lynnwood, USA), recombinant *P. aeruginosa* β-lactamase ampC (Batch Nr.: DA04483a7g0) from Cusabio Technology LLC (Houston, USA), and a recombinant *P. aeruginosa* β-lactamase blend ("SiAl") (Lot: SLBM8806V) from Sigma Aldrich with known hydrolyzing capabilities of benzylpenicillin and cephalosporin C. Recombinant β-lactamases were diluted in PBS to a concentration of 2 nM.

β-lactamase expressing bacterial strains

β-lactamase expressing bacterial strains were purchased from DSMZ – German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). The following strains were used for the experiments: *K. pneumoniae* strain Kp_Goe_828304 (encoding OXA-48, class D carbapenemase), *P. aeruginosa* strain NCCP 12293 (encoding VIM-2, class B metallo-carbapenemase), *A. baumannii* strain RUH2037 (encoding cephalosporinase AmpC, class C cephalosporinase) and *E. coli* strain A511515A (encoding extended-spectrum β-lactamase (ESBL), class A β-lactamase).

K. pneumoniae was cultured in nutrient agar, *P. aeruginosa* and *E. coli* in trypticase soy yeast extract medium and cultivation of *A. baumannii* was performed in LB-medium. 50 mL medium was inoculated with 50 μL of a particular stock culture and incubated at 37 °C. Incubation time varied between 24 and 48 hours.

Isolation of β-lactamases

45 mL of cell suspensions were centrifuged at 3220×*g* at 4 °C for 10 min. The pellets were resuspended in 1 mL phosphate lysis buffer (28.9 mM Na_2HPO_4 $7H_2O$, 21.1 mM NaH_2PO_4 H_2O, 10 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride (PMSF)). The suspensions were sonicated two times for 5 min, in between the samples were kept on ice for 5 min. The cell lysates were centrifuged at 16 000×*g* for 20 min and the supernatants containing the β-lactamases were collected. Specific activity of β-lactamase solutions were obtained and calculated from the substrate turnover rate in the linear range of a nitrocefin substrate assay.

β-lactamase assay for antibiotic susceptibility testing on PDMA plates

65 μl of recombinant β-lactamase (2 nM) respectively 1 U isolated β-lactamase were added to each well of a pretreated 96-well microplate. The plates were incubated at room temperature for 45 minutes to allow for potential hydrolysis of the β-lactam antibiotic respectively β-lactamase inhibitor by the β-lactamases. Subsequently, 35 μL nitrocefin solution was added to each well to determine the activity of β-lactamases. The substrate product formation was monitored as absorbance increase at 492 nm (TECAN Infinite 200 PRO) over 45 minutes. In case of β-lactamases, which are susceptible to specific β-lactam antibiotics respectively β-lactamase inhibitors, no increase in absorption was detected here, as they are inactivated.

For reference measurements, antibiotic susceptibility of β-lactamases was determined in solution. Therefore, 65 μl of recombinant β-lactamase (2 nM) β-lactamase respectively 1 U isolated β-lactamase were added to each well of an untreated 96-well plate and mixed with 10 μl of β-lactam antibiotic

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respectively β-lactamase inhibitor solution. After an incubation of 45 minutes at room temperature 35 μL nitrocefin solution was added to each well to determine the activity of β-lactamases. The substrate product formation was monitored as absorbance increase at 492 nm over 45 minutes.

Microfluidic β-lactamase assay for antibiotic susceptibility testing

35 μl of recombinant β-lactamase (2 nM) respectively isolated β-lactamase (1 U) were added to each lower chamber of a pre-treated microfluidic device. The chips were incubated at room temperature for 45 minutes to allow for potential hydrolysis of the β-lactam antibiotic respectively β-lactamase inhibitor by the β-lactamases. Subsequently, 35 μL nitrocefin solution was carefully added to each lower chamber to obtain a mixture with the β-lactamase samples in both chambers. The substrate product formation by active β-lactamases was visible by colour change of the substrate from pale yellow to dark red.

Results and discussion

Efficiency of PDMA crosslinking and immobilization of β-lactam antibiotics and inhibitors

First, the modified plates were evaluated if proper crosslinking of the PDMA and the immobilization of the β-lactam antibiotics and β-lactamase inhibitors was achieved. For this purpose, IR spectroscopy was used to investigate the plates and the recorded spectra are shown in Fig. 1 and S1.† For PDMA (Fig. S1E†) dominant peaks between 3000–3500 cm^{-1} are present in the recorded IR spectra. All antibiotics/inhibitors likewise feature peaks in this region, so the peaks overlap with those of PDMA to some extent. Nevertheless, significant differences in the peaks are apparent, indicating the presence of the antibiotics on the surface. The expected PDMA–amides can be seen clearly at 1650 cm^{-1}. For antibiotics and inhibitors containing a β-lactam ring (all structures except for avibactam and relebactam), the characteristic peak in the region between 1760–1730 cm^{-1} can be identified. For non-β-lactam–β-lactamase inhibitors (avibactam (Fig. 1C) and relebactam (Fig. S1D†)), the characteristic peak of the diazabicyclooctan can be seen at 1500 cm^{-1}. Thus, the recorded Fourier transform-infrared spectra proved the presence of antibiotic/inhibitors on the microplate surface. As the model material, polypropylene (PP) 96 well plates were coated with the PDMA polymer. Based on previous studies, 3 J cm^{-2} was chosen as an optimum dose of UV irradiation and 150 μg of PDMA per well of a 96 well plate as an optimum amount of PDMA to obtain proper coating stability and coating thickness with minimum unreacted MBP left.[17,18]

The surface morphology of the PDMA–antibiotic/inhibitor coatings was investigated by SEM as shown in Fig. 2. For the

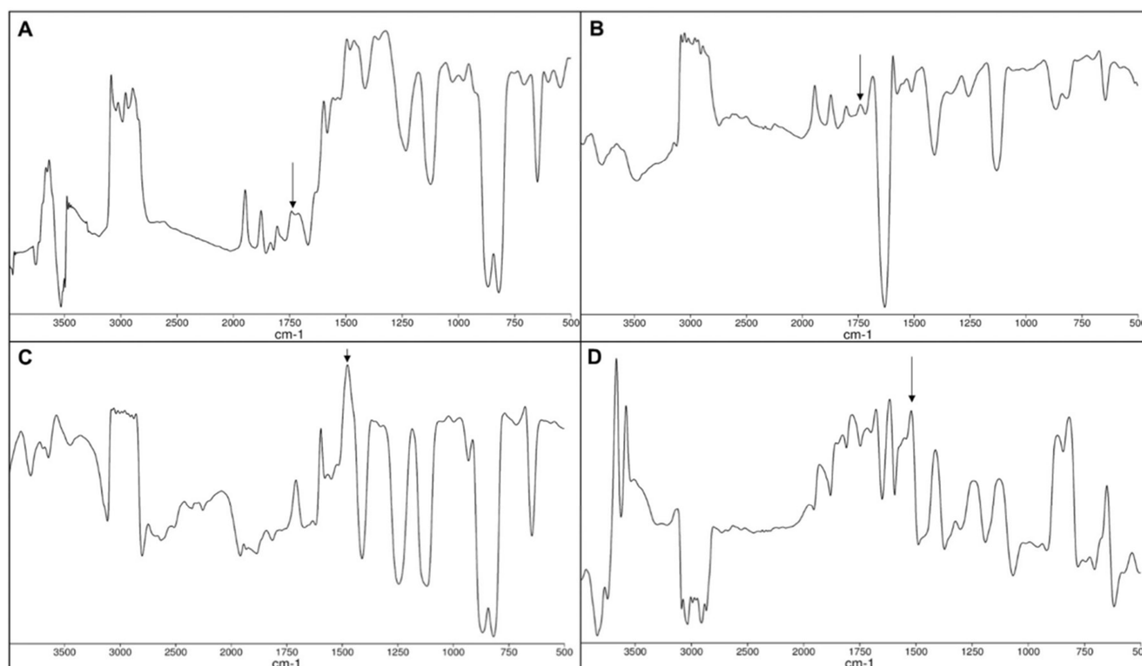

Fig. 1 Recorded Fourier transform-infrared spectra of β-lactam antibiotics and β-lactamase inhibitors crosslinked with PDMA–MBP on polypropylene plates. (A) Meropenem, (B) aztreonam, (C) avibactam and (D) tazobactam. Spectra were recorded in the range of 4000–400 cm^{-1}. Relevant peaks are marked with arrows.

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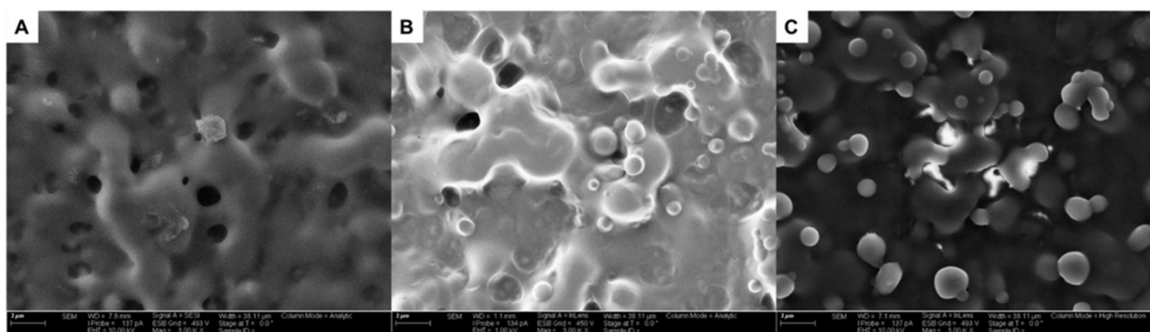

Fig. 2 SEM images of the PDMA coatings crosslinked with a β-lactam antibiotic and β-lactamase inhibitor. SEM images were captured for surfaces crosslinked with (A) PDMA, (B) PDMA and meropenem and (C) PDMA and avibactam.

surface functionalized only with PDMA, in the SEM image (Fig. 2A) a textured surface showing small pores and a sponge-like structure can be observed. Small particles can additionally be detected on the surfaces functionalized with β-lactam antibiotics and β-lactamase inhibitors, supporting successful immobilization. These findings are exemplified for meropenem and avibactam in Fig. 2 (Fig. 2B and C). The SEM images of the other antibiotics/inhibitors are provided in the ESI† (Fig. S2 and S3).

However, presence of the antibiotics/inhibitors on the surface does not necessarily provide assurance that the β-lactamases will interact with them. Several aspects could complicate or even prevent an interaction, such as whether a sufficient amount of antibiotic is present or whether accessibility is limited by steric hindrance. Therefore, inhibition assays with β-lactamases of different classes were carried out. The enzymes were preincubated in wells functionalized with various amounts of antibiotics/inhibitors, followed by a substrate turnover assay using the chromophoric cephalosporin nitrocefin. When the enzymes still had access and could bind to the immobilized antibiotics, inhibition of the enzyme activity was observed. In such a case, the nitrocefin substrate was not hydrolyzed and therefore no increase in absorption could be detected. Thus, the enzyme activity was ~0% relative to the enzyme activity in wells with PDMA only.

The results of the β-lactamase inhibition assays are displayed in Fig. 3–5. Based on the results of the activity test presented, interactions between β-lactamases and antibiotics/inhibitors are possible even after immobilization on the surface.

So far, it was not known whether the amount of antibiotic on the surface is sufficient, therefore, different amounts have been studied. Fig. 3 shows the effect of increasing amounts of immobilized antibiotics/inhibitors on the relative activity of β-lactamases after preincubation in the modified wells. The enzyme activity clearly depends on the amount of antibiotics/inhibitors applied, showing higher enzyme activity at lower antibiotics/inhibitor amounts. As expected, with higher amounts of antibiotics applied, more antibiotics were immobilized on the surface, resulting in greater inhibition of lactamase catalytic activity. The differences in the height of relative activities between the lactamases and antibiotics can be linked to the catalytic properties of the certain lactamases and will be discussed in more detail below. Images of the colour changes of the β-lactamase inhibition assays on PDMA shown in Fig. 3 are provided in the ESI† (Fig. S4).

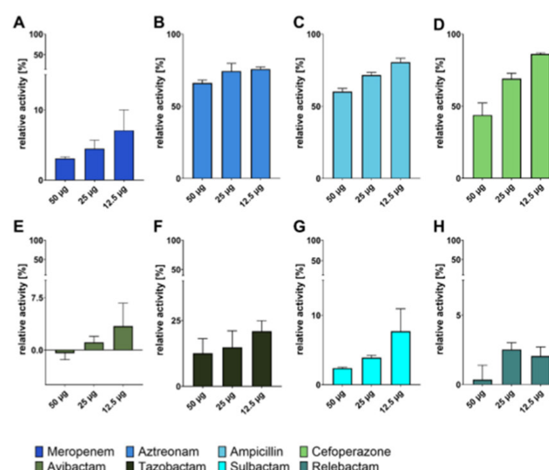

Fig. 3 Effect of different amounts of immobilized antibiotics/inhibitors in inhibition assay. Relative activity in nitrocefin substrate assays of different recombinant β-lactamases after preincubation in microplate wells modified with 50, 25 and 12.5 μg of various β-lactam antibiotics and β-lactamase inhibitors prepared by UV-crosslinking *via* PDMA. (A) ampC incubated on meropenem, (B) OXA-23 on aztreonam, (C) ampC on ampicillin, (D) OXA on cefoperazone, (E) ampC on avibactam, (F) "SiAl" lactamase blend on tazobactam, (G) ampC incubated on sulbactam and (H) ampC on relebactam. As reference for the determination of relative activity, substrate turnover of β-lactamase was measured in a well modified with PDMA only. Shown are the mean values (+/− SD) of three replicates per experimental condition. Negative values are caused by the background subtraction method applied and can be equated with an enzyme activity of 0%.

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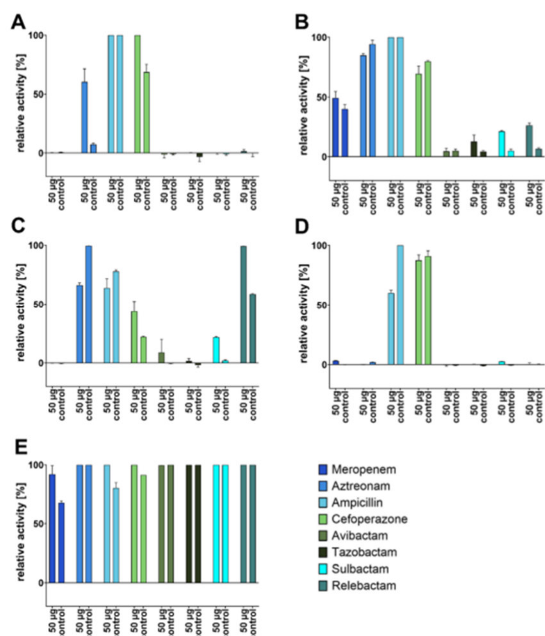

Fig. 4 β-lactamase (recombinant) inhibition assay in microplate wells functionalized with β-lactam antibiotics and β-lactamase inhibitors. Relative activity in nitrocefin substrate assays of different recombinant β-lactamases (A) TEM-1, (B) "SiAl"-lactamase blend, (C) OXA-23, (D) ampC and (E) IMP-4 after preincubation in microplate wells modified with 50 μg of various β-lactam antibiotics and β-lactamase inhibitors prepared by UV-crosslinking *via* PDMA. As reference for the determination of relative activity, substrate turnover of β-lactamase was measured in a well modified with PDMA only. The relative activity of the β-lactamases in the modified wells is compared to the relative activity under incubation conditions in solution ("control"). Shown are the mean values (+/− SD) of three replicates per experimental condition. For measurements with values below 2% relative activity, no increase in absorbance was detected between t = 0 min and t = 45 min. Values are caused by the background subtraction method applied and can be equated with an enzyme activity of 0%. A cutoff of 100% of relative activity was applied that is equivalent to non-inhibited enzyme activity.

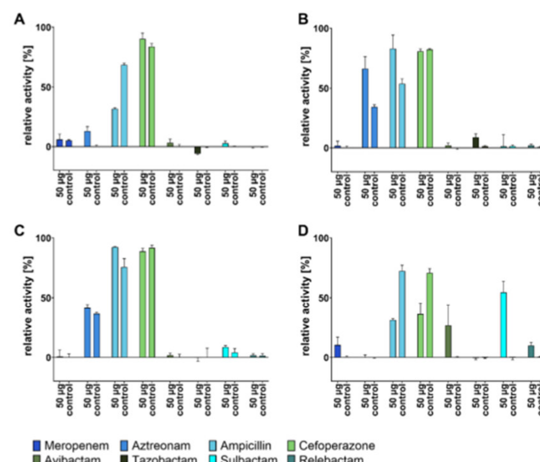

Fig. 5 β-lactamase (isolated) inhibition assay in microplate wells functionalized with β-lactam antibiotics and β-lactamase inhibitors. Relative activity in nitrocefin substrate assays of different isolated β-lactamases from clinically relevant bacterial strains (A) *Acinetobacter baumannii*, (B) *Escherichia coli*, (C) *Klebsiella pneumoniae* and (D) *Pseudomonas aeruginosa* after preincubation in microplate wells modified with 50 μg of various β-lactam antibiotics and β-lactamase inhibitors prepared by UV-crosslinked *via* PDMA. As reference for the determination of relative activity, substrate turnover of β-lactamase was measured in a well modified with PDMA only. The relative activity of the β-lactamases in the modified wells is compared to the relative activity under incubation conditions in solution ("control"). Shown are the mean values (+/− SD) of three replicates per experimental condition. For measurements with values below 2% relative activity, no increase in absorbance was detected between t = 0 min and t = 45 min. Values are caused by the background subtraction method applied and can be equated with an enzyme activity of 0%.

Specifications for the performance of inhibition assays are not consistent and they either use a molar excess of the substrate/antibiotics in relation to the molarity of the enzyme in the assay (~2000×–~20 000×) or in relation to the K_M-values of a β-lactamase for the specific substrate, varying between different β-lactamases and β-lactam antibiotics or β-lactamase inhibitors.[19,20] In this study, as different β-lactamases and antibiotics were investigated, the amounts were not specifically adjusted. The 50 μg investigated as the highest amount is equivalent to 10 times the amount of antibiotics/inhibitors compared to the amount administered in the control experiments in solution, based on our assumption that after the immobilization by UV crosslinking, not 100% of the antibiotic applied is potent. This, too, will be discussed later on. In the following, the experimental results for the assays performed with 50 μg antibiotics/inhibitors will be presented, as these showed most promising results.

Performance of UV-crosslinked β-lactam antibiotics and β-lactamase inhibitors in β-lactamase inhibition assays

The mere presence of antibiotics on the surface does not guarantee that the β-lactamases are able to bind to them. Steric hindrance can be problematic in this context. β-lactamases are diverse enzymes featuring different molecular structures and functional characteristics.[21,22] Here, we investigated β-lactamases of the four main classes Ambler class A (TEM-1), class B (IMP-4), class C (ampC) and class D (OXA-23). Depending on their Ambler class, β-lactamases are able to hydrolyze β-lactam antibiotics of different classes and generations as well as β-lactamase inhibitors. Therefore, in our assay, we evaluated the applicability and performance of meropenem (carbapenem), aztreonam (monobactam), ampicillin (penam) and cefoperazone (cephalosporine) as representatives of different classes of β-lactam antibiotics and moreover, tazobactam and sulbactam as β-lactamase inhibitors as well as avibactam and relebactam as non-β-lactam β-lactamase inhibitor. The results of the inhibition assays are shown in Fig. 4, where different patterns can be identified. Images of the colour changes of the β-lactamase inhibition assays on PDMA shown in Fig. 4 are provided in the ESI† (Fig. S5).

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For the first pattern, there is a consistent inhibition of relative enzyme activity for both the modified wells as well as for the control in solution. This includes TEM-1 incubated on meropenem, avibactam, tazobactam and sulbactam (Fig. 4A), OXA-23 incubated on meropenem and tazobactam (Fig. 4C) and ampC incubated on aztreonam, avibactam and tazobactam (Fig. 4D). For all of them, the determined relative enzyme activities were below 2%. This proves that the β-lactamases have bound to the respective antibiotics/inhibitors and are thus inactivated. In the clinical context, this would be referred to as antibiotics/inhibitors that are still effective inhibiting the particular β-lactamases.

In the second setting, relative activities with comparable heights can be found for both the immobilized antibiotics/inhibitors and the controls. This behaviour can be seen for all lactamases incubated on ampicillin, moreover for "SiAl" lactamase blend incubated on meropenem, aztreonam and avibactam (Fig. 4B), OXA-23 on aztreonam (Fig. 4C) and in case of IMP-4 for all antibiotics and inhibitors tested (Fig. 4E). In principle, two different mechanisms can lead to such a result. First, it is conceivable that the β-lactamases do not bind to the antibiotics and thus a relative activity can be detected. As this was also observed in the controls in solution, we can rule out that this effect is due to an insufficient immobilization of the antibiotics/inhibitors. Second, the β-lactamases are bound to the antibiotic initially but are able to hydrolyze it partially or already completely during the preincubation period. The β-lactamases thereby regain their activity to hydrolyze the nitrocefin substrate in the subsequent inhibition assays.

From the results in Fig. 4, differences in the catalytic activity of the different β-lactamases and antibiotics/inhibitors are also evident. These differences can be ascribed both to structural and functional properties of the various classes of β-lactamases and to the chemical structures of the antibiotics and inhibitors.[21] This influence is discussed in more detail below. Moreover, differences in heights of relative activities among the different antibiotics can, for example, be seen for meropenem (~50%), aztreonam (~90%), ampicillin (~100%) and avibactam (~5%) hydrolyzed by "SiAl" lactamases. These differences can be related to the individual specificity constants (k_{cat}/K_M) of the hydrolysis of the respective antibiotics/inhibitors.[23,24]

In addition to the consistent results described above, β-lactamase-antibiotic/inhibitor combinations can be noted in Fig. 4, where there are occasionally significant higher relative activities in the modified wells than in the controls in solution. However, a distinction must be made at this point in the interpretation of the results, and it must be considered whether the β-lactamase is capable of hydrolyzing the antibiotic under regular test conditions. If the β-lactamase is capable of hydrolysis, the observed differences in relative activity are negligible. This is the case for the incubations of TEM-1 with aztreonam (Fig. 4A) as well as the "SiAl" lactamase blend with tazobactam and sulbactam (Fig. 4B). The results can be attributed to the fact that the β-lactamase in the modified wells was not inhibited as much as in the controls or that the β-lactamase hydrolyzed the antibiotic more rapidly. In the other case, relative activities were detected in the modified wells but not in the controls in solution. This can be seen for the incubation of OXA-23 on avibactam (Fig. 4C) as well as of ampC on meropenem and sulbactam (Fig. 4D) and however, can be explained by the same reasons. Here, not enough antibiotic was present or the accessibility to the antibiotic was limited. Excess lactamase, which was unbound and inhibited, can then cause substrate hydrolysis in the inhibition assays. Nevertheless, even with these combinations, the method was proven to work. If the UV-crosslinking would have not been successful, the relative activity should have been nearly equal to the activity of the respective β-lactamase without antibiotic addition, and thus 100 percent.

The difference in relative activity in the functionalized wells compared to the controls in solution can be linked to several aspects, including the efficiency of UV-crosslinking, and related to this, the amount of antibiotic immobilized as well as the structural characteristics of the antibiotics/inhibitors and the β-lactamases. Several factors influencing the UV-crosslinking efficiency can be assigned to the chemical structure of the antibiotics. The β-lactam antibiotics and β-lactamase inhibitors (except for avibactam (non β-lactam β-lactamase inhibitor)) share the β-lactam ring as pharmacophore unit but differ in the heterocycle that is generally fused to it. Moreover, this bicyclic core is functionalized in several key positions differently.[25,26] The location of C–H bonds in the molecule affects the orientation of the molecule on the PDMA layer and so the accessibility of the β-lactam ring.

Another factor is that the immobilization of the antibiotics during UV-crosslinking occurs randomly *via* C,H-insertion reactions.[14,16] One consequence of this is that antibiotics are not specifically immobilized on the surface but are distributed throughout the PDMA layer. The β-lactamases are unable to penetrate the PDMA layer and thus can only bind to the properly oriented antibiotics on the surface.[16] As a result, not the entire amount of antibiotic applied during the UV-crosslinking can exert an effect. Therefore, less β-lactamase can be bound and inhibited. Another consequence is that the antibiotics with more C–H bonds also have more opportunities for immobilization. This influences the orientation of the antibiotics, which can either enhance or reduce the accessibility of the β-lactam ring to the β-lactamase for binding.

Depending on the orientation of the antibiotics, it is conceivable that the binding is impeded by steric hindrance. This is mainly influenced by two factors, firstly the chemical structure of the antibiotics/inhibitors (bulky *vs.* nonbulky) and secondly the conformation of the active site of the β-lactamases. The initial emergence of β-lactamases and the associated resistance to the first β-lactam antibiotics drove researchers to develop new subclasses of β-lactam antibiotics as well as β-lactamase inhibitors. Strategies for producing

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antibiotics that were still effective involved altering the chemical structure and inserting bulky side groups.[27,28] Another approach was the development of non-β-lactam β-lactamase inhibitors, such as avibactam, which does not contain a β-lactam core but maintains the capacity to covalently acylate their β-lactamase targets.[29,30] As a result, the antibiotics and inhibitors of the various subclasses differ in chemical structure and bulkiness. The β-lactamase classes feature distinct amino acid sequences, which determines their tertiary structure and thus the conformation of the active site as well as influence the formation of the acyl–enzyme complex.[31] Class A, C and D include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine, whereas class B β-lactamases are metalloenzymes that need active-site zinc ion to facilitate β-lactam hydrolysis.[32,33] Dependent on the conformation of their active site, their binding to the immobilized antibiotics is also affected. If we consider the crystal structures of the different β-lactamases, the active site binding pockets are more or less open depending on the subtype.[34,35] This, in combination with the differing bulkiness of the antibiotics/inhibitors, explains the divergence in assay performance with respect to the various lactamase–antibiotic combinations tested. Nevertheless, our results demonstrate that β-lactam antibiotics and β-lactamase inhibitors of different classes can be immobilized on surfaces and that they retain their properties. With regard to the implementation in a diagnostic assay, the method can be optimized in few aspects. First, the UV-crosslinking can be altered in terms of the composition and ratio of PDMA and MBP to obtain steady surface functionalization. Second, the amount of antibiotic can be adapted to optimize crosslinking efficiency specifically for the different antibiotics. Moreover, it can be examined whether the introduction of linker molecules can increase the accessibility of the β-lactamases to the antibiotics/inhibitors and thereby further improve assay performance.

Transferability of PDMA–antibiotic/inhibitor – β-lactamase inhibition assay to clinical samples

The method for immobilization of antibiotics *via* PDMA was further investigated for applicability with clinical samples, especially in anticipation of the development of a potential diagnostic assay. For this purpose, the experiments were also performed with β-lactamases isolated from clinically relevant bacterial strains. The results of the β-lactamase inhibition assays are displayed in Fig. 5. Images of the colour changes of the β-lactamase inhibition assays on PDMA shown in Fig. 5 are provided in the ESI† (Fig. S6).

The results of the inhibition assay show that the method can also be successfully performed with clinical samples. Indeed, the majority of the β-lactamase-antibiotic/inhibitor combinations studied are in agreement with the control experiments. Solely for the incubation of *A. baumannii* β-lactamases on aztreonam (Fig. 5A), *E. coli* β-lactamases on tazobactam (Fig. 5B), and *P. aeruginosa* β-lactamases on meropenem, avibactam and sulbactam (Fig. 5D).

For the isolated β-lactamases, compared to the recombinant β-lactamases, deviations were observed between the modified wells and the controls more frequently. This can be attributed to the isolation method, where no purification was relied upon.

Therefore, the deviations can be caused by the sample matrix and potentially included interfering factors such as other molecules. In terms of application in a diagnostic test, an additional purification step should be considered.

The structural and functional diversity of the β-lactamases complicates their detection at a genotypic or phenotypic level. For example, although certain resistance genes can be detected by sequencing, the presence of these alone does not provide a reliable indication of functionality. The same applies to assays that target phenotypic markers, such as lateral flow assays that use specific antibodies. These can only be used to identify the respective β-lactamase subtype for which the antibodies are specific. Considering the multitude of different subtypes and the rapid evolution of these, such tests are not sufficient. Therefore, the method presented here is a promising approach to the development of an all-encompassing diagnostic assay.

We demonstrated that the method can be successfully applied to β-lactamases of all classes, even when isolated from clinically relevant bacterial strains. It can therefore be assumed that the method can also be applied to clinical samples.

Transferability of PDMA–antibiotic/inhibitor – β-lactamase inhibition assay to a microfluidic device

The method for immobilization of antibiotics *via* PDMA was further investigated for its transferability from 96-well plates to microfluidic devices, especially in anticipation of the development of a potential point-of-care diagnostic assay. Therefore, the method and experiments were performed in 3D printed microfluidic devices. The results are shown in Fig. 6. The results are in consistency with the assays performed in the 96-well plates. The structure and design of the microfluidic devices can be varied in terms of channel geometries, chambers, *etc.* due to the flexibility of 3D printing. This allows the devices to be optimized and transferred to other possible applications. The UV-crosslinking method is, as well, almost universally applicable as C,H groups, which are used as reaction partners for immobilization, are available in abundance at particularly all types of organic surfaces such as biomaterials or polymers. However, the method is not limited to these surfaces. In addition, many other systems such as metallic or inorganic surfaces can also be functionalized. For this, C,H groups must be generated on the surface, which can be simply achieved by binding small molecules. Thus, in principle, almost all kind of

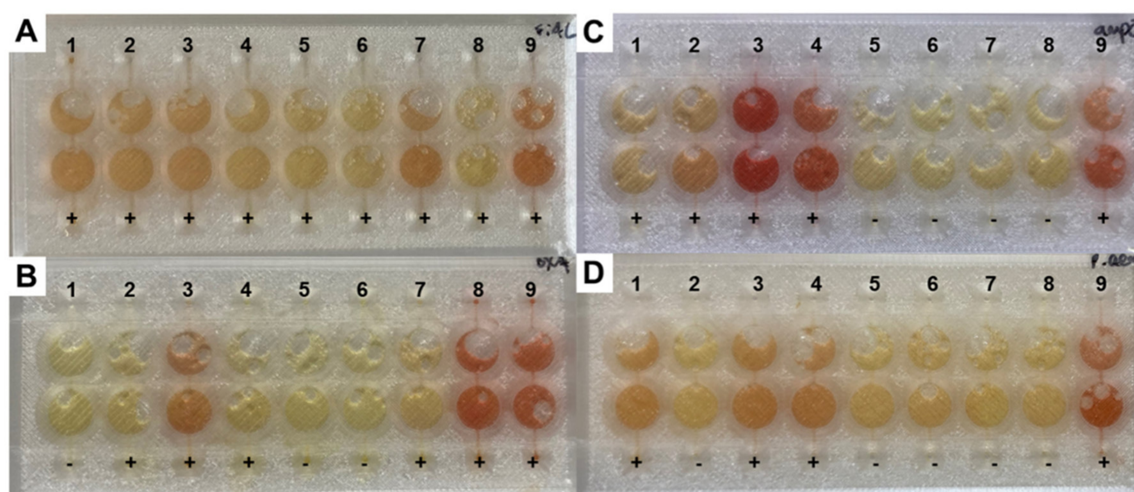

Fig. 6 β-lactamase inhibition assay in 3D printed microfluidic devices functionalized with β-lactam antibiotics and β-lactamase inhibitors. Colour change of nitrocefin substrate turnover of different recombinant β-lactamases (A) "SiAl"–lactamase blend (B) OXA, (C) ampC and (D) *Pseudomonas aeruginosa* after preincubation in the lower reaction chamber of 3D printed microfluidic devices modified with 50 μg of (1) meropenem, (2) aztreonam, (3) ampicillin, (4) cefoperazone, (5) avibactam, (6) tazobactam, (7) sulbactam and (8) relebactam prepared by UV-crosslinking *via* PDMA. No antibiotic was immobilized in chamber (9), serving as positive controls. The chambers in which substrate turnover was detected were marked with "+" and chambers without detectable substrate turnover were marked with "–".

surfaces can be considered in the development of a diagnostic assay based on this method.

The immobilization of antibiotics offers immense advantages regarding the handling and applicability of diagnostic assays. It allows the production of prefabricated devices, saving preparation and handling time and thus enables faster results. By immobilizing a panel of different β-lactam antibiotics and β-lactamase inhibitors onto surfaces, it becomes possible to screen for the presence of a broad spectrum of β-lactamases with a single test. This multiplexing capability significantly improves the efficiency and cost-effectiveness of diagnostic assays, enabling the detection of various β-lactamases in a high-throughput manner.

Furthermore, the immobilization of β-lactam antibiotics on surfaces may enhance the stability and durability of diagnostic assays. The surface-bound antibiotics are less prone to degradation or loss of activity compared to their soluble counterparts. This stability ensures the reliability and longevity of the diagnostic platforms, allowing for repeated and accurate measurements over an extended period. In addition, the method for immobilizing antibiotics offers great potential for a variety of other applications. The method can be extended to other classes of antibiotics and thus to a wider range of bacteria. This can enable further diagnostic tests to be developed or optimized. Other conceivable areas of application include the development of antimicrobial surfaces on implants or wounds dressings. It is also likely, that not only antibiotics, but also drugs in general, can be immobilized on surfaces with this method, contributing among other things to the development of drug delivery systems.

Conclusions

In summary, we have demonstrated the successful application of PDMA networks for the immobilization of β-lactam antibiotics and β-lactamase inhibitors *via* UV-induced C–H insertion reaction. Stable coatings were obtained using benzophenone crosslinker chemistry. The functional coatings were successfully applied in an assay for the determination of susceptibility of recombinant as well as from microbial cell culture isolated β-lactamases to different β-lactam antibiotics and β-lactamase inhibitors.

Overall, this work describes an advantageous approach for the immobilization of β-lactam antibiotics and β-lactamase inhibitors that confers several significant benefits for the creation of specific detection platforms tailored for β-lactamase activity as well as the possibility for the development of high-throughput diagnostic assays and benefits regarding stability and durability of diagnostic assays. Moreover, the method demonstrates potential for multiple other applications ranging from biomedicine and diagnostics to antimicrobial surface functionalization (surgical implants, wound dressings,…) as well as environmental applications (filtration,…).

Author contributions

Conceptualization: H.-P. Deigner; data curation: S. Rentschler; funding acquisition: H.-P. Deigner; investigation: S. Rentschler, M. Borgolte, A. Filbert; methodology: S. Rentschler, M. Borgolte; resources: H.-P. Deigner, S. Laufer, supervision: H.-P. Deigner, S. Laufer, visualization: S.

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Rentschler; writing – original draft: S. Rentschler; writing – review & editing: H.-P. Deigner, S. Laufer.

Conflicts of interest

There are no conflicts to declare.

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6 Concluding remarks

The results of this research highlight the potential of PDMA surface functionalization for optimizing and broadening the applicability and scope of enzymatic applications. The implementation of PDMA surface functionalization into enzymatic reaction systems significantly enhances enzyme performance, resulting in increased substrate conversions, initial velocities, and maximum velocities. The results revealed that substrate conversions were up to three times higher with PDMA incorporation compared to those without. Consequently, this study presents a valuable approach to enhance enzymatic applications to achieve more sustainability and efficiency in bioprocessing by decreasing resource consumption, minimizing waste production, and reducing overall costs.

This beneficial effect was confirmed across various surfaces and enzymatic assays. It is reasonable to assume that similar advantageous outcomes can be attained with other polymers, enzymes, assays, and in various industrial applications. In principle, any surface containing C,H-groups can be modified with this straightforward one-step surface functionalization technique.

The enhanced enzyme performance not only holds implications for industrial settings but also extends to diagnostic applications. The implementation of PDMA in diagnostic assays, with the associated increased substrate conversion and enhanced enzyme kinetics, can provide faster and more sensitive results. The higher substrate conversion generates higher signals that can be detected visually or by instrumentation, allowing detection thresholds to be reached even at low enzyme concentrations. This in turn leads to lower detection limits, enabling for better therapy decisions and patient outcomes.

Another achievement of this work is that the modified surfaces were given additional functionality by integrating β-lactam antibiotics and β-lactamase inhibitors into the PDMA functionalization. The antibiotics and inhibitors were immobilized on support surfaces and these functionalized surfaces were successfully utilized in a chromogenic β-lactamase assay. These assays not only determined the presence of recombinant β-lactamases and β-lactamases isolated from microbial cell culture, but also provided profiles of antibiotic resistance and susceptibility to various β-lactam antibiotics and β-lactamase inhibitors.

By immobilizing diverse β-lactam antibiotics and β-lactamase inhibitors onto surfaces, it becomes feasible to detect a wide range of β-lactamases using a single test. This capability for multiplexing enhances the efficiency and cost-effectiveness of β-lactamase assays and provides the possibility for high-throughput screenings. Moreover, by enabling the production of prefabricated devices, the method of immobilizing antibiotics reduces preparation and handling times and accelerates result acquisition.

Furthermore, another notable accomplishment in this study was the transfer of the antibiotic immobilization method to microfluidic devices, particularly addressing the need for the development of point-of-care tests. POC tests are rapid, cost effective, and user-friendly diagnostic assays that can be easily performed at patient's bedside, even by untrained individuals. A POC test for functional detection of antibiotic resistance and β-lactamases is of pressing need to determine effective antibiotics for adequate treatments. Microfluidic devices are excellent platforms for the development of such a POC test due to their ease of handling. Additionally, microfluidic devices might increase assay performances by requiring less sample volume and enabling the utilization of detection systems with heightened sensitivities. The use of 3D printing of microfluidic devices, as performed in this study, provides significant advantages. It allows for easy and flexible customization of device structures and designs, such as channel geometries and chambers. In this way, the device can be optimized and transferred to other potential areas of application.

The method holds promise for extension to all antibiotics and drugs containing C,H-groups or those in which they can be inserted via linker. This extension could facilitate the development or optimization of diagnostic assays for a wider range of bacteria, as well as the creation of antimicrobial surfaces on implants and wound dressings. Furthermore, the immobilization of drugs can among other things contribute to the progression of drug delivery systems.

In conclusion, this research demonstrates the significant potential of polydimethylacrylamide surface functionalization in enhancing enzymatic applications, offering a valuable strategy to improve sustainability and efficiency in bioprocessing by increasing substrate conversion and minimizing resource consumption. The study confirms the versatility of this approach across various surfaces and assays, suggesting broader applicability in industrial and diagnostic settings. Moreover, the

investigation of PDMA functionalization with β-lactam antibiotics and microfluidic devices holds promise for advancing point-of-care testing and drug delivery systems, with potential implications for a wide array of bacterial diagnostics and antimicrobial applications.

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Appendix

Supplementary Information Publication Project I

S. Rentschler, M. Borgolte, R. Csuk, S. Laufer, H.-P. Deigner, "Toward more Sustainable Enzyme Reactions: Enhancing Kinetics by Polydimethylacrylamide Implementation," *Green Chemistry* **2024**, *26*, 1653-1659

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Supplementary Information

Toward more Sustainable Enzyme Reactions: Enhancing Kinetics by Polydimethylacrylamide Implementation

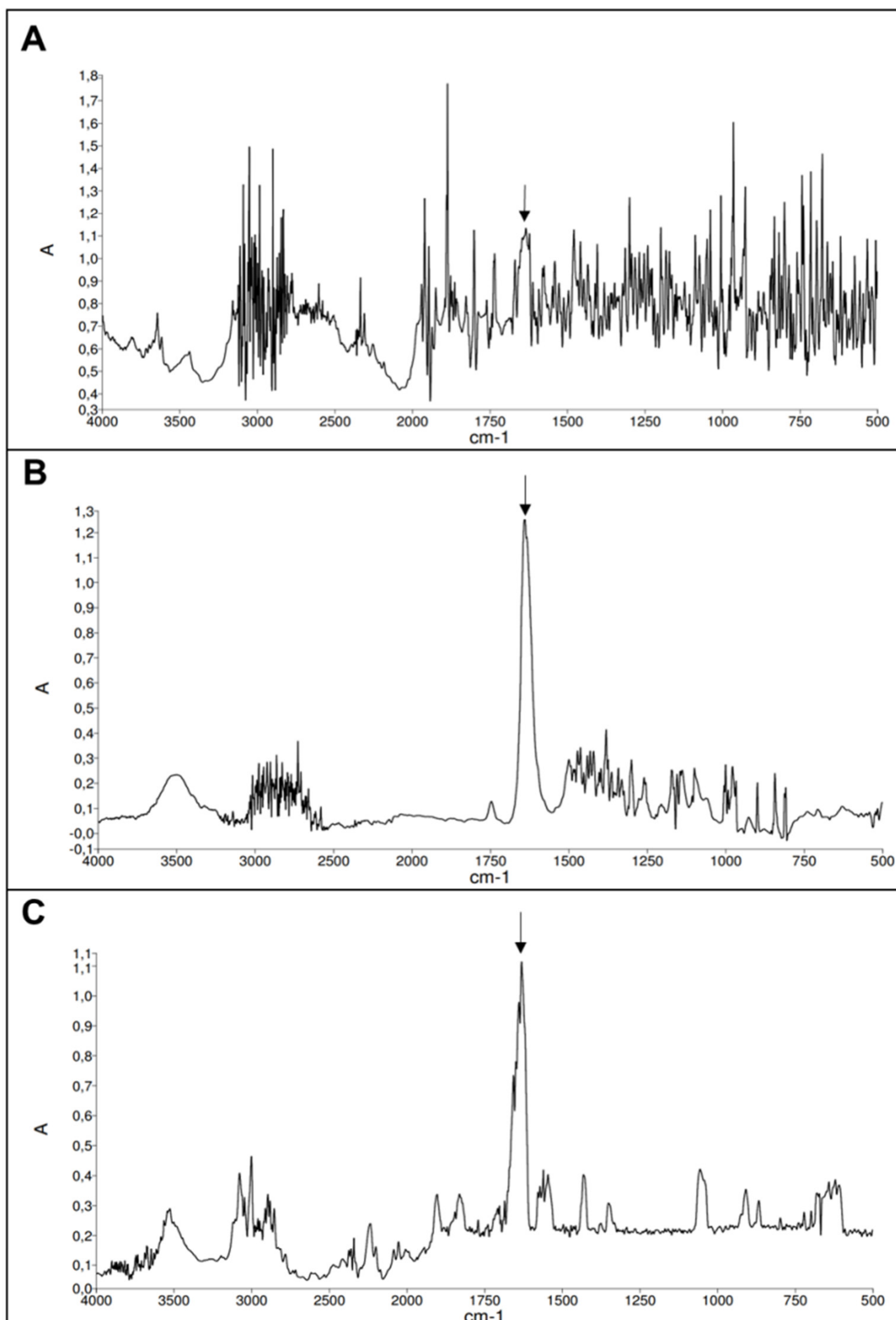

Figure S1 Recorded Fourier transform-infrared spectra of PDMA-functionalized microwell plates. *Spectra of the PDMA coatings on (A) polystyrene. (B) polypropylene and (C) polycarbonate were recorded in the range of 4000 – 400 cm^{-1}. The expected PDMA-amides can be seen clearly at 1650 cm^{-1}(relevant peak marked with arrows). In addition. the dominant peaks between 3000 – 3500 cm^{-1} can be assigned to the PDMA. Thus. the recorded Fourier transform-infrared spectra proved the presence of PDMA on the microtiter plates.*

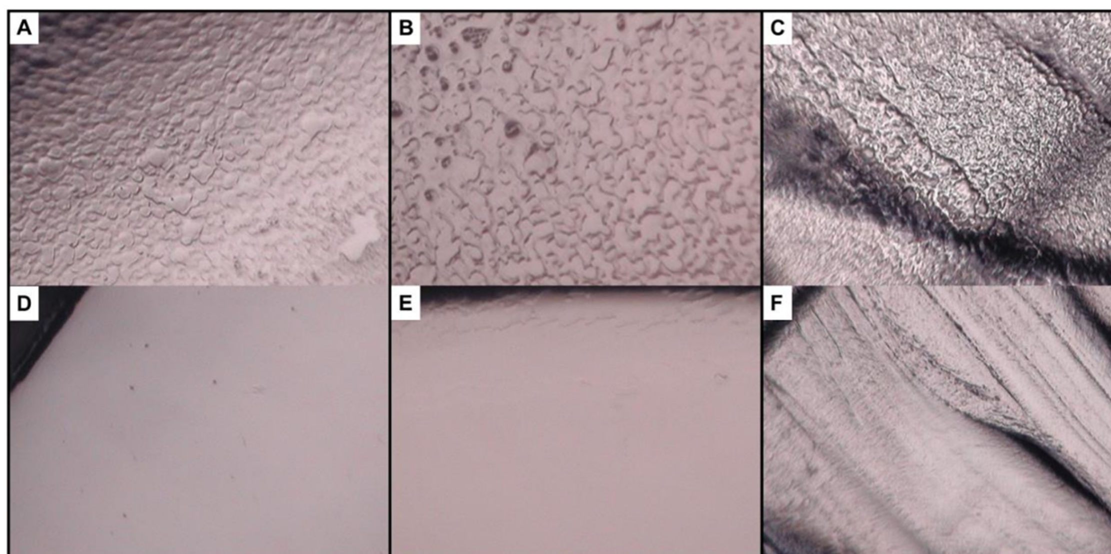

Figure S2 FTIR-Microscope images of PDMA functionalized microwell plates. *Images of (A) polystyrene (B) polypropylene and (C) polycarbonate functionalized with PDMA as well as non-functionalized (D) polystyrene. (E) polypropylene and (F) polycarbonate were caputred with a Spotlight 400 FT-IR microscope (PerkinElmer. Waltham. USA). Plane surfaces can be seen for unfunctionalized PS (D) and PP (E) and a slightly structured surface for unfunctionalized PC (F) originating from the 3D printing. The functionalization of the different polymers with PDMA results in smooth, wavy textured surfaces.*

Table S1 Nitrocefin substrate turnover (µM) of TEM-1 β-lactamase *with varied concentrations of nitrocefin substrate after 60 minutes incubation. Experiments were performed in polystyrene wells functionalized with PDMA and non-functionalized (control). Substrate turnover was measured with a TECAN Infinite 200 Pro at 492 nm. The amounts of substrates converted by the enzyme were determined from the measured absorbance values and the corresponding extinction coefficient of 17,400 M^{-1} cm^{-1} Each data point represents the mean ± SD of three different experiments.*

Nitrocefin [µM]					
	PDMA		control		
	Mean	SD	Mean	SD	p-value
750	349.29	6.02	195.56	15.59	0.0061
500	316.88	7.33	176.38	3.44	0.0019
250	224.27	9.48	102.38	3.88	0.0021
125	104.23	1.95	52.59	3.09	0.0002
62.5	50.04	1.42	33.49	1.11	0.0022
31.25	25.27	0.40	18.20	0.52	0.0022
0	0.00	0.00	0.00	0.00	

Table S2 TMB substrate turnover (µM) of HRP *with varied concentrations of TMB substrate after 60 minutes incubation. Experiments were performed in polystyrene wells functionalized with PDMA and non-functionalized (control). Substrate turnover was measured with a TECAN Infinite 200 Pro at 650 nm. The amounts of substrates converted by the enzyme were determined from the measured absorbance values and the corresponding extinction coefficient of 39,000 M^{-1} cm^{-1} Each data point represents the mean ± SD of three different experiments.*

TMB [µM]					
	PDMA		control		
	Mean	SD	Mean	SD	p-value
117.9	27.09	0.37	19.73	0.85	0.0067
99.46	26.99	0.13	20.95	2.33	0.0456
88.69	21.64	1.22	21.74	0.26	0.8906
81.58	21.71	1.27	21.16	0.59	0.5967
74.48	19.13	0.14	19.56	0.57	0.3848
67.375	18.94	0.70	19.77	0.58	0.1445

Table S3 PNPP substrate turnover (µM) of ALP *with varied concentrations of pNPP substrate after 60 minutes incubation. Experiments were performed in polystyrene wells functionalized with PDMA and non-functionalized (control). Substrate turnover was measured with a TECAN Infinite 200 Pro at 405 nm. The amounts of substrates converted by the enzyme were determined from the measured absorbance values and the corresponding extinction coefficient of 18,000 M^{-1} cm^{-1} Each data point represents the mean ± SD of three different experiments.*

pNPP [µM]	PDMA		control		
	Mean	SD	Mean	SD	p-value
1,000	140.82	18.68	46.25	9.40	0.0279
500	67.55	10.13	22.33	1.62	0.0205
250	37.28	2.67	17.39	0.80	0.0100
125	22.81	0.99	12.77	0.47	0.0069
100	20.29	1.00	11.85	0.20	0.0030
62.5	17.31	1.30	12.54	0.87	0.0391
0	0.00	0.00	0.00	0.00	

Table S4 Absorbance values of nitrocefin substrate turnover by TEM-1 β-lactamase *in polystyrene microtiter plate wells functionalized with different amounts of PDMA. Substrate turnover was measured with a TECAN Infinite 200 Pro at 492 nm. Each data point represents the mean ± SD of three different experiments.*

PDMA [µg]	Mean	SD
400	0.840	0.018
350	0.828	0.007
300	0.761	0.037
250	0.767	0.016
200	0.741	0.016
150	0.666	0.021
100	0.609	0.079
50	0.444	0.006
0	0.379	0.007

Table S5 Absorbance values of TMB substrate turnover by HRP *in polystyrene microtiter plate wells functionalized with different amounts of PDMA. Substrate turnover was measured with a TECAN Infinite 200 Pro at 650 nm. Each data point represents the mean ± SD of three different experiments.*

PDMA [µg]	Mean	SD
800	1.416	0.029
700	1.376	0.020
600	1.354	0.035
500	1.339	0.013
400	1.303	0.024
300	1.316	0.024
200	1.267	0.006
100	1.226	0.029
0	1.069	0.004

***Table S6 Absorbance values of pNPP substrate turnover by ALP** in polystyrene microtiter plate wells functionalized with different amounts of PDMA. Substrate turnover was measured with a TECAN Infinite 200 Pro at 405 nm. Each data point represents the mean ± SD of three different experiments.*

PDMA [µg]	Mean	SD
400	0.639	0.042
350	0.619	0.063
300	0.530	0.072
250	0.505	0.075
200	0.450	0.031
150	0.344	0.053
100	0.256	0.043
50	0.183	0.053
25	0.181	0.014
12.5	0.193	0.023
0	0.158	0.013

***Table S7 Initial reaction rates of nitrocefin substrate turnover by TEM-1 β-lactamase** in polystyrene microtiter plate wells. Substrate turnover was monitored as absorbance increase with a TECAN Infinite 200 Pro at 492 nm in 10 second intervals. Initial reaction rates were calculated from the linear range of substrate turnover at different substrate concentrations. Each data point represents the mean ± SD of three different experiments.*

Nitrocefin [µM]	V_0				
	PDMA on PS		control		
	Mean	SD	Mean	SD	p-value
600	3.308	0.074	1.753	0.220	0.0108
400	3.418	0.037	1.733	0.037	0.0006
200	3.101	0.016	1.319	0.032	0.0002
100	2.240	0.032	0.953	0.072	0.0006
50	1.402	0.072	0.756	0.047	0.0030
25	0.911	0.018	0.415	0.007	0.0005
0	0.000	0.000	0.000	0.000	

***Table S8 Initial reaction rates of TMB substrate turnover by HRP** in polystyrene microtiter plate wells. Substrate turnover was monitored as absorbance increase with a TECAN Infinite 200 Pro at 650 nm in 15 second intervals. Initial reaction rates were calculated from the linear range of substrate turnover at different substrate concentrations. Each data point represents the mean ± SD of three different experiments.*

TMB [µM]	V_0				
	PDMA on PS		control		
	Mean	SD	Mean	SD	p-value
117.9	0.952	0.005	0.670	0.038	0.0059
99.46	0.861	0.007	0.674	0.057	0.0270
88.69	0.697	0.039	0.686	0.004	0.6796
81.58	0.668	0.029	0.643	0.015	0.3232
74.48	0.572	0.016	0.574	0.014	0.9104
67.375	0.553	0.025	0.561	0.017	0.7522

Table S9 Initial reaction rates of pNPP substrate turnover by ALP *in polystyrene microtiter plate wells. Substrate turnover was monitored as absorbance increase with a TECAN Infinite 200 Pro at 405 nm in 10 second intervals. Initial reaction rates were calculated from the linear range of substrate turnover at different substrate concentrations. Each data point represents the mean ± SD of three different experiments.*

pNPP [µM]	V_0				
	PDMA on PS		control		
	Mean	SD	Mean	SD	p-value
500	0.229	0.021	0.068	0.005	0.0085
250	0.140	0.007	0.051	0.003	0.0040
125	0.081	0.002	0.018	0.005	0.0041
100	0.063	0.007	0.012	0.002	0.0104
62.5	0.044	0.006	0.016	0.002	0.0105
0	0.000	0.000	0.000	0.000	

Table S10 Nitrocefin substrate turnover (µM) of TEM-1 β-lactamase *with varied concentrations of nitrocefin substrate after 60 minutes incubation. Experiments were performed in polypropylene wells functionalized with PDMA and non-functionalized (control). Substrate turnover was measured with a TECAN Infinite 200 Pro at 492 nm. The amounts of substrates converted by the enzyme were determined from the measured absorbance values and the corresponding extinction coefficient of 17,400 M^{-1} cm^{-1} Each data point represents the mean ± SD of three different experiments.*

	Nitrocefin [µM]				
	PDMA on PP		control		
	Mean	SD	Mean	SD	p-value
750	385.33	56.25	193.43	13.21	0.0206
625	411.77	36.80	163.33	29.36	0.0151
500	330.98	30.66	132.16	6.21	0.0113
375	281.39	12.14	125.33	10.24	0.0040
250	195.57	13.39	90.00	0.30	0.0051
125	115.95	9.85	65.10	8.52	0.0369
62.5	60.55	6.16	48.93	4.04	
0	0.000	0.000	0.000	0.000	

Table S11 Nitrocefin substrate turnover (µM) of TEM-1 β-lactamase *with varied concentrations of nitrocefin substrate after 60 minutes incubation. Experiments were performed in polypropylene wells functionalized with PDMA and non-functionalized (control). Substrate turnover was measured with a TECAN Infinite 200 Pro at 492 nm. The amounts of substrates converted by the enzyme were determined from the measured absorbance values and the corresponding extinction coefficient of 17,400 M^{-1} cm^{-1} Each data point represents the mean ± SD of three different experiments.*

	Nitrocefin [µM]				
	PDMA on PC		control		
	Mean	SD	Mean	SD	p-value
750	277.35	34.79	80.59	18.12	0.0139
625	238.95	4.23	81.76	8.65	0.0021
500	207.05	9.21	49.72	6.47	0.0032
375	168.37	5.73	49.47	5.56	0.0010
250	129.38	10.97	42.86	4.89	0.0032
125	78.82	5.32	18.60	6.86	0.0020
62.5	36.55	1.21	13.01	5.19	0.0182
0	0.000	0.000	0.000	0.000	

***Table S12 Initial reaction rates of nitrocefin substrate turnover by TEM-1 β-lactamase** in polypropylene microtiter plate wells. Substrate turnover was monitored as absorbance increase with a TECAN Infinite 200 Pro at 492 nm in 10 second intervals. Initial reaction rates were calculated from the linear range of substrate turnover at different substrate concentrations. Each data point represents the mean ± SD of three different experiments.*

Nitrocefin [µM]	V_0				
	PDMA on PP		control		
	Mean	SD	Mean	SD	p-value
750	3.112	0.440	1.786	0.095	0.0232
625	3.118	0.156	1.576	0.227	0.0055
500	2.584	0.092	1.330	0.041	0.0037
375	2.348	0.020	1.264	0.142	0.0045
250	1.815	0.098	0.973	0.017	0.0046
125	1.522	0.077	0.824	0.084	0.0168
62.5	1.101	0.037	0.682	0.094	0.0307
0	0.000	0.000	0.000	0.000	

***Table S13 Initial reaction rates of nitrocefin substrate turnover by TEM-1 β-lactamase** in polycarbonate microtiter plate wells. Substrate turnover was monitored as absorbance increase with a TECAN Infinite 200 Pro at 492 nm in 10 second intervals. Initial reaction rates were calculated from the linear range of substrate turnover at different substrate concentrations. Each data point represents the mean ± SD of three different experiments.*

Nitrocefin [µM]	V_0				
	PDMA on PC		control		
	Mean	SD	Mean	SD	p-value
750	2.179	0.206	0.847	0.197	0.0287
625	2.076	0.037	1.085	0.107	0.0017
500	1.967	0.043	0.859	0.092	0.0047
375	1.477	0.096	0.694	0.024	0.0071
250	1.288	0.061	0.659	0.019	0.0017
125	1.005	0.026	0.445	0.043	0.0046
62.5	0.614	0.047	0.262	0.010	0.0027
0	0.000	0.000	0.000	0.000	

Supplementary Information Publication Project II

S. Rentschler, M. Borgolte, A. Filbert, S. Laufer, H.-P. Deigner, “Highly efficient β-lactamase assay applying Poly-Dimethylacrylamide- based surface functionalization with β-lactam antibiotics and β-lactamase inhibitors,” *Lab on a Chip* **2023**, *23*, 5120-5130

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Supplementary Information

Highly efficient β-lactamase assay applying Poly-Dimethylacrylamide-based surface functionalization with β-lactam antibiotics and β-lactamase inhibitors

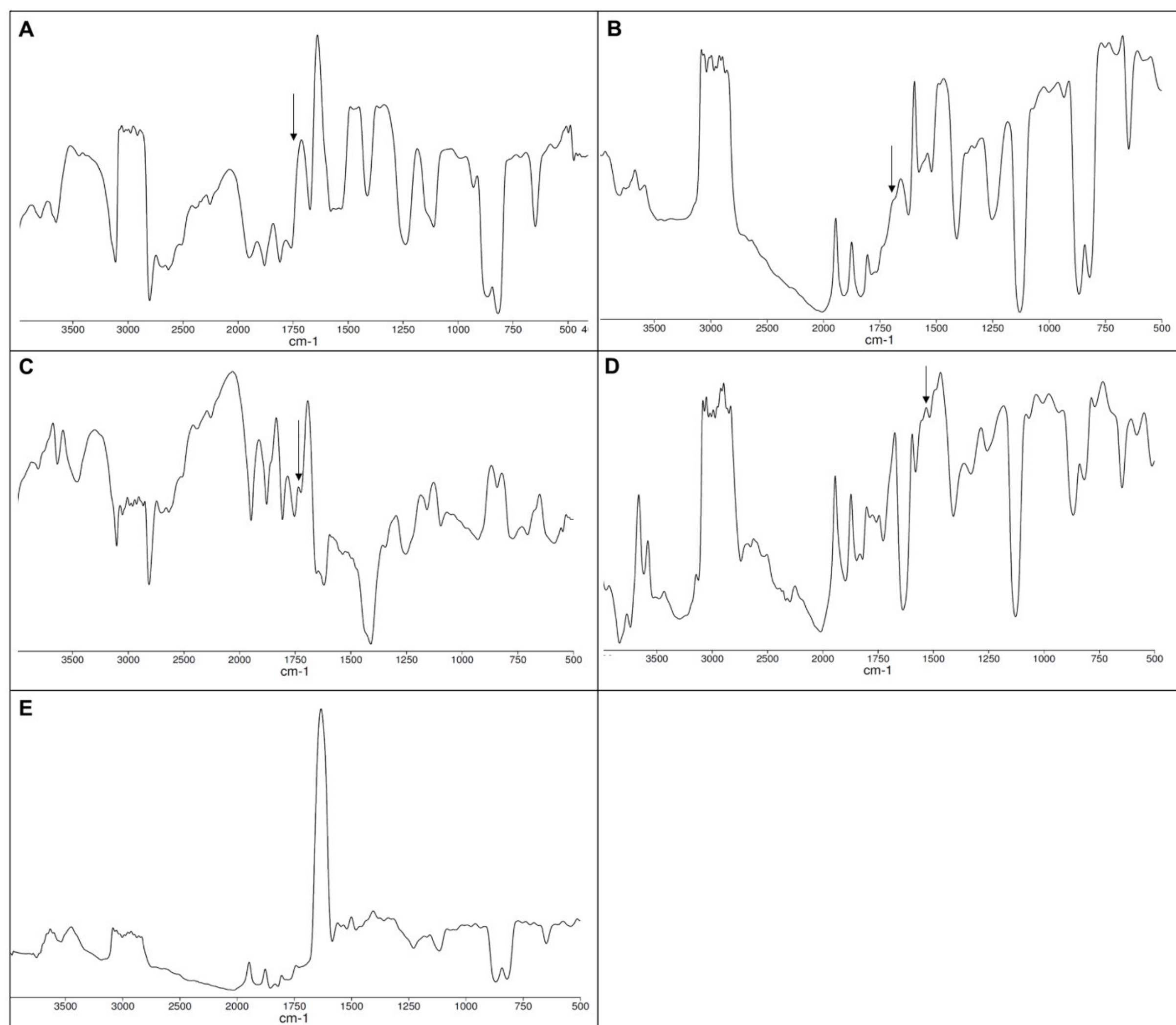

Fig. S1 Recorded Fourier transform-infrared spectra of β-lactam antibiotics and β-lactamase inhibitors crosslinked with PDMA-MBP on polypropylene plates. (A) ampicillin, (B) cefoperazone, (C) sulbactam and (D) relebactam. Spectra were recorded in the range of 4000 – 400 cm $^{-1}$. Relevant peaks are marked with arrows.

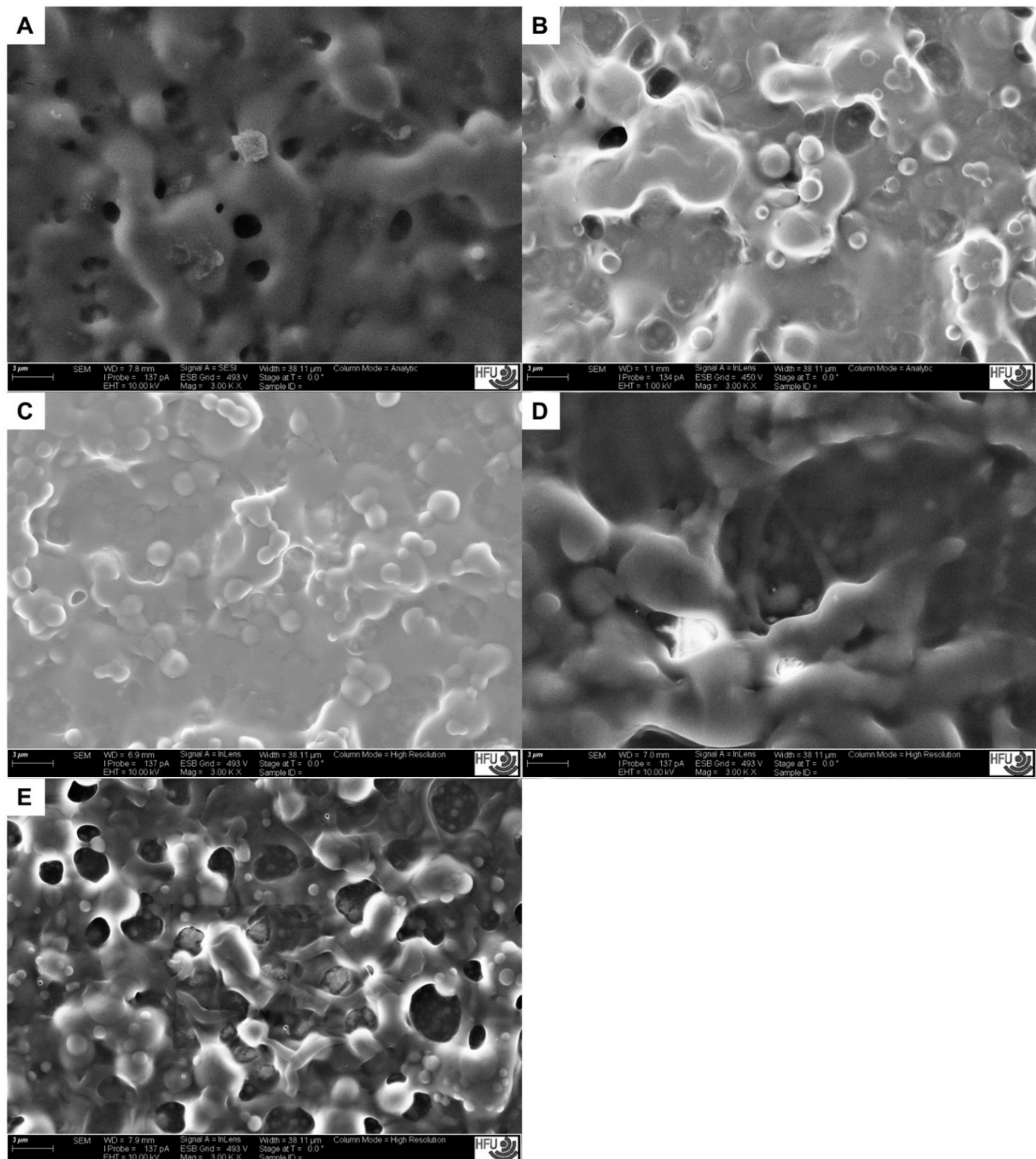

Fig. S2 SEM images of the PDMA coatings crosslinked with different β-lactam antibiotics and β-lactamase inhibitors. (A) PDMA, (B) PDMA with meropenem, (C) PDMA with aztreonam, (D) PDMA with ampicillin, (E) PDMA with cefoperazone.

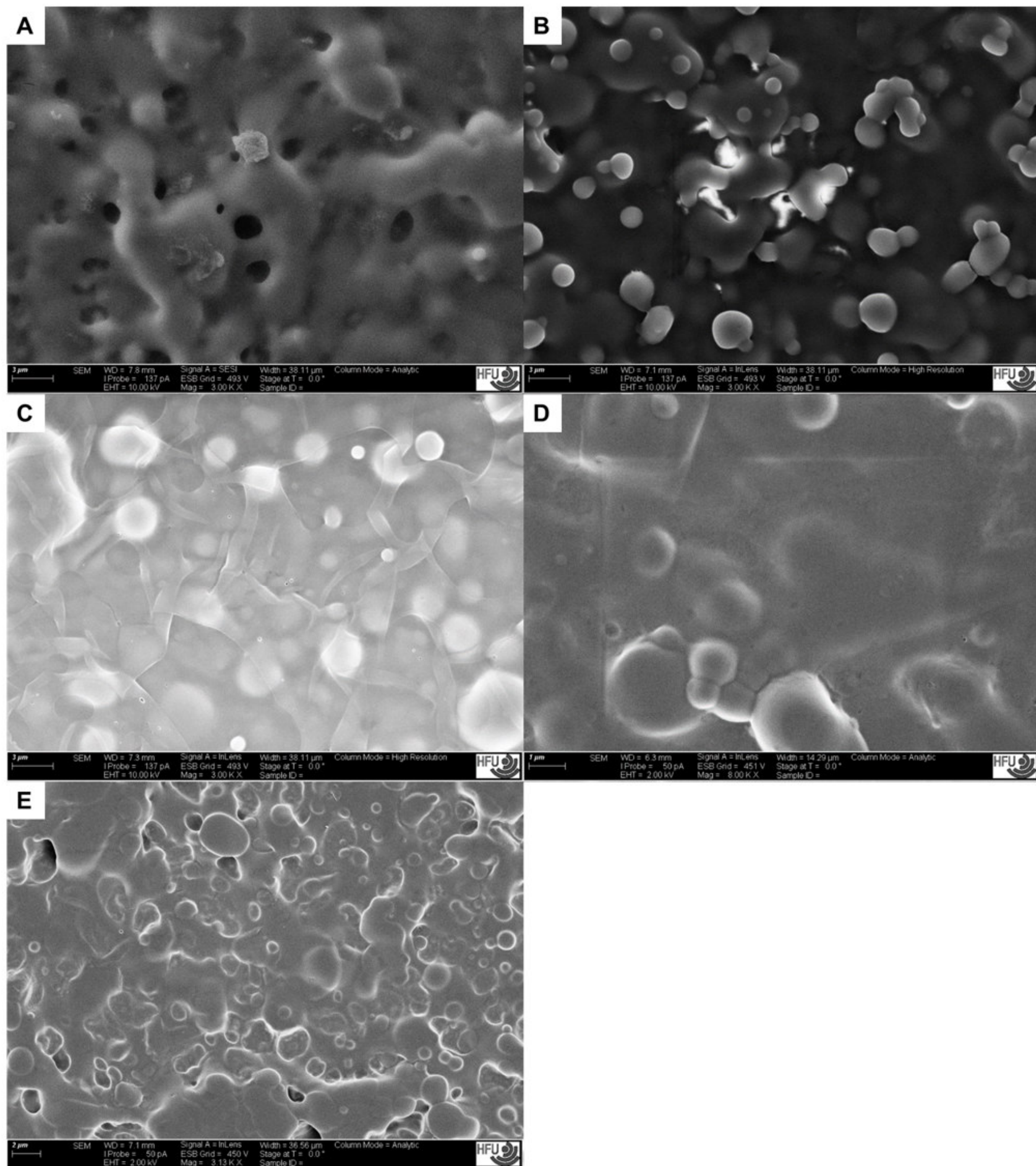

Fig. S3 SEM images of the PDMA coatings crosslinked with different β-lactamase inhibitors. (A) PDMA, (B) PDMA with avibactam, (C) PDMA with tazobactam, (D) PDMA with sulbactam and (E) PDMA with relebactam.

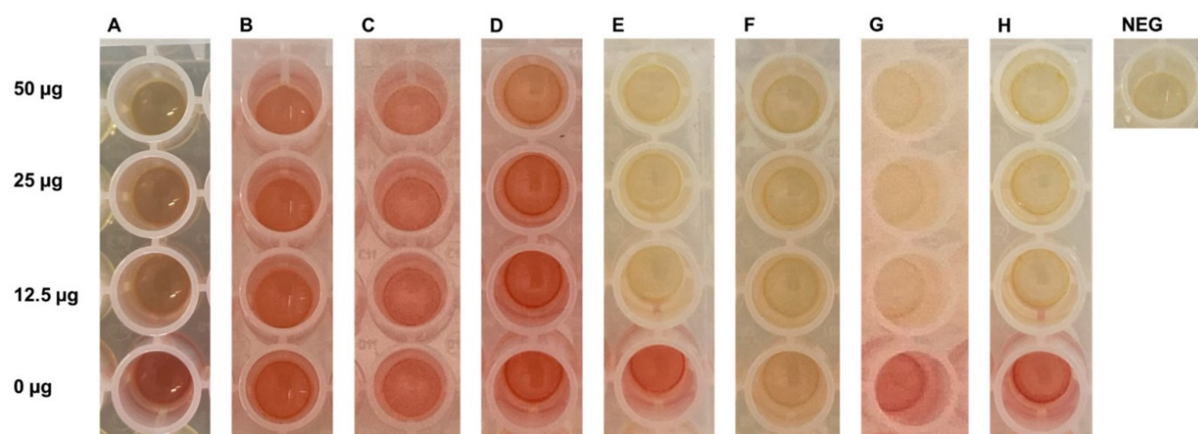

Fig. S4 Images of the β-lactamase inhibition assays for the determination of the effect of different amounts of immobilized β-lactam-antibiotics and β-lactamase inhibitors. Colour change of nitrocefin substrate turnover of different recombinant β-lactamases after preincubation in microplate wells modified with 50, 25 and 12.5 µg of various β-lactam antibiotics and β-lactamase inhibitors prepared by UV-crosslinking via PDMA. (A) ampC incubated on meropenem, (B) OXA-23 on aztreonam, (C) ampC on ampicillin, (D) OXA on cefoperazone, (E) ampC on avibactam, (F) "SiAl" lactamase blend on tazobactam, (G) ampC incubated on sulbactam and (H) ampC on relebactam.

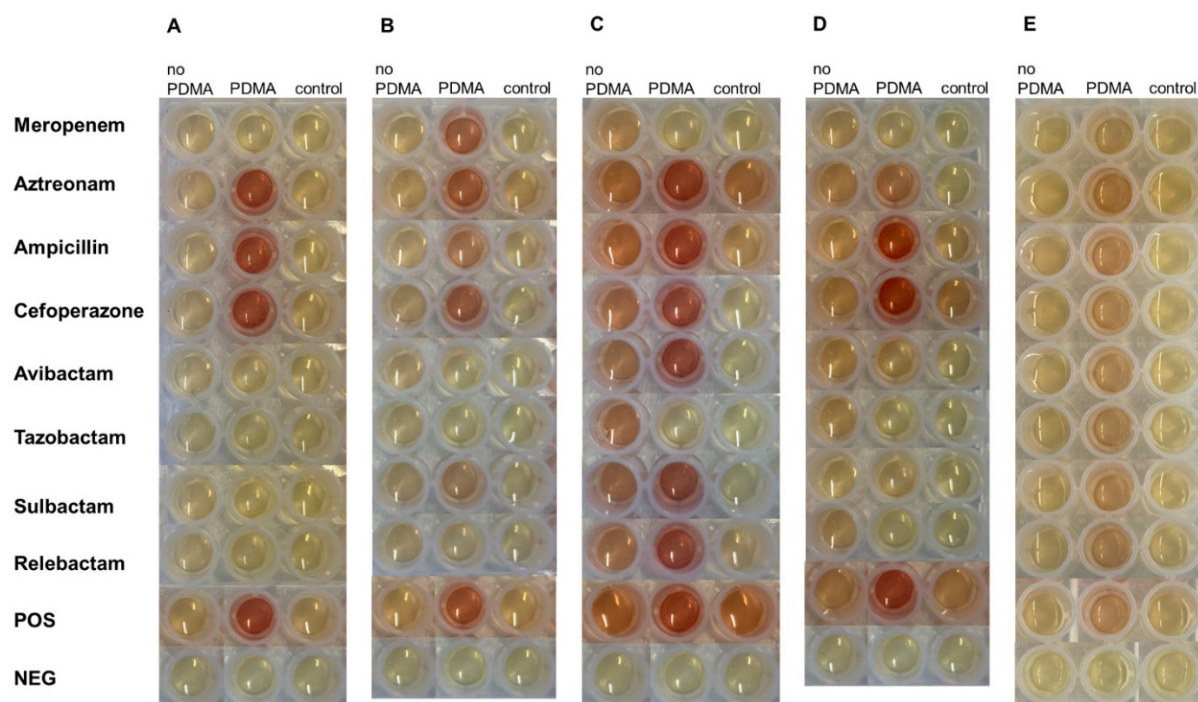

Fig. S5: Images of β-lactamase (recombinant) inhibition assay in microplate wells functionalized with β-lactam-antibiotics and β-lactamase inhibitors. Colour change of nitrocefin substrate turnover of different recombinant β-lactamases (A) TEM-1, (B) "SiAl"-lactamase blend, (C) OXA-23, (D) ampC and (E) IMP-4 after preincubation in microplate wells modified with 50 µg of various β-lactam antibiotics and β-lactamase inhibitors prepared by UV-crosslinking via PDMA ("PDMA"). To verify the functionality of the wells modified with PDMA and β-lactam-antibiotics/β-lactamase inhibitors, inhibition assays in solution were performed as reference experiments ("control"). Therefore, β-lactamases were added to each well of an untreated 96-well plate and pre-incubated with β-lactam antibiotic/β-lactamase inhibitor in solution, followed by the addition of nitrocefin. To exclude nonspecific binding of the β-lactam antibiotics/β-lactamase inhibitors to the wells, wells without PDMA were prepared as controls ("no PDMA"). Therefore, only the β-lactam antibiotics/β-lactamase inhibitors were added and crosslinked with UV in the wells. High activity of lactamases was detected in each of these "no PDMA"-control wells. Therefore, nonspecific binding of the antibiotics/inhibitors to the wells can be excluded.

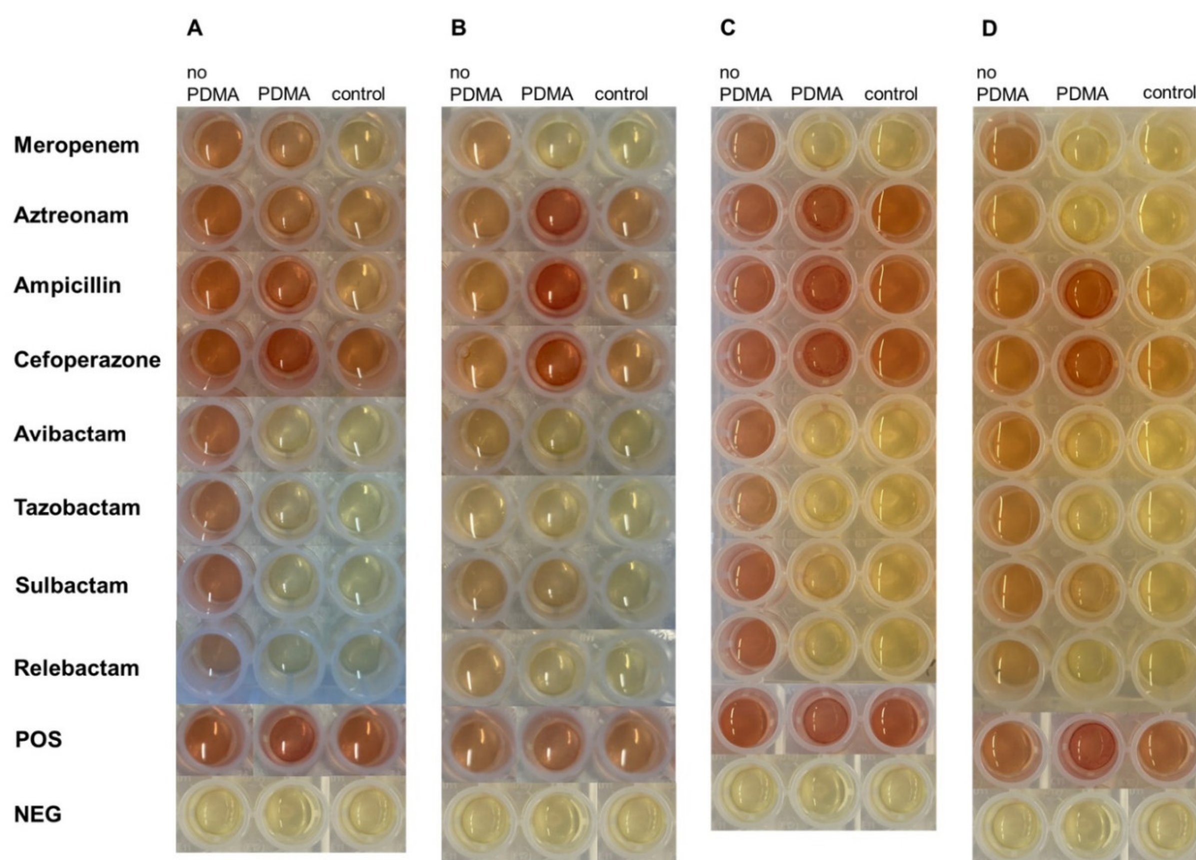

Fig. S6 Images of β-lactamase (recombinant) inhibition assay in microplate wells functionalized with β-lactam-antibiotics and β-lactamase inhibitors. Colour change of nitrocefin substrate turnover of different recombinant β-lactamases (A) *Acinetobacter baumannii*, (B) *Escherichia coli*, (C) *Klebsiella pneumoniae* and (D) *Pseudomonas aeruginosa* after preincubation in microplate wells modified with 50 µg of various β-lactam antibiotics and β-lactamase inhibitors prepared by UV-crosslinking via PDMA ("PDMA"). To verify the functionality of the wells modified with PDMA and β-lactam-antibiotics/β-lactamase inhibitors, inhibition assays in solution were performed as reference experiments ("control"). Therefore, β-lactamases were added to each well of an untreated 96-well plate and pre-incubated with β-lactam antibiotic/β-lactamase inhibitor in solution, followed by the addition of nitrocefin. To exclude nonspecific binding of the β-lactam antibiotics/β-lactamase inhibitors to the wells, wells without PDMA were prepared as controls ("no PDMA"). Therefore, only the β-lactam antibiotics/β-lactamase inhibitors were added and crosslinked with UV in the wells. High activity of lactamases was detected in each of these "no PDMA"-control wells. Therefore, nonspecific binding of the antibiotics/inhibitors to the wells can be excluded.

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