An Acoustic Trap for Bead Injection Attenuated Total Reflection Infrared Spectroscopy

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ABSTRACT: In this work, we introduce a system combining an acoustic trap for bead injection with attenuated total reflection (ATR) infrared (IR) spectroscopy. By mounting an acoustofluidic cell hosting an ultrasound source on top of a custom-built ATR fixture, we were able to trap beads labeled with the enzyme alkaline phosphatase without requiring any mechanical retention elements. Sequential injection analysis was employed for reproducible sample handling and bead injection into the acoustic trap. To showcase potential applications of the presented setup for kinetic studies, we monitored the conversion of *p*-nitrophenylphosphate into *p*-nitrophenol and phosphate via beads carrying the immobilized enzyme using ATR-IR spectroscopy. Retaining the labeled beads via ultrasound particle manipulation resulted in excellent experimental reproducibility (relative standard deviation, 3.91%). It was demonstrated that trapped beads remained stably restrained with up to eight changes of liquid passing through the acoustofluidic cell. Beads could be discarded in a straightforward manner by switching off the ultrasound, in contrast to systems containing mechanical retention elements, which require backflushing. Multiple experiments were performed by employing different substrate concentrations with the same batch of trapped beads as well as varying the amount of enzyme present in the cell, enabling enzyme kinetic studies and emphasizing the application of the proposed setup in studies where enzymatic reuse is desired. This proves the potential of the acoustic trap combined with ATR-IR spectroscopy to monitor the activity of immobilized enzymes and its ability to perform complex bead-based assays.

Fourier transform infrared spectroscopy (FT-IR) is a powerful analytical technique that provides molecule-specific qualitative and quantitative information in a non-destructive and label-free manner by probing molecular vibrations.[1,2] This technique is applied in a large variety of fields ranging from biology to materials chemistry.[3] For measurements in water, which is a strong absorber in the mid-IR region, thus allowing interaction lengths of just a few tens of micrometers, attenuated total reflection (ATR) configurations are the most commonly employed. Using this robust technique, the light is totally reflected within the optically denser ATR element, forming an evanescent field that interacts with the sample at penetration depths of typically up to 2 µm.[4]

Ultrasound (US) particle manipulation using a standing ultrasound wave has evolved as a promising method in recent years for separating suspended particles from their host liquid,[5] and it has shown potential in various analytical tasks, such as cell isolation, process monitoring and particle characterization. [6-10] In this technique, a standing US wave is generated via a piezo-element-driven transducer as the US source, operated at frequencies of approximately 2 MHz. When the initial US wave is reversed by a reflector, superposition occurs between the reflected and the incident wave, resulting in a standing US wave. Acoustic radiation forces allow particles within this standing wave to be manipulated.[11] The typical size of the particles handled in such experiments ranges from 1 to 20 µm.[12] In combination with IR spectroscopy, analysis of conglomerates formed by ultrasonic exposure of different particle suspensions (e.g., yeast,[13,14] *Escherichia coli*[15]) has led to the development of US-enhanced ATR-IR spectroscopy. In US-ATR-IR spectroscopy, the ATR element itself is the reflector as well as the sensitive surface for probing the suspension. Particles present in the cavity between the US source and the ATR element may be caught by switching the ultrasound off or on, and they may be steered to specific locations by tuning the ultrasound frequency. Active movement of biological particles of interest into or out of the evanescent field at the ATR element has already been successfully reported.[13,16]

Most common systems for liquid handling include flow injection analysis (FIA) and sequential injection analysis (SIA) to improve accuracy, reproducibility, speed, and robustness of analytical procedures.[17] In combination with those techniques, FT-IR spectroscopy has been employed for the determination of enzyme activity[18-21] and the examination of foodstuffs (analysis of tomatoes,[22] sugars[23] or red wines[24]). Flow analysis systems combined with bead injection (BI), where precise volumes of bead suspension are handled via SIA, enabling both liquid−solid interactions such as solid-phase extraction and reactions with, or catalyzed by, active groups present on the surface of the beads. Furthermore, the application of a

mobile active surface circumvents problems associated with aging or other alterations of the surface by discarding the beads after each analysis.[25] BI has been successfully employed in combination with a vast variety of detection techniques, including spectroscopic methods (atomic absorption spectrometry, ultraviolet, visible, fluorescent chemiluminescence, and Raman spectroscopy), voltammetric detection, chromatography, radiometry, and mass spectrometry.[26]

The jet ring and rotating-rod flow cell approaches are based on inserting a solid rod into the flow system to hold the beads in the desired space while allowing liquid to pass through it. In the so-called lab-on-a-valve manifold approach, optical fibers are used to retain aspirated beads. Magnetic particle manipulation and custom-built setups using, for instance, glass wool for bead retention have also been reported.[27] Finally, US particle manipulation might be employed to guide particles in a continuous flow system, i.e., acoustophoresis,[8,12] or retain particles via an acoustic trap.[28,29] In the latter technique, trapping systems are designed to hold objects against the liquid flow, typically utilizing the lateral component of the ultrasound radiation force, to counteract the Stokes drag on the manipulated particles.[30] In the field of spectroscopy, only one application, which uses a SIA system to retain beads via US particle manipulation and subsequently probes the surface of retained beads via Raman microscopy, has been reported.[31] So far, the principle of US particle manipulation is predominantly used in microfluidic devices and microscopic studies, for instance, for cell sorting and the enhancement of bead-based bioassays.[7,29]

In this work, we demonstrate the implementation of an acoustic trap for bead injection ATR-IR spectroscopy To showcase the applicability of the presented approach for monitoring enzymatic reactions, the substrate conversion induced by the trapped beads labeled with the enzyme alkaline phosphatase (AP) was monitored by ATR-IR spectroscopy. To the best of our knowledge, this is the first time that ultrasound particle manipulation has been utilized for bead injection ATR-IR spectroscopy.

Experimental

Reagents and Samples. Polyoxyethylene (20) sorbitan monolaurate (Tween 20), diethanolamine, and alkaline phosphatase (3262 U mg^{-1}) were purchased from Sigma-Aldrich (Steinheim, Germany); bovine serum albumin was obtained from Fluka (Buchs, Switzerland); *p*-nitrophenylphosphate (pNPP) disodium hexahydrate was obtained from Boehringer-Mannheim (Mannheim, Germany); magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\,H_2O$) and zinc sulfate heptahydrate ($ZnSO_4 \cdot 7\,H_2O$) were sourced from Merck (Darmstadt, Germany); and *p*-nitrophenol was purchased from Alfa Aesar (Karlsruhe, Germany). Immobilization of alkaline phosphatase was accomplished using the dynabead antibody coupling kit (bead diameter = 2.8 µm, epoxy-functionalized) and a tube rotator from Thermo-Fisher Scientific (Darmstadt, Germany). Reverse osmosis water was used for all solutions. The vial containing the obtained alkaline phosphatase was subsequently filled to 1 mL with an aqueous solution of 0.1 mmol L^{-1} Zn^{2+} and 1 mmol L^{-1} Mg^{2+}. AP-labeled beads (20 µg enzyme per 1 mg beads) were prepared by carrying out all immobilization steps as suggested by the acquired dynabead kit. The final bead concentration was adjusted to 0.5 mg mL^{-1} (~3.5 × 10^7 beads mL^{-1}). A stock substrate solution was obtained by dissolving 50 mmol L^{-1} pNPP in a 2 mol L^{-1} diethanolamine buffer containing 0.1 mmol L^{-1} Zn^{2+}, 1 mmol L^{-1} Mg^{2+}, and 0.02% Tween 20. The final substrate solutions, consisting of 5−30 mmol L^{-1} pNPP, were prepared by diluting the stock solution with the same ethanolamine buffer. The pH of the final substrate solution was adjusted to 9.8 using hydrochloric acid. A wash solution was prepared by dissolving 1% BSA in water containing 0.1 mmol L^{-1} Zn^{2+}, 1 mmol L^{-1} Mg^{2+}, and 0.02 % Tween 20. Reverse osmosis water containing 0.02% Tween 20 was used as the carrier solution.

Experimental setup. The experimental setup is shown in Figure **1**.

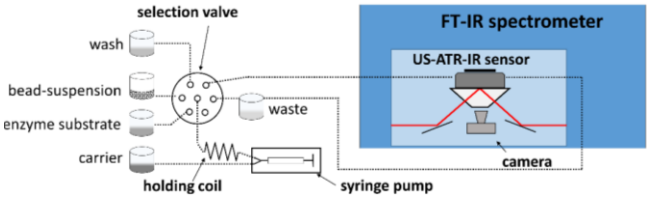

Figure 1. Schematic of the experimental setup for ultrasound-enhanced bead injection ATR-IR spectroscopy.

For the ultrasound-enhanced bead injection ATR-IR experiments, the IR beam of a Tensor 37 FT-IR spectrometer (Bruker Optics, Ettlingen, Germany) was guided through a single-bounce custom-built ATR setup; the design was inspired by a recently published similar design.[32] The employed zinc sulfide ATR element (25 x 20 x 10 mm , 60)was purchased from Crystran (Poole, United Kingdom). The transparency of ZnS allowed real-time visual observation of the acoustofluidic cell. The camera system by which ongoing US experiments were monitored was assembled using a Raspberry Pi 2 coupled to a Picamera module from the Raspberry Pi foundation (Cambridge, UK). A light-emitting diode (LED) was mounted next to the camera, creating ideal filming conditions in the sample compartment of the FT-IR spectrometer. The FT-IR spectrometer was equipped with a liquid-nitrogen-cooled mercury cadmium telluride (HgCdTe) detector. Prior to spectrum acquisition, the spectrometer was flushed with dry air. Spectra were recorded with a spectral resolution of 4 cm^{-1} in double-sided acquisition mode. A total of 128 scans were averaged per spectrum (acquisition time, 28.4 s), which was calculated using a Blackmann-Harris 3-term apodization function and a zero-filling factor of 2. All spectra were acquired at room temperature. The aperture was set to 4 mm, restricting the IR beam to the profile of the acoustofluidic cell. Spectra were analyzed using the software package OPUS 7.5 (Bruker Optics, Ettlingen, Germany). The SIA system for liquid handling consisted of a 10-port selection valve from VICI (Schenkon, Switzerland) and a Carvo XC syringe pump from Tecan (Männerdorf, Switzerland) equipped with a 100 µL glass syringe; 1/16” PTFE tubing with an inner diameter of 0.18 mm from VICI (Schenkon, Switzerland) was used to connect the selection valve and the syringe pump and for the holding coil. The ports of the selection valve were linked to the wash buffer solution, the enzyme substrate solution, the bead suspension solution, the acoustofluidic cell, and a waste cell (see Figure 1). The entire SIA setup was controlled via LabView (National Instruments, Austin, TX, USA) with a server-client program structure.[33]

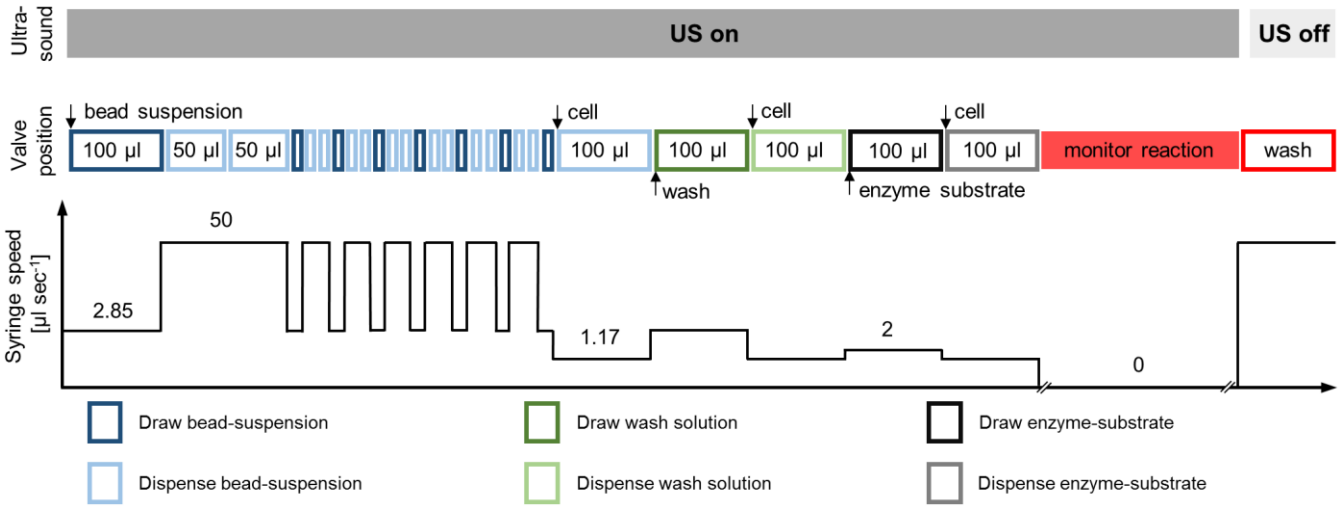

Figure 2. Schematic of the experimental sequence employed for bead injection and IR measurements. 10 minutes of sample handling is followed by 30 minutes of reaction monitoring.

Acoustofluidic cell. A more detailed schematic of the acoustofluidic cell on top of the ATR-element is shown in Figure 3.

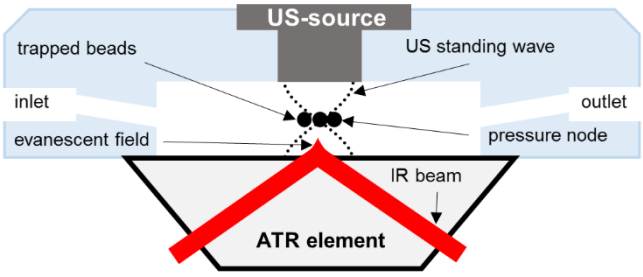

Figure 3. Schematic of the acoustofluidic cell.

The acoustofluidic cell was manufactured by Protolabs (Feldkirchen, Germany) following self-developed blueprints. The body of the cell is made of aluminum and hosts the US transducer (US source); the sample compartment has a volume of approximately 20 µL and a height of 500 µm. The transducer, a composite component, consists of a 10 mm piezo disc (lead zirconium titanate, Type 181; PI Ceramics, Lederhose, Germany) with wraparound silver electrodes glued to a Macor cylinder with a two-component epoxy resin (Polytec PT, Karlsbad, Germany). For thermal management, a cooling fan was mounted above the acoustofluidic cell. A custom-made driver (Frequency Power Synthesizer FPS 4025, Psi, Austria) was used to operate the US transducer, with a driving signal of approximately 0.8 W electrical power input. The US operating frequency was adjusted to 2.2 MHz. Prior to sample handling, the US was turned on for 10 minutes to allow the acoustofluidic cell to stabilize thermally to prevent thermal drifts from influencing the IR spectra.

Bead injection sequence. An optimized liquid handling sequence was devised and is depicted in Figure 2. The bead suspension was aspirated and returned to the reservoir eight times via the syringe pump (blue). This crucial step prevented inhomogeneity of the bead suspension, which is prone to sedimentation, thus ensuring a homogeneous suspension for reproducible bead injection. Subsequently, 100 µL of the bead suspension was injected into the acoustofluidic cell, where the beads were retained acoustically while their hosting liquid was discarded. Pumping 100 µL through the 20 µL flow cell while trapping the beads led to an additional concentration step, resulting in approximately 50 µg of beads in the acoustofluidic cell. Subsequently, wash buffer solution was passed through the cell to remove unstable retained beads (green). In the next step, 100 µL enzyme substrate solution was injected into the cell (grey). Directly after completion of the injection step, continuous acquisition of IR spectra was started (red). Parallel to monitoring the enzymatic conversion via ATR-IR spectroscopy, the holding coil was rinsed. After successfully monitoring the reaction, the US was switched off and the acoustofluidic cell was rinsed thoroughly with carrier solution. The US was turned on during the entire experimental sequence with the exception of the final washing procedure. The time needed from bead homogenization to the start of the enzymatic reaction was approximately 10 minutes.

Results and Discussion

US-ATR-IR spectroscopy for monitoring enzymatic conversion within the acoustofluidic cell. AP-labeled beads were injected into the acoustofluidic cell by the in-house-built SIA system following the sequence depicted in Figure 2. Ultrasound particle manipulation retained the beads within the cell, above the evanescent field, while enzyme substrate was pumped into the cell. Figure 4 depicts a scheme of the monitored enzymatic reaction.

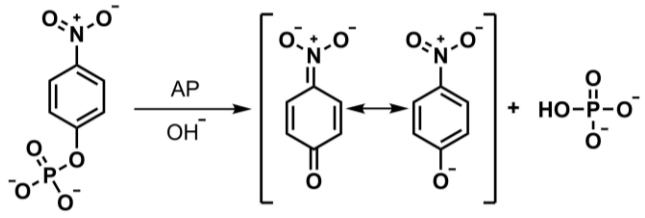

Figure 4. Reaction scheme of the conversion of *p*-nitrophenylphosphate to *p*-nitrophenol and phosphate ion via the enzyme alkaline phosphatase.

IR spectra of the conversion of the enzyme substrate pNPP into the products *p*-nitrophenol and phosphate ion via the

acoustically trapped AP-labeled beads were recorded over the course of 60 min with a time resolution of 1 min. The first of these consecutively recorded spectra was used as the background. IR spectra of the ongoing conversion of substrate into products are shown in Figure 5. The spectra show increases of the product-related bands at 1280, 1170, 1075, and 990 cm^{-1}, whereas the substrate-related bands at 1345, 1130, and 980 cm^{-1} decrease over time. These IR band positions in the recorded spectra of the ongoing enzymatic conversion are in good agreement with the literature.[34]

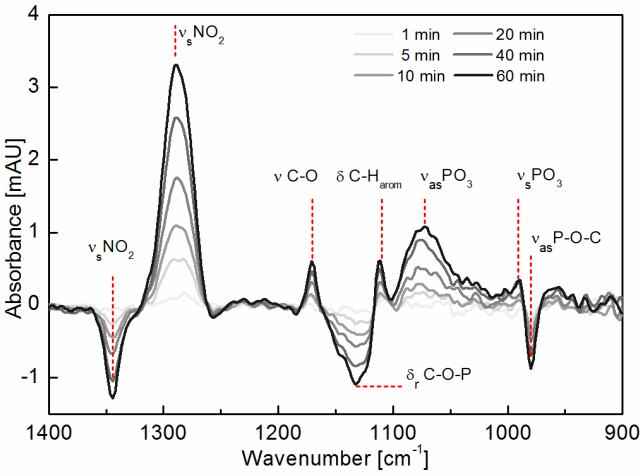

Figure 5. Mid-IR spectra monitoring the conversion of the enzyme substrate *p*-nitrophenylphosphate (30 mmol L^{-1}) to *p*-nitrophenol and phosphate ion recorded between 5 and 60 min.

As shown in Figure 5, the bands between 1260 and 1350 cm^{-1} can be related to the symmetric stretching vibration of the nitro group (ν_s NO_2) of the product and substrate, respectively.[34] Cleavage of the phosphate moiety results in a phenolate ion, which is a strong electron donor compared to the moderately donating phosphate ester group. Consequently, the increased electron density of the aromatic ring induces a red shift of the NO_2 vibrational mode.[35] The band at 1170 cm^{-1} is related to stretching vibrations (ν C–O) assigned to the C–O group of the nitrophenol product.[35] An increase in the aromatic C–H deformation vibration (δ C–H_{arom}) can be seen at 1110 cm^{-1}.[36] At 1130 cm^{-1}, the decreasing band is correlated to the rocking vibration of the aryl phosphate ester (δ_r C–O–P).[36,37] The bands at 1075 and 990 cm^{-1} can be related to the asymmetric (ν_{as} PO_3) and symmetric (ν_s PO_3) stretching vibration of the inorganic phosphate ion, respectively.[38] The decrease of the band at 980 cm^{-1} corresponds to the asymmetric stretching vibration of the phosphate ester (ν_{as} P–O–C),[36] which largely masks the symmetric stretching vibration (ν_s PO_3) of the inorganic phosphate band at 990 cm^{-1}.

In summary, these spectra verify the ability of the developed acoustic trap to retain beads within the acoustofluidic cell. Moreover, the potential of our custom-built ultrasound-enhanced ATR-IR setup to monitor bead-bound enzymatic reactions is demonstrated through the monitoring of the conversion of pNPP into *p*-nitrophenol and phosphate ion via alkaline phosphatase.

Bead retention capabilities of the acoustic trap. In conventional bead injection manifolds, the beads of interest are retained via mechanical systems, such as jet ring cells or optical fibers.[27] When these methods are employed, backflushing or removal of the retention element is required to remove the beads from the flow system. In contrast, the acoustic trapping system retains the beads only via the acoustic radiation force.[30] The washing process is easily initiated by turning off the US. These benefits make the introduced method an excellent alternative for bead injection setups. To demonstrate the reproducibility of the developed setup, multiple experiments were performed by repeating the complete bead injection sequence (see Figure 2) as described before, enzyme-labeled beads were retained in the cell by ultrasound particle manipulation. Then, the acoustically trapped beads were supplied with enzyme substrate and the subsequent conversion was monitored for 20 min, during which a sequence of 20 spectra were acquired using ATR-IR spectroscopy. All experiments used a substrate concentration of 30 mmol L^{-1}. After each injection, the beads were discarded and the acoustofluidic cell was thoroughly rinsed with wash solution. Analysis was based on evaluation of the band height at 1280 cm^{-1}, which originates from the nitro-group of the product, after 20 min of enzymatic conversion; this duration was chosen as the signal reached a readily detectible level after this reaction time (see Figure 5). Figure 6A shows the increase of the IR band intensity attributed to the product of eight consecutively performed bead injection procedures and enzymatic reactions. The relative standard deviation of 3.91% of the analytical signal confirms the high reproducibility of the bead injection procedure with regard to the injected bead concentration and initial bead trapping efficiency. The observed variations between the experimental repetitions may be due to aspiration of unequal amounts of beads with the syringe pump, caused by heterogeneity in the bead suspension reservoir despite the initial optimized routine to disperse the beads homogeneously in the bead-suspension reservoir.

Among the other benefits of the presented approach, not only can beads be trapped in a reproducible way, but it is also possible to retain and reuse them in multiple reaction cycles. A second set of experiments was performed to demonstrate the retention performance of the US trap with regard to fresh substrate passing through the cell.

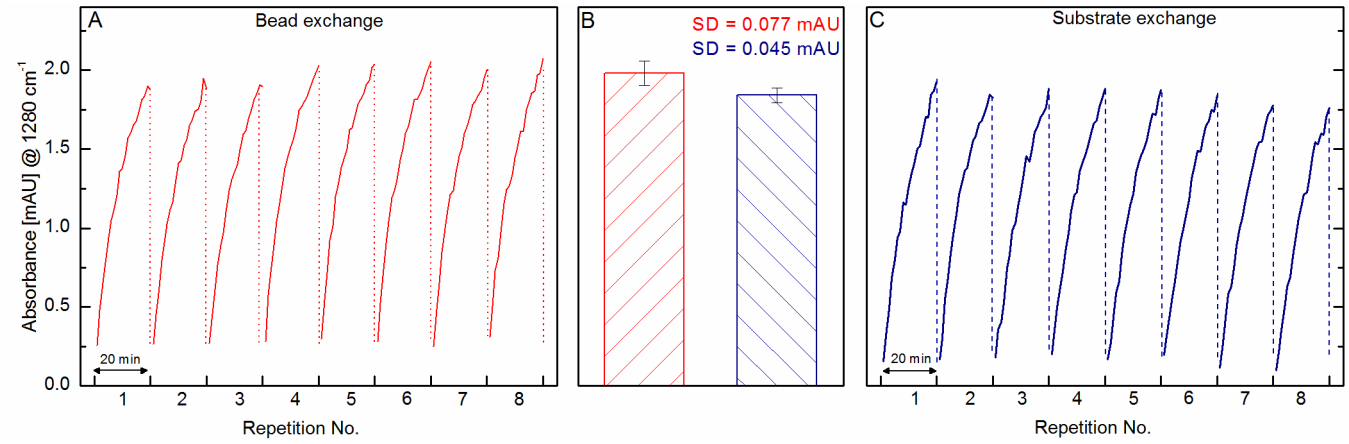

Figure 6. Bead retention experiments. (A) Reaction kinetic (red solid line) monitored at 1280 cm^{-1} for 20 min through eight repetitions of the entire experimental sequence, including bead and substrate exchange (red dotted line). (B) Comparison of the average final IR band intensity and standard deviation of experiments shown in (A) and (C). (C) Reaction kinetic (blue solid line) monitored at 1280 cm^{-1} for 20 minutes after initial bead supply and retention followed by eight repetitions of substrate exchange (blue dashed line).

The beads were initially injected into the acoustofluidic cell, followed by introduction and exchange of the enzyme substrate, and the enzymatic reaction was performed eight consecutive times. During this experiment, enzyme substrate equivalent to 40 times the volume of the cell was pumped through the acoustofluidic cell while the beads were retained. As previously, the band height at 1280 cm^{-1} was evaluated after 20 min of enzymatic conversion. Figure 6C shows the progression of the product band of eight consecutively performed enzymatic conversions with retained beads. The low relative standard deviation of 2.46% demonstrates the high bead retention efficiency of the acoustic trap even when high volumes of liquid are passed through the sampling.

Studying enzyme kinetics using an acoustic trap for bead injection ATR-IR spectroscopy. Finally, we investigated the enzyme kinetics of the AP-labeled beads with regard to the effect of varying substrate and enzyme concentrations. In the first step, the developed setup was calibrated by mimicking the total conversion from substrate into product by recording difference spectra. To achieve this, substrate spectra at defined concentrations were employed as background and the absorbance spectra of the product were calculated at the corresponding concentration (data not shown), allowing evaluation of the analytical signal reached at different product concentrations. Then, an entire measurement run was performed and the enzymatic conversion was monitored at six different substrate concentrations, ranging from 5–30 mmol L^{-1}. The bead suspension was injected into the acoustofluidic cell once, and the enzyme-labeled beads were retained using acoustic trapping and the host liquid was discarded. Each of the six different substrate concentration solutions were injected three times, while retaining the beads throughout all eighteen experiments. ATR-IR spectra were recorded after injection of the enzyme substrate (see Figure 7A). As with previous experiments, the height of the IR band of the product nitro-group at 1280 cm^{-1} was used as the analytical signal. The absorbance measured after 20 min of substrate conversion was translated via the calibration into enzyme velocity and enzyme activity. It was found that different substrate concentrations lead to different enzyme velocities (see Figure 7A).

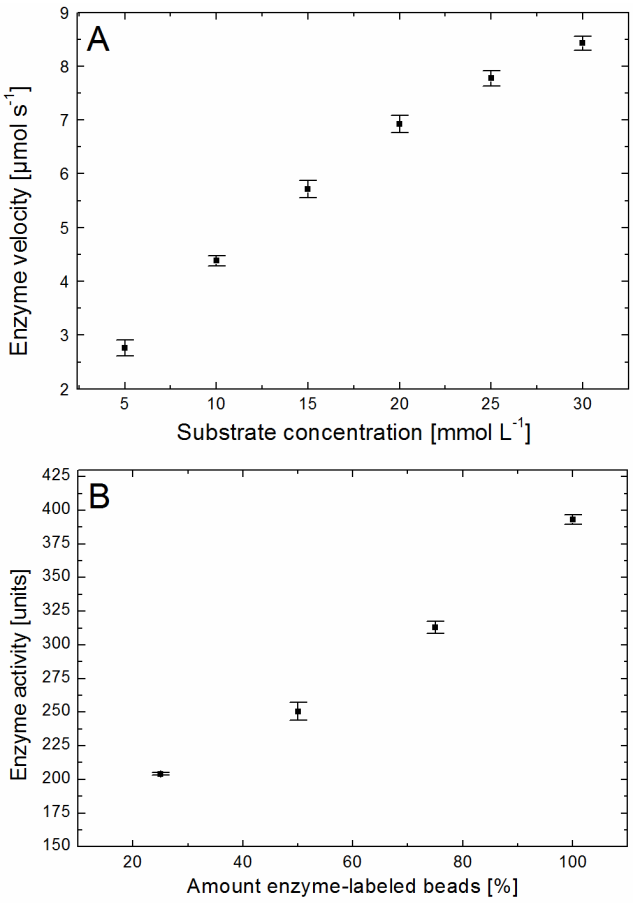

Figure 7. (A) Enzyme velocity monitored via ATR-IR spectroscopy at different substrate concentrations of acoustically trapped enzyme-labeled beads. (B) Enzyme activity monitored via ATR-IR spectroscopy at different amounts of AP-labeled beads. One unit is defined as the conversion of 1 µmol min^{-1} pNPP at room temperature in 2 mol L^{-1} diethanolamine buffer, pH 9.8.

These findings are in good accordance with the literature,[39] describing non-linear behavior of enzyme velocity at tenfold substrate concentrations of the k_m value (k_m = 1.5 mmol L^{-1}).[40] These measurements demonstrate the applicability of the developed setup for monitoring enzyme kinetics. As discussed before, these experiments also demonstrate the possibility of using retained beads several times, for example when enzyme reuse is desired. Over the course of this experiment run, a total volume of 1.8 mL enzyme substrate at different concentrations was pumped through the cell while still retaining the beads, resulting in a total liquid exchange of 90 times the cell volume. Finally, to study the effect of varying enzyme concentrations, unfunctionalized and AP-labeled beads were mixed at different ratios. Four different suspensions containing varying amounts of AP-labeled beads were injected three times into the acoustofluidic cell. The retained beads were supplied with substrate containing 15 mmol L^{-1} pNPP. The total bead concentration was kept constant (0.5 mg mL^{-1}) in the different suspensions, whereas the proportion of enzyme-labeled beads ranged from 25−100%. Adjustment of the desired AP-labeled bead concentration was accomplished by using non-labeled bead suspension (0.5 mg mL^{-1}). As before, the substrate conversion was monitored by ATR-IR spectroscopy, and the height of the IR band of the product nitro-group at 1280 cm^{-1} was evaluated after 20 min reaction time. The results of these experiments revealed increasing enzyme activity with the presence of increasing amounts of AP in the acoustofluidic cell (see Figure 7B). Hence, these results corroborate the suitability of the employed method for kinetic studies of immobilized enzymes.

Conclusions and Outlook

This work introduced the combination of an acoustic trap with ATR-IR spectroscopy, which is a promising alternative to established bead injection manifold approaches. In the presented approach, particles are retained by ultrasonic radiation forces, circumventing the need for conventional mechanical retention techniques and thus enabling elegant liquid handling wherein removing retained particles can be achieved by simply switching off the ultrasound. Enzyme-labeled beads were employed for characterizing the setup. It was demonstrated that both bead injection and bead retention can be performed in a highly reproducible manner. To showcase the potential and versatility of the presented setup, we monitored the conversion of pNPP via the well-studied enzyme alkaline phosphatase immobilized onto beads. ATR-IR spectroscopy is a perfect match for the acoustic trap as the ATR element acts not only as a reflector for particle manipulation via the standing ultrasound wave but also as the sensing element. The next steps to further optimize the presented method involve incorporating a multiple-bounce ATR element for higher sensitivity as well as integrating the system into a quantum cascade laser-based setup. Regarding potential application to biologically relevant questions, the presented results pave the way for enzyme kinetic studies exploring non-chromogenic natural enzyme-substrates via ATR-IR spectroscopy. Traditional assays for studying enzyme kinetics are limited to chromogenic or fluorescence substrates due to the use of UV-VIS or fluorescence spectroscopy as detection methods. In contrast, the bead retention capabilities of the introduced acoustic trap allow it to be combined with UV-VIS or fluorescence spectroscopy to perform bead-based assays employing traditionally used enzyme substrates. Thus, further biological or life science applications of the presented setup may include enzyme kinetic studies and bead-based assays combining the acoustic trap with ATR-IR or UV-VIS spectroscopy. In addition, future studies may address the real-time observation of solid-phase extraction or classic catalytic reactions induced by acoustically trapped beads.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement no. 731778.

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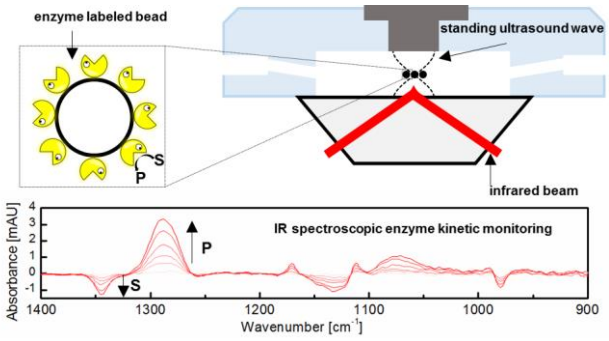

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