Development and characterization of temperature-controlled microreactors for protein crystallization

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Physico-chemical properties of the crystallization of biological macromolecules are of particular interest for an efficient way to get high-quality crystals. Concept and realization of a novel temperature-controlled microreactor to study these parameters is revealed. The characterization of the device is focussed on the temperature distribution across the reaction chamber, its long-term stability and accuracy as well as the regeneration of the surface inside the chamber after contamination with a hydrophilic protein (rGFP). We conclude that a microreactor for *in situ* observation of a temperature-controlled batch crystallization is in place.

Keywords: protein batch-crystallization; microreactor; temperature-control

1. Motivation

In parallel to high-throughput approaches in the crystallization of small amounts of biological macromolecules such as proteins (Cachau, R. E.. *et al.*, 2000; Moran & Stewart, 2001; Blundell, T., 2002), basic research on their physico-chemical properties and molecular interactions has increasingly gained on importance again in recent years (Gripon *et al.*, 1997; Vekilov & Rosenberger, 1998; Tsekova *et al.*, 1999; Ruppert *et al.*, 2001; Budayova-Spano *et al.*, 2002; Brzozowski, 2002). The development of phase diagrams is of particular importance in order to understand the processes involved in crystallogenesis (Haas & Drenth, 1998; Mühlig *et al*., 2001). Our aim is to improve the success of any crystallization attempts significantly as well as to find new methods of predicting it. Today it is assured that most of the fundamental effects and mechanisms governing the crystal growth of small (inorganic) molecule systems also exist for macromolecular systems (Rosenberger, 1996; McPherson, 1999; Tsekova *et al.*, 1999). Both domains exhibit remarkable similarities, yet the crystal growth of biopolymers takes place almost exclusively from aqueous solution around room temperature and atmospheric pressure (Bergfors, 1999; McPherson, 1999). Such conditions can easily be realized in experiments and allow the use of some powerful *in situ* observation techniques such as the atomic force microscopy (AFM) (Astier, 2001; McPherson *et al*., 2000), Michelson interferometry (DeLucas, 2001) or the confocal laser scanning microscopy (cLSM) (Mühlig *et al.*, 2001). Even though cLSM is seldomly used in the analysis of crystallogenesis, it seems particularly well suited. On the one hand, this observation technique allows the non-invasive study of the surface topology of crystals by characteristic diffraction patterns. While Michelson interferometry can analyze growth of crystals of about 100 µm or larger besides concentration gradients in solution, with cLSM crystals as small as 1 to 10 µm can be tracked. In addition, due to its confocality, cLSM is capable of scanning through the crystal in order to detect morphological imperfections with a vertical resolution of down to 100 nm while interferometric techniques are limited to the measurement of the thickness of growth layers.

Numerous methods for the crystallization of proteins have been reported, such as vapor diffusion ('sitting or hanging drop'), batch crystallization or free interface diffusion, to name the most popular ones only. In complement, several methods have been invented to alter and thus optimize the conditions of how to get crystals that are appropiate for X-ray diffraction analysis. Parameters in question for this screening approach are e.g. pH, temperature, ionic strength of the bulk solution ('salting in/ out') or the chemical properties of the protein molecule itself (Bergfors, 1999; Blagova, 1999; McPherson, 2001). Research on the dependence of protein crystallogenesis with respect to the variation of the temperature has been somewhat neglected by the majority of crystallographers, although it has been realized to play a pivotal part in the process of the crystallization of biological macromolecules (DeMattei & Feigelson, 1993; Nadarajah *et al.*, 1995; Christopher *et al.*, 1998; Galkin & Vekilov, 1999). For instance, polymorphisms were studied and phase diagrams were set up for BPTI (bovine pancreatic trypsin inhibitor) and α-amylase with regard to temperature changes (Lafont *et al.*, 1997; Veesler & Astier, 2002). Lysozyme was found to possess a well-defined temperature at which the solubility drastically change. The change of phase can be introduced by comparably small temperature changes in the order of $\Delta T \approx 0.5$ K (Haas & Drenth, 1998; Haas & Drenth, 2000). In general, the temperature-dependence of the solubility, i.e.the sensitivity of the enthalpy of dissolution Δh_{dis}, of many proteins may range in the order of about 10% per K. On the other hand, the growth characteristics of protein crystals can be influenced by introducing relatively small variations of the temperature of about $\Delta T < 0.5$ K, often leading to defects in the lattice and hence resulting in a higher mosaicity. This is often attributed to cooperative processes due to inter- and intra-molecular interactions of the protein molecules leading to conformational changes.

In this report, a new device will be presented for studying the temperature-dependence of the protein crystallogenesis in a batch process. Although the microreactor is operated close to the physiological temperature range, particularly suited for the research on the crystallogenesis of biological macromolecules. Design, characterization and its application are a central part of the initiative 'Jena BioCrystallognesis Centre' (JBCC) launched in 2001, which is connected to the 'European Bio-Crystallogenesis Initiative' (EBCI), also coordinated in Jena (Hilgenfeld *et al.*, 2001; IPHT, 2002; JBCC, 2002). JBCC couples the regional capabilities of biochemists in the area of the crystallography of biological macromolecules at the 'Institute of Molecular Biotechnology' (IMB), Jena with experience of physicists and engineers on crystal growth, optical microscopy and microreaction technology at the 'Institute for Physical High Technology' (IPHT), Jena. We ultimately aim at the design of an experimental set-up, currently being under development, which allows for a defined alteration of parameters like ionic strenght, pH, forced convection etc. by running experiments in a dynamic batch process. In combination with cLSM, this procedure may display a promising tool for getting access to crucial physico-chemical properties of the crystallization of biological macromolecules in order to get parameters for setting up phase diagrams and thus modelling and understanding the behaviour of biopolymers (Hilgenfeld *et al.*, 2001; Mühlig *et al.*, 2001; Mayer & Berg, 2002).

Preliminary experiments were carried out in the microreactor in order to assess the feasibility of the device to be able to perform studies of the crystal growth as envisaged. At this preliminary stage, we successfully used the well characterized hen-egg-white lysozyme (HEWL) as model protein in order to prove the functionality of the microreactor, in principle. Yet, experiments on the crystallogenesis

of several proteins will be emphasized on in a subsequent publication. In the present paper, we focus on the development and characterization of the novel temperature-controlled microreation device.

2. Concept and realization

On the first stage of the project, the technological background of a novel microreactor is evaluated which enables to perform batch crystallization of proteins by variation of temperature. In order to be able to study the crystallogenesis and growth of protein crystals and to assemble reproducible phase diagrams, an accurate temperature control better than at least ΔT = +/- 0.5 K is a necessary requirement (Lorber & Giegé, 1992; DeMattei & Feigelson, 1993; Haas & Drenth, 2000; Mühlig *et al.*, 2001; McPherson, 2002; Veesler & Astier, 2002). The growth rate of small protein crystals (diameter ≈ 3 to 30 µm) is registered for different temperatures using cLSM. Via extrapolation to zero growth, the temperature can be evaluated for a given protein concentration. This concentration can be assumed to remain constant as long as the crystals do not grow larger than about 50 µm. This assumption has been shown to be valid as the processes are still limited by the growth kinetics and has not entered the diffusion-controlled region (Mühlig *et al.*, 2001; Chernov, 1984). The microreator offers the possibility to analyze minute amounts of protein solutions (V_{total} < 15 µl) as well as to control the conditions inside the reaction chamber.

For the second and final stage of the project, the device is currently under redesign driving at a micro-flow module (Vekilov & Rosenberger, 1998; IPHT, 2002).

In order to meet the requirements of crystallogenesis, we developped a temperature-controlled microreactor (see Fig. 1) based on Si-microsystem technology such as photolithography, etching techniques or thinfilm technology, adapted from the microelectronics industries. Thinfilm transducers integrated on the chip-reactor work in combination with Peltier-coolers controlled by a specifically designed controller as well as a software program for a convenient operation. The growth of protein crystals is studied by *in situ* and non-invasive observation method using confocal laser scanning microscopy (cLSM) via an optical window (Mühlig *et al.*, 2001). For the preparation of thermodynamic phase diagrams, pivotal requirements are an exact and long-term stable control of temperature at ΔT < +/- 0.1 K in the range of 8°C < ϑ < 50°C as here, the vast majority of proteins reveals for crystallographers particuliarly informative behaviour (McPherson, 1999; Hilgenfeld *et al.*, 2001). Due to the fragile nature of protein crystals and the sensitivity towards pressure differences during the crystallisation process (McPherson, 1999; Bergfors, 1999), the reactor needs to be operated in a pulse-free mode. Further, economical reasons demand the regeneration of the reaction chamber after use. Therefore a cleaning protocol was designed and optimized in order to avoid cross-contamination in experimental series.

The technological roots of the chip-reactor with integrated thinfilm elements lie in the development of chip-thermocyclers for rapid PCR (polymerase chain reaction) designed at the IPHT, Jena. The integration of thin-film transducers (heaters and sensors) in these devices allow local temperature measurements and a temporal and spatial thermostabilization due to the excellent thermal properties of the functional materials Si, Si_3N_4 and Pt as well as appropriate geometries. Consequently, much faster heating and cooling rates (15 to 40 K/s) can be achieved in comparison to commercial table-top thermocyclers (e.g. 'Eppendorf® Mastercycler® gradient', Eppendorf, Germany: 2 to 3 K/s). Furthermore, these chipreactors enable researchers to perform PCR in volumes as small as 15 µl compared to a minimum of 200 µl in

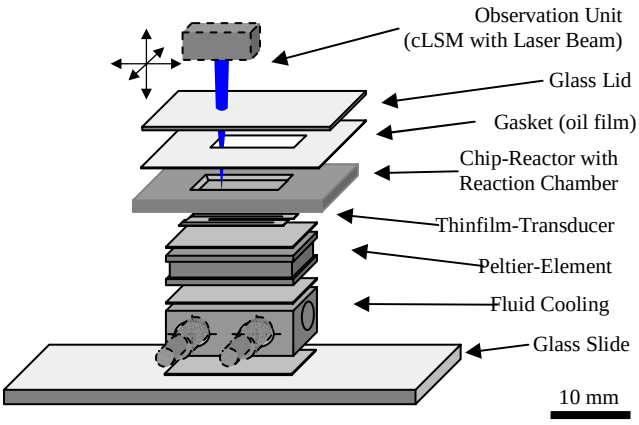

Figure 1

Schematic drawing of a temperature-controlled microreactor designed for *in situ* observation (cLSM) of protein crystallization

Eppendorf's device (Poser *et al.*, 1997; Poser *et al.*, 1998; Schneegaß & Köhler, 2001).

The preparation of this device involves classical processes in micromechanics, such as photolithography, wet and dry etching or thinfilm technologies. The reaction chamber is worked out of the silicon wafer (Si <100>) by Si-deep etching using NaOH. This anisotropic process yields rectangular cavities, due to the inherent properties of Si crystals (Büttgenbach, 1994; Köhler, 1999). Around the reaction chamber, longish cavities are located, the so-called air-gaps (see Fig. 2). These gaps act as a barrier for an (undesired) thermal conduction and therefore improve the dynamics of the temperature management by virtually isolating the chamber from the bulk chip. The heater and sensor elements are prepared by sputtering metal films (Pt and Al) onto a Si_3N_4-layer which acts as a dielectric separating the functional structures from the bulk silicon. The lateral dimensions of these elements go down as small as 10 µm whereas their thickness amount to 100 nm in the case of the Pt-structures (heaters and sensors), and 1 µm for Al-structures. The latter is installed as electrical conductors due to its geometry and lower resistance compared to Pt.

The preparation process for the current chip-device was modified in order to meet the requirements of long-term protein crystallization. The dimensions were adjusted to our needs forming a reaction chamber measuring 4 x 8 mm laterally with corresponds to a total volume V_{total} = 14 µl at a depth of up to 450 µm. This parameter can easily be adapted if the total volume of the reaction chamber is of interest due to a limited amount of protein, for instance. Whereas the chip-thermocyclers exhibit extraordinarily fast dynamics to shorten cycle times, priorities for the design of a microreactor for the studying the crystallogenesis of biological macromolecules had to be switched. Due to the characteristics of protein crystallization, processes are relatively slow but require an accurate temperature control over several days. As a consequence, both design and peripheral components of the chip-reactor had to be altered and adjusted accordingly. Whereas heating the reaction chamber is still managed by thinfilm transducers, the cooling has to be taken care of by a Peltier-element supported by a fluid cooling to get rid of excess heat produced by the element. Another important aspect displays evaporation control during the crystallization experiments. In a non-saturated environment, an aqueous solution of 15 µl evaporates within about 30 min out of the reaction chamber on the chip-reactor. In order to get the opportunity of running long-term experiments, in combination with the size restriction of the cLSM lead to a top cover consisting of an optical glass slide and a gasket of

paraffin oil. Similar oils are used as matrix for evaporation-free batch crystallization of biological macromolecules in small volumes of V_{total} = 1 to 2 µl (Chayen *et al.*, 1990; Blagova, 1999). Therefore, the growth of protein crystals is suspected not to be affected negatively by direct contact of oil and protein solution. Figure 2 displays the procedure of manually loading and covering the reaction chamber which shuts off the solution from the environment thus preventing the solvent from evaporating during the experiment.

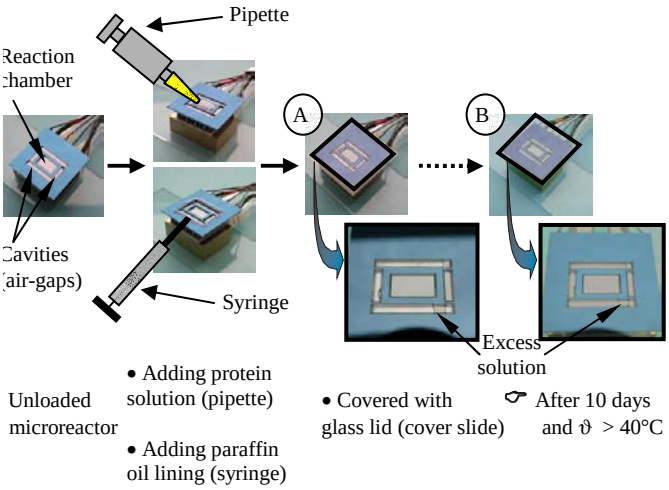

Figure 2

Loading the microreator: photographic documentation of manually feeding and covering the reaction chamber – experimental results of evaporation control after loading (A), after 10 days and operation at elevated temperatures temporarily above ϑ > 40°C (B)

After adding an excess amount (1.1 x V_{total}) protein solution into the reaction chamber with a pipette and applying a film of paraffin oil with a syringe onto the chip surface, the lid is slowly manipulated down towards the reaction chamber. Excess solution will drain off into cavities around the chamber (see Fig. 2). Therefore air bubbles are avoided inside the chamber. Pictures A) and B) in Fig. 2 display that over 10 days at elevated temperatures (ϑ > 40°C) neither bubbles nor evaporation can be depicted. Therefore the procedure of covering the reaction chamber suppresses evaporation effectively.

In order to use the instrumental set-up for crystallization experiments, the microreactor device has to be evaluated, which is set out below.

3. Results and discussion

The characterization of the microreactor device for protein crystallization is split into the analysis of the temperature control and regeneration of the reaction chamber.

3.1. Temperature control

The temperature control was evaluated elucidating two aspects: 1) the temperature distribution inside the reaction chamber, 2) the long-term temperature stability during the experiments. As for many crystallization processes of practical interest the relevant temperature range is around or below room temperature, we chose to concentrate here on the presentation of the cooling mode. However, the results gained during the tests of the heating mode were qualitatively similar.

With regard to the temperature distribution, an IR-camera was used to take images of the homogeneity of the temperature across the chip-reactor and the reaction chamber, in particular (see Fig. 3, A). Although the emission coefficient of each material contributes to the IR-images and therefore distorts the overall quality of the measurement, their influence were not separated by calibration in before hand. A coarse estimation of its influence showed, that the misrepresentation amounts only to about ΔT = +/- 1 K and happens well outside the region of interest (reaction chamber).

In comparison to this practical experiment, a simulation was performed applying the method of 3D-FEM (finite element method) using a commercial software tool (ANSYS® - Engineering analysis system, Rel. 6.0). In principal, the model was already in place as similar devices had been modelled before (Köhler *et al.*, 1999; Poser *et al.*, 1998). Minor changes regarding the geometry as well as the adjustment of some modelling parameters were sufficient to get appropriate results. As Figure 3 exhibits, the simulation (B) corresponds qualitatively very well with the representation of the IR-image (A). Still, the temperature gradient across the reaction chamber predicted by the theoretical model are larger than the ones revealed by IR-measurement. This was attributed to the simplifications of the physical model, assuming water to be a solid (no thermal convection) and neglecting surface effects.

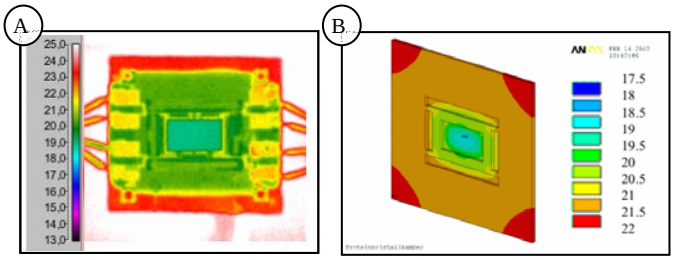

Figure 3

Temperature distribution inside the reaction chamber (cooling mode): A) picture from IR-camera; B) 3D-FEM simulation

Finally, the geometry of the cooling element was modelled to end at the edges of the chamber, whereas in reality it exceeds this barrier leading to an actually more uniform distribution. The temperature gradient across the chamber just underneath the lid measured with the IR-instrument exhibits a value of ΔT < 0.5 K at a temperature level of ϑ = 19°C, compared to a slightly larger value calculated to be ΔT < 0.8 K. So the simulation can be regarded as a tool for an estimation of the temperature distribution across the chip-reactor.

As the 'cold spot' of the chamber is located in the center of the chamber, the formation of nuclei is probably favored there. This effect could contribute to an improvement of the quality of protein crystals as there less surface around the crystals which could affect its growth negatively compared to when growing in the edges or corners of the reaction chamber. As surfaces can introduce stress in the lattice of the crystals, which is then relieved by changing the relative position of the building blocks towards each other. This may lead to less uniformly growing crystallites and thus yielding crystals of low quality for X-ray diffraction analysis (McPherson, 1999).

As outlined above, the second point deals with the long-term stability of the temperature-control during the cooling experiments. The time-scale of the crystallization process is located in the dimension of hours and days. Therefore, it was tested whether a drift in temperature occurred. Figure 4 illustrates one of the trials performed at different temperature levels. This particular experiment was picked as we here introduced a defined temperature change (ΔT < 10 K) and the return to the initial level at ϑ = 10°C.

The deviation of the actual temperature T_{act} and the nominal value T_{set} varied across the overall range at $\Delta T < 0.03$ K. This was suggested to be due to inaccuracies during the calibration process of the sensor elements. Still, it can be seen that T_{act} follows T_{set} without significant delay. This means that the thermal dynamics of the system fulfills the dynamic requirements of the crystallization experiments, and that control parameters are well adjusted. The random error, i.e. the maximum and minimum deviations from the nominal value or the the inaccuracy of the control loop, respectively, was found to be $\Delta T < 0.08$ K. As this value gets close to the threshold interval of ΔT = +/- 0.1 K, minor enhancement of the control parameters will follow. Additional tests were perfomed keeping the temperature constant at ϑ = 18°C for more than 250 h (> 10 days). The temperature remained inside the required interval without any time during the experiment.

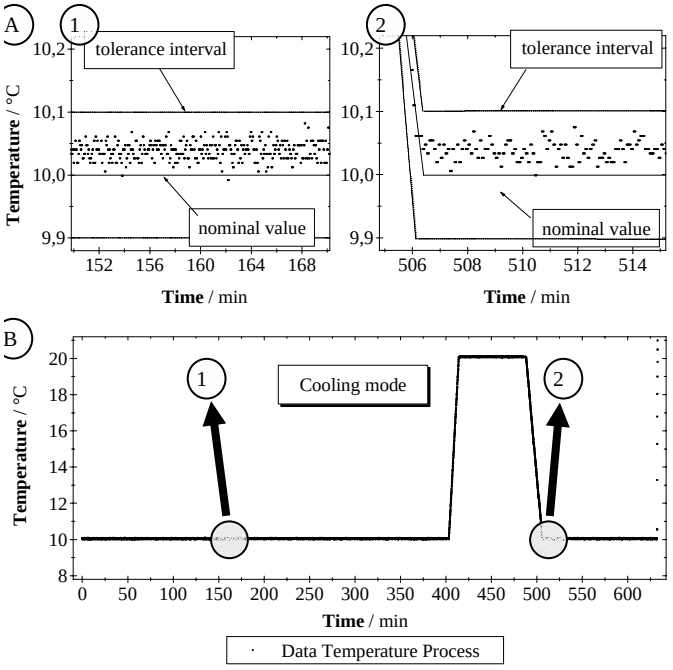

Figure 4

A) Detailed illustration of B): 1), 2) comparison of T_{act} and T_{set} at different times to manifest a possible drift in temperature B) Temperature stability in the cooling mode during long-term experiments: recording of temperature data

The heating experiments were performed in analogy to the tests run in the cooling mode. Particular attention was paid on the temperature range from room temperature up to ϑ = 35°C. Even though these investigations are not illustrated here in detail, the central aspects should not go unmentioned. The long-term experiments exhibited that the inaccuracy ranged at a lower level ($\Delta T < 0.06$ K) as running in the cooling mode. This fact was assigned simply to the fact that the control paramters had already been optimized more thoroughly. Other aspects such as the faster dynamics of thinfilm-transducers compared to Peltier-elements were also discussed. But with regard to our application these features were found to be of minor importance. Addressing the homogeneity of the temperature inside the reaction chamber, the microreactor performed worse in the heating than in the cooling mode. In the centre of the chamber, a 'hot spot' occurred flattening out towards the edges forming a temperature gradient of $\Delta T < 0.8$ K (IR-images). In turn, the simulations resulted in a value of $\Delta T < 1.2$ K which approximates well the measured values taking into account the simplifications of the model as described above. In comparison to cooling taking advantage of a Peltier-element, the thinfilm-transducers heat more locally. This is not surprising as these elements originally had to be adapted geometrically to the lateral dimensions of the reaction chamber of the chip-thermocyclers (Poser, 1997). This challenge could be overcome in future designs by extending the heater structures well across these geometrical limits.

The excess amount of protein solution possibly caught in the cavities during the loading procedure of the reaction chamber (see Fig. 2) was suspected to influence the thermal behaviour of the device. For both heating and cooling, IR-measurements did not reveal any disturbances from uniformity of the temperature distribution. Modelling the extreme case of completely filled cavities in comparison to empty ones, the temperature niveau across the chamber was slightly higher in the case of cooling and lower for heating, respectively ($\Delta T \approx 0.02$ K). This was attributed to the higher heat transport coefficient of aqueous solutions ($\lambda_{aq.} \approx 0.60$ W/m K) with regard to air (λ_{air} 0.024 ≈ W/m K). As the entire chamber is affected by this deviation, the influence on the temperature gradients ranges well below the periodic fluctuations introduced by the temperature control itself set to be ΔT = +/- 0.1 K at maximum. Despite the above mentioned uncertainties of the simulation, the influence of excess solution drained into the cavities on the temperature distribution was therefore assumed to be neglectable.

Overall, the temperature inside the reaction chamber of the microreactor can be tuned according to the constraints for temperature-sensitive measurements.

3.2. Regeneration of reaction chamber

Another pivotal aspect is the capability to fully regenerate the microreactor after having performed an experiment. In order to see eventually remaining particles or protein clusters on the surface of the reaction chamber, we chose cLSM which allowed a confocal fluorescence analysis. This way we could directly scan over the bottom and the side walls of the chamber which would not have been as easily realized with techniques, e.g. AFM or high resolution light microscopy. As a hydrophilic fluorescent protein, the well characterized rGFP (c_{stock} = 1 mg/ml; Roche Diagnostics/ D) was picked. This protein is highly soluble in aqueous buffer solutions (1 M Tris HCL, pH = 8; 0.5 M EDTA, pH = 8). rGFP exhibits a strong absorption maximum at λ_{ext} = 395 nm at a molar extinction coefficient ε = 24000 1/mol, as well as a minor maximum at λ_{ext} = 475 nm, ε = 7150 1/mol. The excited fluorophore emits around λ_{em} = 509 nm with a quantum yield of Q ≈ 0.77 (Spector *et al.*, 1998; Kahana & Silver, 1996). Even though the fluorescence properties rGFP are less favorable than those of FITC, it is common practice to use the filter set-up for this fluorophore. The ratio of the molar extinction coefficients using an FITC-filter (λ_{ext} = 485 nm) is about ε ($rGFP_{FITC\text{-}Filter}$) : ε ($FITC_{FITC\text{-}Filter}$) = 1 : 10. Consequently, the wavelength of the Ar/Kr-laser of λ_{em} = 488 nm was used (Spector *et al.*, 1998; Kahana & Silver, 1996). At the cLSM ('TCS', Leica/ D) we used an immersed objective ('Planapo', Leica/ D; magnification 20x, NA = 0.7), a standard immersion oil (^{n}D (20°C) = 1.520, fluorescence-free at λ = 488 nm; Carl Zeiss Jena/ D) and a zoom factor of 2.25. In order to get enough fluorescence signal from potentially small quantities of fluorescent particles sticking to the surface, a trade-off was made reducing the confocality by tuning the pinhole to a value of 100. The degree of sensitivity gained by tuning the optical parameters is illustrated by Fig. 6 A) and C): a disparity in the fluorescence signal was measured for immersion oil layers of

a difference in thickness $\Delta d \approx 450$ µm, even though this oil is claimed by the manufacturer to be 'non-fluorescent'. Due to this highly sensitive experimental set-up and the favourable photometric properties of rGFP (see above), the protein concentration was adjusted to c_{rGFP} = 500 µg/ml. The focus was adjusted to the maximum signal and the amplification voltage U_{amp} was varied in order to get comparable grey values. In calibration experiments it was found, that U_{amp} could directly be correlated to the concentration of the fluorescing substance. To prevent bleaching of the fluorophore, the laser power of the cLSM was turned down to its minimum value. For the experiments, the Si-chamber was hydrophilized/ activated with H_2SO_4 : H_2O_2 (4:1) to get a strong interaction of the surface with the hydrophilic protein.

The measurement were carried out at 3 conditions: A) non-contaminated microreactor, B) 'contaminated' with rGFP (V = 2 µl, c_{rGFP} = 500 µg/ml) and C) regenerated microreactor with an optimized protocol: After delidding the microreactor, the protein solution is removed from the reaction chamber by sucking up with a pipette (20 µl). The chamber is washed first with sodium acetate solution (0.05 M) in order to denature remaining proteins and make them more soluble towards aqueous solutions. In the following, the contaminated surface of the reation chamber is immersed with various diluted aqueous SDS-solutions (10%, 5% and 1%, respectively). Here, each volume (V = 20 µl) is repeatedly sucked up and harshly released back into the chamber, thus actively producing foam. The reaction chamber is then 5 x rinsed with aqua bidest. (V = 20 µ) and finally cleaned with water of spectroscopic grade to effectively get rid of eventually remaining streaks of impurities.

The amplification voltage was adjusted to an optimum value U_{amp} = 765 V to be able to compare the results. After pipetting the protein solution onto the activated Si-surface, it immediately spread to the edge nearest to the initial position of the drop. From there all edges at the bottom of the chamber were wet by the solution and sticked to the edges until dryed out after about 30 min. This wettening of the edges was attributed to the strong hydrophilic interaction of the aqueous protein solution, the protein itself and the activated Si-surface. An extremely large fluorescent signal was therefore detected via the fluorescence microscope in the edges at the bottom of the reaction chamber. Therefore, this region was chosen to be analyzed primarily (see Fig. 5).

For each condition cLSM-images were taken as shown in Figure 6. In case A), no fluorescent particles were detected. At the maximum amplification voltage U_{amp} = 1000 V, a weak fluorescence signal was measured. This was assigned to the fluorescent behaviour of the immersion oil which covered the whole chip-reactor as

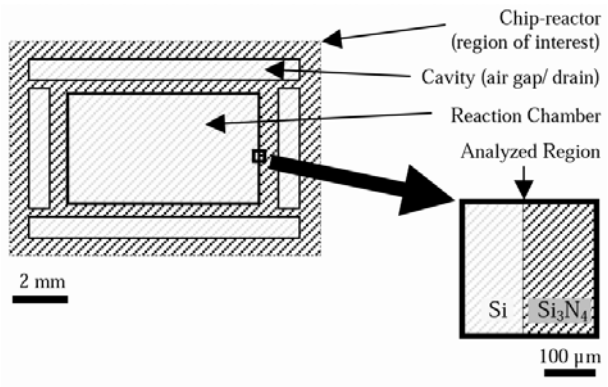

Figure 5

Schematic drawing of the reaction chamber: region analyzed using a cLSM.

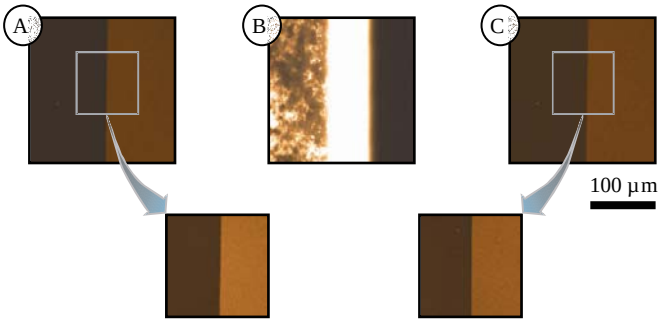

Figure 6

Regeneration experiments: fluorescence measurements with cLSM A) uncontaminated Si- and Si_3N_4-surface (edge of reaction chamber); B) contaminated with rGFP; C) after cleaning with optimized protocol

mentioned before. The sharp line in intensity represents the upper edge of the reaction chamber, where the brighter area corresponds to the Si_3N_4-surface. The slightly higher fluorescence signal may be attributed to the interaction of traces of contaminants in the immersion oil with the surface (thickness of layer of interaction ≈ 1.5 µm). No fluorescence signal neither from the Si-, nor from the Si_3N_4-surface was detected when using an objective ('Planapo', Leica/ D; magnification 20x, NA = 0.7) without immersion oil as a control. We therefore conclude that the Si-surface of the reaction chamber was clean. In case B), the image reveils an extremely bright signal. Even at U_{amp} < 250 V, a particulate fluorescent structure could clearly be seen (results not shown). This corresponds to the qualitative observation using the fluorescence microscope and the optical observation of a non-shining surface. After cleaning the surface using the optimized protocol, no contamination of the surface could be detected as illustrated in case C). Comparing the images taken for case C) with those of A), we conclude that the original state of the Si-surface was completely recovered.

4. Conclusions

In this report, a novel thermo-controlled microreactor was presented and characterized. A chip-reactor has been redesigned in order to meet the requirements of in situ analyzing the crystallization of proteins in a batch process. Evaporation is effectively suppressed for at least 5 days at elevated temperatures. However a simplified lid handling is under development. The temperature distribution, long-term stability and accuracy range within the claimed limits. Finally, the regeneration of reaction chamber is realized effectively applying an optimized cleaning protocol which allows reusing the microreactor after running experiments with soluble proteins. First crystallization experiments which were performed in the microreactor using HEWL as a model protein, proved the functionality of the device and feasibility of our concept (details not addressed in this paper).

We conclude that the described micro (batch-) reactor is ready for use in studying the temperature-dependence of protein crystallization.

Research on the crystallogenesis of other model proteins (e.g. human insulin) as well as diverse scientifically more relevant proteins (still to be determined) using the microreactor is currently under consideration and will be focussed on in the next months. Future improvements of the device will be put forward dealing with the conversion of the batch reactor towards a micro flow-module. This apparatus, currently being redesigned, will give us the opportunity to alter crystallization conditions by coupling different

agents into a slow but continuous flow of solution through the reaction chamber in a closed loop (Vekilov & Rosenberger, 1998; IPHT, 2002). By performing dynamic batch crystallization experiments, it will be possible to adjust, besides temperature, further crucial parameters such as protein concentration, ionic strength, pH or natural convection.

This study was performed as part of the Jena BioCrystallogenesis Centre (JBCC), a project supported by the Thuringian government (TMWFK) (grant No. B 378-00024). We also thank Prof. Dr.-Ing. Clemens Posten (University of Karlsruhe (TH)) as well as Prof. Dr. Michael J. Köhler (Technical University of Ilmenau) for many fruitful discussion.

References

Astier, J. P., Bokern, D., Lapena, L. & Veesler, S. (2001). *Journal of Crystal Growth*, **226**, 294-302.
Bergfors, T. M. (1999). *Protein Crystallization*. La Jolla: IUL Press.
Blagova, E. V. (1999). *Crystallogr. Rep.* **44**, 513-531.
Blundell, T. (2002). *High-Throughput crystallography for lead discovery in drug design*, presented at the *Drug Discovery Technology Europe, Stuttgart*, 15-19/04/02, p. 40.
Brzozowski, A. M. (2002). *Is there any alternative to high-throughput screening?* presented at the *ICCBM9*, Jena, 23- 28.03.02.
Budayova-Spano, M., Bonneté, F., Aster, J. P. & Veesler, S. (2002). *J. Cryst. Growth*, **235**, 547-554.
Büttgenbach, S. (1994). *Mikromechanik - Einführung in Technologie und Anwendung*. 2nd ed. Physik/Elektrotechnik. Braunschweig: Teubner, Stuttgart.
Cachau, R. E., J.R., C.-F. & Sanjoh, A. (2000). *Automated crystallization of polar biomolecules in the metastable regime using microfluidic silicon devices and a variable temperature incubator*, presented at the *19th European Crystallographic Meeting (ECM19), Nancy*, 25-31.08.00.
Chayen, N. E., Steward, P. D., Maeder, D. L. & Blow, D. M. (1990). *J. Appl. Crystallogr.* **23**, 297.
Chernov, A. A. (1984). *Modern Crystallography III: Crystal Growth*, vol. 36, pp. 208. Springer, Berlin.
Christopher, G. K., Phipps, A. G. & Gray, R. J. (1998). *J. Cryst. Growth*, **191**, 820-823.
DeLucas, L. J. (2001). *Drug Discovery Today*, **6**, 14.
DeMattei, R. C. & Feigelson, R. S. (1993). *J. Cryst. Growth*, **128**, 1225-1231.
Galkin, O. & Vekilov, P. G. (1999). *J. Phys. Chem.* **103**, 10965-10971.
Gripon, C., Legrand, L., Rosenman, I., Vidal, O., Robert, M.-C. & Boué, F. (1997). *J. Cryst. Growth*, **178**, 575-584.
Haas, C. & Drenth, J. (1998). *J. Phys. Chem. B*, **102**, 4226-4232.
Haas, C. & Drenth, J. (2000). *J. Cryst. Growth*, **196**, 388-394.
Hilgenfeld, R., Klupsch, T., Mühlig, P. & Mesters, J. R. (2001). *Zeitschrift der Deutschen Gesellschaft für Kristallwachstum und Kristallzüchtung*, **73**, 16-20.
IPHT (2002). http://www.ipht-jena.de/BEREICH_3/index_b3.html
JBCC (2002). http://www.imb-jena.de/JBCC/index.html
Kahana, J. A. & Silver, P. A. (1996). *Use of the A. Victoria Green Fluorescent Protein to Study Protein Dynamics in vivo*, in *Current Protocols in molecular biology*, F. M. Ausubel, Editor., pp. 9.7.22-9.7.28. John Wiley & Sons, New York.
Köhler, J. M. (1999). *Etching in Microsystem Technology*. 1st ed. Weinheim: Wiley-VCH.
Köhler, J. M., Baier, V., Dillner, U., Zieren, M., Ehricht, R., Poser, S. & Schulz, T. (1999). *Sensor '99, 9th International Trade Fair and Conference for Sensors, Transducers Systems, Nürnberg*, pp. 583 - 588.
Lafont, S., Veesler, S., Astier, J. P. & Boistelle, R. (1997). *J. Cryst. Growth*, **173**, 132-140.
Lorber, B. & Giegé, R. (1992). *J. Cryst. Growth*, **122**, 168-175.
Malkin, A. J., Kuznetsov, Y. G. & McPherson, A. (1999). *J. Cryst. Growth*, **196**, 471-488.
Mayer, G. & Berg, M. (2002). *Annual Report IPHT 2001*, pp. 56-59. IPHT, Jena.
McPherson, A. (1999). *Crystallization of biological Macromolecules*. Cold Spring, New York: Cold Spring Harbor Laboratory Press.
McPherson, A., Malkin, A. J. & Kuznetsov, Y. G. (2000). *Annu. Rev. Biophys. Biomol. Struct.* **29**, 361-410.
McPherson, A. (2001). *Protein Sci.* **10**, 418-422.
McPherson, A. (2002). Personal communication.
Moran, S. & Stewart, L. (2001). *Drug Discovery World*, Winter 2000/1, pp. 41-46.
Mühlig, P., Klupsch, T., Schell, U. & Hilgenfeld, R. (2001). *J. Cryst. Growth*, **232**, 93-101.
Nadarajah, A., Forsythe , E. L. & Pusey, M. L. (1995). *J. Cryst. Growth*, **151**, 163-172.
Poser, S., Schulz, T., Dillner, U., Baier, V., Köhler, J. M., Schimkat, D., Mayer, G. & Siebert, A. (1997). *Sensors and Actuators A - Physical*, **62**, 56-59.
Poser, S., Schulz, T., Dillner, U., Baier, V., Köhler, J. M., Mayer, G., Siebert, A. & Schimkat, D. (1998). *Process Miniaturization: IMRET 1*, pp. 294-301. Frankfurt/ M: Springer.
Rosenberger, F. (1996). *J. Cryst. Growth*, **166**, 40-54.
Ruppert, S., Sandler, S. I. & Lenhoff, A. M. (2001). *Biotechnol. Prog.* **17**(1), 182-187.
Schneegaß, I & Köhler, J.M. (2001). *Rev. Mol. Biotechnol.* **82** 101-121.
Spector, D. L., Goldman, R. D. & Leinwand, L. A. (1998). *Optical Detection of GFP*, in *Cells - A Laboratory Manual*, D. L. Spector, R. D. Goldman, and L. A. Leinwand, Editors., pp. 78.16-78.21. Cold Spring Harbor Laboratory Press: New York.
Tessier, P. M., Lenhoff, A. M. & Sandler, S. I. (2002). *Biophys. J.* **821**(3), 620-631.
Tsekova, D., Dimitrova, S. & Nanev, C. (1999). *J. Cryst. Growth*, **196**, 226-233.
Veesler, S. & Astier, J. P. (2002). *In-situ studies of protein crystallization*, presented at the *ICCBM9*, Jena, 23- 28.03.02.
Vekilov, P. G. & Rosenberger, F. (1998). *J. Cryst. Growth*, **186**, 251-261.