# Investigations on the effect of autoclaving on the properties of lipid nanodispersions by asymmetrical flow field-flow fractionation (A4F)

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Lipid nanoparticle dispersions are under investigation as delivery systems for poorly water-soluble drugs. For parenteral formulations, sterility is required and autoclaving is the preferable option to sterilize lipid nanoparticle dispersions. For poloxamer 188-stabilized lipid nanoparticle dispersions an increase in the mean particle size after autoclaving has been described [1]. Recent size investigations on poloxamer 188-stabilized trimyristin particles by laser diffraction with PIDS technology (LD-PIDS) confirmed the increase in particle size after autoclaving but not the overall better homogeneity as indicated by results from photon correlation spectroscopy (PCS) [2].

Asymmetrical flow field-flow fractionation (AF4) provides a broad separation range over the whole colloidal size range (from ~5 kDa up to a few µm [3]).Accurate size information is obtained when the fractionation system is combined with multi-angle laser light scattering (MALLS). In the present study, AF4 was used to evaluate the changes in particle size and sample homogeneity induced by autoclaving of poloxamer-stabilized lipid nanoparticle dispersions in more detail. Trimyristin nanoparticles both in liquid (nanoemulsion) and solid (solid lipid nanoparticles) state differing in particle size as well as initial poloxamer concentration were analyzed by AF4/MALLS before and after autoclaving. In addition, determination of unbound poloxamer in the dispersion was addressed.

Four differently sized nanodispersions were prepared by high-pressure melt homogenization (75 °C) as described earlier and characterized by PCS and LD-PIDS [2]. All dispersions contained 10% trimyristin as lipid phase (Dynasan® 114); the aqueous phase consisted of 2.25% glycerol as isotonizing agent, 0.05% sodium azide as preservative and 5 or 12% poloxamer 188 (Kolliphor P 188®) as emulsifier, dissolved in bidistilled water. The dispersions were autoclaved at 121 °C for 15 min. One part of the dispersions was stored at room temperature and another part at 4 °C to obtain nanoemulsions and solid lipid nanoparticles, respectively.

Samples were analyzed by an Eclipse3+ AF4 system connected with MALLS and differential refractive index detectors (all from Wyatt). The AF4 channel was equipped with a trapezoidal spacer and a membrane of regenerated cellulose (MWCO 5 kDa, Nadir). 100 µl of diluted nanoparticle dispersions (1:100 v/v, except E/S45 which was diluted 1:10 v/v) were injected into the channel.

Particle sizes and size distributions were calculated based on the MALLS data applying the Mie model. The amount of free poloxamer was quantified by analyzing the dRI detector signals.

In the A4F elution profiles of nanoemulsion before and after autoclaving the free poloxamer was clearly detected in addition to the nanoparticle fraction. Due to the small size of the poloxamer molecules, this fraction was, however, only detectable in the dRI detector. While the elution behavior of the poloxamer fraction was not altered by autoclaving, the lipid nanoparticles eluted distinctly later in the autoclaved sample indicating an increase in particle size. Moreover, the small-sized structures, which were detected by the dRI detector between 20 and 30 min in dispersions before autoclaving, were not present in the autoclaved nanoparticle dispersions. The disappearance of the small-sized structures and an increase in particle size was found for all autoclaved samples. Size distributions of the lipid nanoparticle fractions based on the MALLS data confirmed the increase in size by autoclaving but no distinct change in width of distribution. This is in good agreement with previous LD PIDS measurements [2]. However, the fraction of small-sized particles was not included in the MALLS size evaluations due to the insufficient light scattering within this fraction. The increase in size caused by autoclaving was observed in all studied samples. Due to their platelet-like shape, solid nanoparticles showed a slightly larger particle size. The amount of free poloxamer clearly depended on both particle size (homogenization conditions or autoclaving) and shape (crystallization) due to the reduced specific particle surface with increasing size and the larger specific particle surface of the anisometric solid particles compared to spherical emulsion droplets.

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# LC-MS method using monolithic silica column for the determination of hypoglycemic agents in mixture

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In this work a LC-ESI-MS method has been developed and validated for the simultaneous determination of metformin, alogliptin, sitagliptin, vildagliptin and linagliptin in mixture. The method is applicable for the quality control of a number of pharmaceutical products since metformin is usually combined in one dosage form with either of the above dipeptidyl peptidase-4 inhibitors for the treatment of type II diabetes mellitus. The LC analyses were performed using an Agilent 1100 LC system with second-generation monolithic silica column Chromolith® HighResolution RP-18e column (100 × 4.6 mm, Merck). A mobile phase consisting of acetonitrile/ ammonium formate buffer (20:80, v/v) at a pH 3 was used at a flow rate of 0.4 mL/min. A single-quadrupole mass spectrometer (Agilent 1620) equipped with an electrospray ionization source was used. After optimization, the best conditions were set with a nebulizer pressure of 30 psi, drying gas temperature of 250 °C, capillary voltage of 3 kV and a fragmentor voltage of 70 V under the positive ion mode. After identification of the individual molecular ions using the scan mode, which gave excellent mass accuracy, the MS was set on selected-ion monitoring (SIM) mode using target ions at m/z $[M+H]^+$ 130.1 for metformin, m/z $[M+H]^+$ 304.2 for vildagliptin, m/z $[M+H]^+$ 340.2 for alogliptin, m/z $[M+H]^+$ 408.1 for sitagliptin and m/z $[M+H]^+$ 473.2 for linagliptin. The method was linear over the concentration range of 0.039-20 μg/mL for metformin and 0.19-100 μg/mL for the others. The correlation square of the linear regressions were better than 0.99 for the five quantitated compounds. RSDs % of peak areas and retention times were less than 0.028 % and 2.5 %, respectively. This precise method would be further tested for stability and bioanalysis investigations.

# Bestimmung der Beladungsgeschwindigkeit einer Trimyristin-Nanoemulsion mittels dynamischer Differenzkalorimetrie

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Die schlechte Wasserlöslichkeit vieler neuer Arzneistoffe kann durch ihre Auflösung in Nanopartikeln aus Lipiden überwunden werden. Um aus der Vielzahl an verfügbaren Lipidnanopartikeln für einen gegebenen Arzneistoff schnell und materialsparend die richtige Zubereitung auszuwählen, wurde das Screeningverfahren mittels passiver Beladung entwickelt [1]: Verschiedene Nanopartikel werden mit Arzneistoff im Überschuss inkubiert, bevor nach einer ausreichend langen Beladungszeit ungelöster Arzneistoff abgetrennt wird. Abschließend wird die Menge Arzneistoff bestimmt, die sich während der Inkubation in den Nanopartikeln gelöst hat. Vorhergegangene Untersuchungen haben gezeigt, dass die Geschwindigkeit der passiven Beladung in verschiedenen Modellsystemen sehr unterschiedlich ist und einige Arzneistoffe überhaupt keine passive Beladung zeigen, obwohl sie eine hohe Löslichkeit in den Lipidpartikeln aufweisen. Ziel ist es daher, die Einfluss-faktoren auf die Geschwindigkeit der passiven Beladung zu ermitteln und den zu Grunde liegenden Mechanismus zu verstehen. Dazu muss zunächst eine effiziente und genaue Methode zur Bestimmung der Beladungsgeschwindigkeit entwickelt werden.

Konventionell wird zur Ermittlung der Beladungsgeschwindigkeit zu definierten Zeitpunkten eine Probe der Nanodispersion durch Filtration vom ungelösten Arzneistoff getrennt und der Arzneistoffgehalt in der filtrierten Nanodispersion dann z.B. photometrisch bestimmt. Nachteile dieser Methode sind das benötigte Probenvolumen von 1 ml und die aufwändige Probenpräparation. Als material- und zeitsparende Alternative für Nanopartikel aus dem Triglycerid Trimyristin (TM) wurde daher die Kinetikuntersuchung mittels dynamischer Differenzkalorimetrie entwickelt: TM zeigt im nanopartikulären Zustand eine so starke Unterkühlung, dass es bei Raumtemperatur flüssig vorliegt und erst unterhalb der Raumtemperatur auskristallisiert. Die Kristallisationstemperatur der TM-Nanoemulsion verhält sich dabei proportional zur Menge an Arzneistoff, die sich in den Nanopartikeln befindet [2]. Da nur der in den TM-partikeln gelöste Arzneistoff die Kristallisations-temperatur absenkt, ist das Abfiltrieren von ungelöstem Arzneistoff nicht nötig und 20 µL Probe reichen aus. Mit Hilfe dieser neuen Methode wurden verschiedene Einflussgrößen getestet und die Arzneistoffoberfläche als ein entscheidender Parameter identifiziert.

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# Micro manufacturing for Organs-on-Chips

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The realization of organ-on-chip systems creates novel requirements for material selection and tailored structuring processes in the field of microtechnology. Due to the wide variety of different organoid cultures used by various cooperating research groups, different constraints are imposed on the manufacturing process of each new system. The requirements led to the development of two different fabrication routes, requiring microtechnology: femtosecond-laser-ablation and precision machining. These methods have led to the production of novel, one of a kind systems. Here we present both of them aimed at a) the cultivation of islets of Langerhans of the pancreas, b) epithelial and stromal cells of the human cornea.

a) Within the PVZ (Zentrum für Pharmaverfahrenstechnik), a research cooperation between the Institute of Pharmacology, Toxicology and Clinical Pharmacy and the Institute of Microtechnology was initiated. As a result of this collaboration, the concept of a system which permits the culturing of Langerhans cell islets and the measurement of their oxygen consumption was developed. These cell cultures are sensitive to shear stress caused by circulating nutrient solutions inside the system. To protect them against this environment, small wells of different depths were part of the micro fluidic design for first prototypes. The wide variety in the depths of the micro wells, which need to be milled into the material, pose a challenge to conventional micro production methods. We addressed this challenge by employing laser ablation using a femtosecond laser in conjunction with a subsequent bonding of the two system halves. Besides cell compatibility and transparency, a low permeability to oxygen is required for this system. The material of choice therefore was BOROFLOAT® produced by SCHOTT AG.

b) In cooperation with the Institute of Pharmaceutical Technology (TU Braunschweig), we developed a modular fluidic system suitable for the cultivation of three-dimensional, barrier-forming cell structures. It bears the name Dynamic Micro Tissue Engineering System (DynaMiTES). The focus of DynaMiTES is the optimal cultivation of various cell types in a dynamic micro fluidic environment. Its modular concept allows exchanging individual components designed for the highly variable demands of different cell cultures. Furthermore, DynaMiTES provides integrated electrodes for online TEER (Trans Epithelial Electrical Resistance) measurements to validate the barrier-forming properties of the cells. It is designed to be used in a laboratory environment and does not require the use of additional equipment. These features are expected to increase the acceptance of DynaMiTES in many research labs and laboratories of the pharmaceutical industry. To allow transfer from a prototype to mass production, polycarbonate was chosen as the main material. The shaping process can therefore easily be changed from current precision machining to micro injection molding.

With the availability of different fabrication routes, such as precision engineering, laser micro machining and photolithographic multilevel structuring, and with the different materials like glass, PDMS and polycarbonate, much tailored microfluidic systems could be successfully realized. These systems are optimized for specific cell types with divergent environmental requirements and already allow a taste of the variety of options microtechnology can offer for further organ-on-chip research.

First cell cultivation tests were already carried out by the cooperation partners for all systems described above. The results prove that the cells cultivated inside the DynaMiTES show no signs of lower cell viability. In addition the first TEER-value trends under fluidic flow could be recorded and show the known drop to the change from cell culture medium to buffer solution.

The microfluidic system for the cultivation of islets of Langerhans of the pancreas allowed first measurement results of the autoflorescence of nicotinamide adenine dinucleotide phosphate. This allows the direct insight in the cell metabolism of single cell islets.

While these systems are still in development they already indicate the possibilities resulting from cooperations of skilled and motivated people from different fields of research.

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# Combining SAX and CGE for 2D protein analysis

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The analysis of complex protein-mixtures is challenging when using just one dimension. Therefore, the following two techniques were combined, HPLC with a strong anion exchanger (SAX) column and capillary gel electrophoresis (CGE). Both are used for protein analysis on their own. The characterisation of biotechnological products such as protein based biopharmaceuticals could be a task for this approach. Classical two dimensional gel electrophoresis (2D-GE) is often used to achieve a separation of such samples.

The combination of SAX and CGE was chosen to achieve comparable results. In the first step the proteins were separated according to their charge at a certain pH using SAX-HPLC. The separation of the second dimension is based on the molecular size. This was performed by using CGE. These two steps are very similar to 2D-GE, where isoelectric focusing is combined with SDS-PAGE.

A mixture of five proteins was used to determine the suitability and precision of this approach. These proteins were namely Myoglobin, β-Lactoglobulin, Ovalbumin, bovine serum albumin and a monoclonal antibody. The first dimension is conducted at pH 8.5 with gradient elution. The sodium chloride concentration of the eluent was increased from 0.0 M to 0.75 M over 40 min. 20 fractions were collected. As second dimension CGE was used with a SDS-gel buffer.

The goal is to provide a faster two dimensional separation with a higher precision than classic 2D-GE.

*We thank Polymicro™ for providing the capillaries.*

# Handling small sample volume for capillary electrophoresis investigations

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Capillary electrophoresis is an appropriate technique for analysing pharmaceuticals and biopharmaceuticals [1-2] for which using small volume samples is advantageous.
There are numbers of benefits involved in handling small sample volume in capillary electrophoresis, among which using less material, generating less waste, and applying high concentrations of valuable samples which are all very important from an economic perspective stand out [3-4].
Most of the conventional CE instruments need approximately 50 µL of the sample in the injection vial to perform the analysis, even though the injected sample volume in capillary electrophoresis is only in the nanoliter range.
Hence, in order to fully profit from the low injection volumes, smaller vial volumes are required.
Furthermore the immersion depth of the capillary of most CE instruments is not properly adjustable. Even if it is, one cannot ensure that the capillary does not break at the end or at the detection window if it comes against the bottom of the vial in lower settings. Therefore it is helpful to fill the dead volume of the sample vials, which is inaccessible for the capillary with a chemically inert liquid.
Thus experiments were performed using silicone oil as a filler of the vial dead volume and the results were compared to those performed without this filling.
As study example five standard proteins namely beta-lactoglobulin, BSA, HSA, Myoglobin and Ovalbumin, and one of the coagulation cascade involved proteins called vitonectin were investigated using capillary electrophoresis. The experiments were performed once without the addition of silicone oil in the sample vial and once in the presence of silicone oil in the sample vial. The equation of migration times of EOF-marker and proteins (mobility ratios) as well as peak areas were compared. However no significant changes were observed (RSDs% for mobility ratios and peak areas were better than 0.9% and 5.8% respectively).
Afterwards, an affinity capillary electrophoresis method was used to investigate the interactions of two proteins, namely HSA and vitronectin, with three ligands namely enoxaparin sodium, unfractionated heparin and pentosan polysulfate sodium (PPS). Mobility shift precision results over 12 hours analysis showed that the employment of the filling has no noticeable effect on any of the protein-ligand interactions. Accordingly, the employed silicone oil is suitable as a water immiscible and inert liquid for filling the dead volume of sample vials. Using a commercial instrument and an autosampler the required sample volume is reduced down to 10 µL, and almost this complete volume can be subsequently injected during repeated experiments.

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# Improved liquid antibody formulations by combination of in silico and analytical methods

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Biopharmaceuticals, especially monoclonal antibodies, are essential in the treatment of various diseases such as diabetes and cancer. At present, more than 35 antibodies are approved in the European Union and many more are in the pipeline [1, 2]. The development of new antibody preparation is limited by their stability during storage. The subsequent occurrence of aggregates in antibody formulation can cause severe adverse effects and therefore needs to be carefully monitored [3]. In order to reduce aggregation, many different excipients have been applied. Despite the high structural homology of antibodies and the large number of possibly stabilizing excipients, the mechanisms of stabilization are still not sufficiently understood. A rational strategy to improve antibody stability is needed.

In this study computer models have been used to investigate the interactions of excipients and antibodies. Some of the results suggest possibly better formulations. These will be tested in stability studies with regard to oligomerization and fragmentation using size exclusion chromatography, capillary gel electrophoresis and capillary isoelectric focusing. First results from in silico screenings showed a decrease in the calculated aggregation propensity for excipients such as L-proline and L-histidine. These screenings also give scores to rank excipients according to their suitability for a certain antibody.

Using these in silico studies in combination with analytical experiments, the right combination of excipients for future drug developments can be selected in a more rational manner. In silico works will reduce the number of necessary experiments, and analytical results can refine the models at the same time. The development of formulations will be shortened and its cost efficacy increased.

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# Untersuchung des Wirkstofftransfers in Lipidnanopartikeln – Entwicklung einer neuen Methode mittels dynamischer Differenzkalorimetrie

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Lipidnanopartikel werden schon länger als kolloidale *drug delivery*-Systeme eingesetzt, um neue, meist schwer wasserlösliche Wirkstoffe zu formulieren. Aufgrund ihrer geringen Größe können diese Partikel auch in die Blutbahn appliziert werden. Um die Eignung eines solchen Systems einschätzen zu können, sind Untersuchungen zum Wirkstofffreisetzungs-verhalten wichtig. Aufgrund der geringen Teilchengrößen kann die Durchführung solcher Freisetzungsuntersuchungen bei kolloidalen Partikeln jedoch schwierig sein, da eine Abtrennung der entsprechenden Partikel erschwert ist. Als vielversprechende Methode hat sich die dynamische Differenzkalorimetrie erwiesen: Nanopartikel aus dem Triglycerid Trimyristin (TM) zeigen im nanopartikulären Zustand eine starke Unterkühlung, wodurch die Partikel nach der Herstellung flüssig vorliegen und erst unterhalb der Raumtemperatur auskristallisieren. In wirkstoffhaltigen Triglycerid-Partikeln verhält sich die Kristallisations-temperatur proportional zur enthaltenen Wirkstoffmenge: mit steigendem Wirkstoffgehalt sinkt die Kristallisations-temperatur [2]. Auf dieser Grundlage wurde eine neue Methode für Wirkstofftransferuntersuchungen mittels dynamischer Differenz-kalorimetrie entwickelt. Dabei wurde davon ausgegangen, dass es bei einer Freisetzung des Wirkstoffes zu einem Anstieg der Kristallisationstemperatur kommen müsste. Für entsprechende Untersuchungen wurden TM-Nanopartikel hergestellt, mit einem schwer wasserlöslichen Arzneistoff passiv beladen und anschließend mit einem Freisetzungsmedium gemischt. Als Freisetzungs-medium wurde kein rein wässriges System eingesetzt, um die Bedingungen möglichst realitätsnah zu gestalten. Stattdessen wurde eine Rapsölemulsion verwendet, deren Tropfen als Akzeptor für den freigesetzten Wirkstoff fungieren sollen, ähnlich wie Lipoproteine oder zelluläre Bestandteile im Blut. Rapsöl kristallisiert unter den gewählten Messbedingungen nicht aus, so dass es die Auswertung der Kristallisation der TM-Partikel nicht beeinflusst. Durch die Betrachtung der Kristallisations-temperatur der TM-Partikel über die Zeit konnte ein schlagartiger Transfer von Fenofibrat aus den Trimyristin-Partikeln hin zu den Rapsölpartikeln gezeigt werden, ohne vorherige Trennung der beiden Partikeltypen.

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# Comparison of two binding kinetic assays in long term studies for performance qualification using Biacore X100

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Surface Plasmon Resonance (SPR) is a dominant tool for biomolecular interaction characterization. This technique facilitates label-free binding analysis studies of biomolecules such as affinity, kinetic, thermodynamics and specificity analysis in real-time. Therefore SPR is an important application in drug discovery and proteomics.
In this study the model system for measuring binding kinetics was $\beta_2$ microglobulin from human urine and the antibody anti-$\beta_2$ microglobulin produced in mouse. The antibody was covalently bound by amine coupling on the surface of the gold chip and the antigen was flowed over the chip.
Two different assay designs were implemented to determine kinetic rate constants ($k_d$, $k_a$), dissociation constants ($K_D$), residuals from the experimental to the optimal fitted curve (RU) and the maximal Response Units ($R_{max}$) as an indicator for ageing of the chip/antibody.
The first method was a titration series called Single Cycle Kinetic (SCK) where subsequently five different concentrations of $\beta_2$ microglobulin (2, 4, 8, 16 and 32 nM) were injected. One regeneration step followed subsequently to unhitch the $\beta_2$ microglobulin from the antibody. The second method was a Multi Cycle Kinetic (MCK) where all concentrations were injected in their own cycle with two regeneration steps after each. As a further variation the randomization of the injection sequence was evaluated in MCK. The dilution series was prepared freshly every day with a HEPES-buffer pH 7.4 and the regeneration solution was glycine-HCl 10 mM pH 2.5.
The investigation of this antibody/antigen system by using both SCK and MCK methods reveals that the parameter that represents best the aging of the chip was $R_{max}$. In SCK the $R_{max}$ was decreasing slightly after 20 runs without affecting the quality of the other kinetic parameters. Interestingly in contrast to SCK in MCK $R_{max}$ was stable over 30 cycles although there are five times more regeneration steps for one data set than in SCK. That implies that the ageing of the chip is not only caused by the harsh regeneration steps but mainly by the time period it is used. The most stable parameter measured was the $k_d$ with a relative percental standard deviation (RSD %) of 2.5 to 6.2 % over a minimum of 30 measurements. In contrast the $k_a$ was more unstable within the range of 6.5 to 30 %.

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# Quartz-crystal-microbalances for protein detection

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Commercially available sandwich ELISA assays allow high sensitivity quantification of serum proteins. Here we present a quartz-crystal-microbalance (QCM) biosensor which uses antigen-antibody interactions to measure immune interactions directly. This method provides measurement results within minutes because it is only limited by the kinetics of the immune interaction. No time consuming incubation periods are necessary, automation is possible and it can be integrated into lab-on-chip devices. We were also able to show its reusability. Compared to ELISA, less analyte and detection chemicals are needed [1].

The presented method uses microfabricated QCM mass sensors and is able to measure protein concentrations down to a few micrograms per milliliter sample. The clinically important inflammation marker C-reactive protein (CRP) was used as reference substance. A detection sandwich formed by two especially engineered CRP-specific antibodies is the core element of this detection method. The first detection antibody is immobilized by a self-assembled-monolayer (SAM) onto the gold electrode of the QCM. After blocking of the remaining bare electrode surface, the antigen (CRP) can be added, and then the second CRP-specific antibody is injected. Its attachment leads to a significant and very specific increase of the signal because of an increase of the resonant mass of the sensor. The recombinant antibodies were generated by phage display [2]. Their biochemical properties were engineered by an iterative process to match the requirements of the mass sensor. The sensitivity is high enough to visualize all process steps that take place on the QCM in real time.

For diabetes diagnostics, the serum concentration of c-peptide and proinsulin in addition to the concentration of insulin are of interest. Beside their absolute quantities, their time dependent variation is also relevant. Therefore we present a concept to enhance the sensitivity of our QCM array by applying bio-functionalized, superparamagnetic nanoparticles [3]. The aim is to expand the limit of detection to the range of single digit nanograms per milliliter and further to develop a 3-parametric point of care device for diabetes diagnostics.

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# Development of microtechnology based rapid tests for the diagnosis of Buruli ulcer and tuberculosis

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Tuberculosis is an infectious disease caused by the bacterium *Mycobacterium tuberculosis*. The disease is a major source of global mortality. The tremendous ongoing burden of tuberculosis is mainly due to 1. the lack of reliable and fast diagnosis tools, 2. inefficient drugs, and 3. ineffective vaccine. Similarly, Buruli ulcer caused by *Mycobacterium ulcerans* is an emerging diseases with high prevalence in several African countries. Novel diagnostic tests are badly needed for these two diseases, especially for the low-resource endemic countries in Africa and Asia. Several considerations must be taken into account for the development of cheap but efficient rapid tests for Buruli and tuberculosis. Next to outstanding accuracy, the test must be adapted to social and economic conditions of the area of application. LIONEX has been highly involved in research in this area fort he last 15 years and has participated in a number of projects supported by the European Union (POCKET, IP4-Plasma, RAPPID, DEMO-NOPERSIST) and the BMWi/BMBF . All these projects use modern technologies for the development of rapid tests. Our emphasis is on developing non-invasive, point-of-care diagnostic tests for buruli and tuberculosis. Few examples shall be presented.

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