New trends of individualised medicine at the Center of Pharmaceutical Engineering

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Ensuring and improving medical care with simultaneous establishment of cost-effective therapies are major global challenges. An efficient development based on new pharmaceutical and procedural methods and the provision of low-cost and effective medicines are prerequisites for meeting these needs. The new methods and processes to be developed are the key to future personalized therapy with individualized medicinical products including individualized compositions of medicines which will result in significantly improved therapeutic results in the treatment of diseases like cancer.

Promising new drug candidates commonly exhibit poor solubility, low membrane permeability and a delicate structure. Important objectives in the development and manufacturing of future products are therefore:

(1) providing customized, gentle and cost-effective manufacturing processes and layout methods at different scales as well as corresponding scale-up methods as essential basis for the production of cost-effective medicines,
(2) efficient formulations and related processes as well as analytical methods for poorly soluble drugs and delicate biopharmaceuticals for development and production of efficient medicines,
(3) the design of miniaturized production equipment including analytical and quality control methods for medicinal products in small scale for individualized medicines.

The Center of Pharmaceutical Engineering PVZ complements the nationally unique combination of university and non-university research institutions in Lower Saxony, integrated in the biomedical Translational Alliance in Lower Saxony (TRAIN) that mainly works in the fields of systems biology, biotechnology, drug development and infection research.

By developing cost-effective manufacturing processes and tailor-made formulations of active pharmaceutical ingredients (API) the PVZ bridges the gap between the identification and characterization of the API on the one hand and the subsequent GMP manufacturing (Good Manufacturing Practice), testing and application of the drug on the other hand. Thus, the value-added chain for the development and exploration of cost-effective, efficient and personalized medicine is completed by the PVZ in a unique way.

In the paper examples of strategies to design, characterize and produce drugs for an individualised medicine are presented. One possibility is the individual printing of orodispersible films. Another way of producing small quantities of drugs is the use of microsystems, for example for the production of individualised drug carries as lipid nanoparticles.

Mikrosysteme in Analytik und Produktion – Micro systems in analysis and production

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Micro technologies offer many advantages over conventional systems in particular in the field of health care applications. Microsystems for fluid manipulation are small, light and cheap. Their dimensions offer cells more natural environments, intensify heat and mass transfer and have almost perfect control of local energy input. Such systems also known as Lab-on-a-chip systems (LOC), bio-microelectromechanical systems (BioMEMS) or miniaturized total analysis systems (μTAS) allow us bridging the gap between volumes which are familiar in classical laboratories or pilot factories and the micro-scale volumes common in biology. They can be the key to automated drug formulation, fast screening, nanoparticle precipitation, organ-on-chip and many other applications [1]. Two examples for the realization of these microsystems and their functioning will be presented.

The precipitation of nanoparticles is an important process for the formulation of drugs in order to improve their bioavailability. Precipitation can be controlled in small fluidic micro compartments like droplets or fluid plugs in which the concentration gradients are equilibrated extremely rapidly. The physical process of microfluidic droplet or plug formation and the resulting sizes of the dispensed droplets are determined by the local capillary forces and the ratio between sample flow and flow of gases or liquids in which compartments are produced. The balance of the viscous forces and interfacial tension force defines the droplet and plug sizes at the moment they separate. First results on nanoparticle formation shall be discussed.

For analyzing the absorption of drugs through barrier forming cell tissues under dynamic conditions a novel microfluidic cell culture model was designed. Three-dimensional cell tissue structures cultivated on standard inserts can be integrated in these and be exposed to dynamic micro flow conditions in which two fluid compartments are separated by the barrier forming tissue structure. The dilution rate of the tested drug inside the donor compartment can be adjusted in a dynamic flow, while taking samples out of the acceptor compartment at any given time. To evaluate barrier formation and to compare measured drug absorption under dynamic flow conditions, online monitoring of the TEER-values (transepithelial electrical resistance) of inserted cell tissue barriers is performed. The functionality of this new organ-on-chip test platform which can easily be adapted to mimic other cellular barriers for drug absorption such as the human nasal mucous membrane, the blood-brain and blood-retina barrier could already be demonstrated.

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In vivo performance of Nano-Drug Delivery Systems – how much control do we have?

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Nanoscaled Drug Delivery Systems (Nano-DDS) were and are in the focus of industrial and academic research. Their applications range from basic research to clinical use. Nano-DDS include different sizes, shapes and charges. They cover a very broad range of different materials with liquid, solid and liquid-crystalline properties. In many applications, a specific and controlled release is desired, which means to control the location, the time and the amount of the drug which is released. Although controlled release from Nano-DDS has been claimed for many systems, the goal remains still very challenging. Drug diffusion from the core of the Nano-DDS to the interface will be fast even for small diffusion coefficients due to the very small distance. Therefore, diffusion controlled release is - in contrast to microparticles - difficult or almost impossible to achieve. Biodistribution strongly depends on the size, shape, surface charge and flexibility of the carrier. Although PEG-PLGA did accumulate in tumour tissue in preclinical models, they also showed a rather high accumulation in liver and spleen [1], which was also seen in healthy animals [2]. The desired accumulation of Nano-DDS in tumour tissue was better with stimulus-sensitive HPMA-conjugates based on pH- [3] or redox- [4] activated drug release. Moreover, recent data show that chemotherapy resistance can be overcome by stimulus sensitive HPMA conjugates [5]. Surprisingly, a high ovarian accumulation of PLGA-nanoparticles was observed [6]. Ovarian and adrenal accumulation of multiple labelled lipid Nano-DDS has also been described [7-8], which show that the unintended biodistribution of nanomaterials in small, but important organs might not be an artefact, but rather a common phenomenon.

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Thermoanalytische Charakterisierung von Triglycerid-Nanodispersionen

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Wässrige kolloidale Dispersionen von Triglyceriden können als Trägermedien für schwer wasserlösliche, lipophile Arzneistoffe genutzt werden, um diese für die Anwendung am Patienten, z.B. mittels Injektion, verfügbar zu machen. Für Trägerpartikel mit Phasenübergängen zwischen etwa 0 und 90°C ist die DSC ein häufig verwendetes Hilfsmittel, um die Partikel zu charakterisieren und die gewünschten Eigenschaften (v.a. Aggregatzustand, Kristallmodifikation) sicherzustellen [1]. Über solche Routineanwendungen hinaus kann die DSC in bestimmten Fällen auch dazu verwendet werden, weitere Informationen über die Partikel und ihr Verhalten in pharmazeutisch bedeutsamen Prozessen zu gewinnen. Ein Beispiel ist die Nutzung des partikelgrößenabhängigen Schmelzverhaltens feiner Triglycerid-Partikel, um Veränderungen der Partikelgrößenverteilung z.B. bei einer Hitzebehandlung zu erkennen [2]. Im Gegensatz zu üblichen Partikelgrößenmessverfahren erlaubt die DSC auch noch nach dem Einschluss der Partikel in eine Gelmatrix eine Aussage über die Partikelgrößenverteilung der Trägerpartikel. Einflüsse des Einschlussprozesses auf die Integrität der Partikel können auf diese Weise sichtbar gemacht werden [3]. Die Interaktion der Triglycerid-Partikel mit Arzneistoffen führt zu einer Veränderung ihres Schmelz- und Kristallisationsverhaltens. Die konzentrationsabhängige Reduktion der Kristallisations-temperatur kann z.B. dazu genutzt werden, die Aufnahme bzw. Abgabe von Arzneistoffen im Rahmen der Herstellung oder im Hinblick auf die Anwendung der Wirkstoffträger zeitaufgelöst zu charakterisieren.

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Flow field-flow fractionation for the characterization of lipid-based colloidal formulations

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Among the large family of field-flow fractionation techniques, asymmetrical flow field-flow fractionation (A4F) is one of the most commonly used technique due to its high versatility [1-2]. In this technique, the sample is fractionated depending on hydrodynamic size by a cross flow applied perpendicularly to the main flow in a separation channel. Comparatively small molecules up to particles with a diameter of about 1 µm can be analyzed whereas the lower limit is determined by the molecular weight cut off of the membrane (i.e. 5 kDa) used as accumulation wall in the separation channel. When the fractionation system is coupled with multi-angle laser light scattering (MALLS), information about the molar mass and size of the colloids at each elution time is obtained, thus allowing accurate determination of molar mass and size distributions of even heterogeneous samples [3-4]. In addition, sample fractions with high size homogeneity can be obtained by collecting the eluting sample.

After short introduction of the A4F principle and size determination by MALLS, results of selected recent and on-going projects on lipid-based colloidal formulations will be presented. The first example will explore the potential of AF4 to study on drug release and transfer form liposomes [5] and the second example is directed on investigations on structural changes in poloxamer-stabilized nanoemulsions induced by autoclaving [6].

These results will illustrate the broad range of applications of A4F/MALLS for the characterization of lipid-based colloidal formulations together with the high reproducibility of these measurements but also disclose some difficulties and limitations.

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Application of Tailored Magnetic Nanoparticles for Protein Purification

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Magnetic nanoparticles have become highly promising in diverse fields of biomedicine, in particular for *in vivo* applications such as hyperthermia cancer therapy or magnetoassisted drug targeting. However, they are also attractive for a diversity of *in vitro* applications in diagnostics and downstream processing due to their highly selective manipulation possible by applying external magnetic fields. For all of these applications, homogeneity of the particle collective is of crucial importance, allowing a uniform response and defined chemical and physical characteristics. In addition to defined particle properties such as size, composition and crystallinity, the surface chemistry must be tailored and optimized for the desired application to both ensure stability of the particles against agglomeration in the desired medium as well as the desired interaction with the biological system.

We have established a multi-step process for the fabrication of biofunctionalized iron oxide nanoparticles, and studied their application for the purification of recombinant model proteins. The non-aqueous synthesis of iron oxide nanoparticles in different media was thoroughly studied [1-4] to gain an understanding of particle formation mechanisms. Additionally, this synthesis allows to tailor particle properties, enabling more defined particle characteristics than for the classical precipitation synthesis. Subsequently, the surface chemistry of the particles was optimized by modification with a tailored ligand system consisting of a silane terminus for covalent linkage to the particle surface as well as a nitrilotri(acetic acid) terminus that is capable of forming stable metal complexes with histidine (His), which is a standard strategy for the purification or recombinant proteins [5]. The successful modification of the nanoparticles resulted in colloidal stability in aqueous biological media and could be systematically varied [6].

Subsequently, extensive purification experiments were performed. His_6-tagged Protein A as well as ABF D1.3 scFv antibody fragment also fused to a His_6-tag recombinantly secreted by *Bacillus megaterium* served as the model proteins that should be purified from the growth medium. The protein binding capacity and separation efficiency of the nanoparticles were measured in a number of consecutive cycles of cultivation. Analysis was performed using ELISA (protein activity), SDS-PAGE (protein quantity), gravimetry and dynamic light scattering. Thereby, *in situ* purification was studied by adding the particles directly to the cultivation broth of *B. megaterium* in shaking flasks. By establishing a complex cycle of application and regeneration, we could successfully utilize the nanoparticles for separation of the recombinant model proteins using handheld magnets, achieving separation efficiencies of > 80 % with purities exceeding 99.5 % regarding protein components over five consecutive cycles. The presented approach thus allows facile and highly efficient purification of recombinant proteins and appears promising also for large-scale applications.

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In-vitro Biotesting of Nanoparticles using Mammalian Cells

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Nanoparticles are widely employed for many applications and the number of consumer products, incor-porating nanotechnology, is constantly increasing. A novel area of nanotechnology is the application inmedical implants. The widespread use of nanoparticles leads to their higher prevalence in our environ-ment. This, in turn, raises concerns regarding potential risks to humans. Previous studies have shownpossible hazardous effects of some nanoparticles on mammalian cells grown in two-dimensional (2D)cultures. However, 2D in vitro cell cultures display several disadvantages such as changes in cell shape,cell function, cell responses and lack of cell-cell contacts. For this reason, the development of bettermodels for mimicking in vivo conditions is essential.In the present work, we cultivated A549 cells and NIH-3T3 cells in three-dimensional (3D) spheroidsand investigated the effects of zinc oxide (ZnO-NP) and titanium dioxide nanoparticles (TiO2-NP). Theresults were compared to cultivation in 2D monolayer culture. A549 cells in 3D cell culture formed looseaggregates which were more sensitive to the toxicity of ZnO-NP in comparison to cells grown in 2Dmonolayers. In contrast, NIH-3T3 cells showed a compact 3D spheroid structure and no differences inthe sensitivity of the NIH-3T3 cells to ZnO-NP were observed between 2D and 3D cultures. TiO2-NP werenon-toxic in 2D cultures but affected cell-cell interaction during 3D spheroid formation of A549 andNIH-3T3 cells. When TiO2-NP were directly added during spheroid formation in the cultures of the twocell lines tested, several smaller spheroids were formed instead of a single spheroid. This effect was notobserved if the nanoparticles were added after spheroid formation. In this case, a slight decrease in cellviability was determined only for A549 3D spheroids. The obtained results demonstrate the importanceof 3D cell culture studies for nanoparticle safety testing, since some effects cannot be revealed in 2D cellculture.

Part of this research work was performed in cooperation with Prof. Kwade and Dr. Hesselbach from iPAT, TU Braunschweig]

Microfluidic technologies for nanoparticle characterization and microscopy

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Micro- and nanofluidic technologies provide exciting opportunities for the characterization of nanoparticles and biomolecular complexes.

In this talk, I will describe a new method for the measurement of mass and size of nanoparticles in solution by fluctuation analysis using microfluidic detectors. By using a unique class of nanomechanical mass sensors as the detector, it is possible to characterize the change in mean mass of nanoparticles with a precision at the 100 zg level (1 zg = 10^{-21} g). As an example of an application, we have used this technique to monitor the formation of insulin amyloids from monomers to mature fibrils. There are many interesting possibilities to generalize this technique to multimodal measurements using different types of flow-through detectors in micro- and nanofluidic systems. For example, combinations of mass sensitive detection with fluorescence and light scattering may provide higher sensitivity and yield more detailed information about the composition and size of specific nanoparticles; I will discuss some of these directions in the presentation.

The second part of my talk will focus on work of our group towards connecting light and electron microscopy (EM) through a new mode of cryofixation based on microfluidics. Cryofixation is widely regarded as the gold standard in stabilizing biological samples for ultramicroscopy. We recently introduced a new concept for the cryofixation of cells directly in the light microscope with millisecond time resolution. Formation of crystalline ice is suppressed by the high cooling rate (~10^4 K/s) that can be achieved in a microfluidic system that is optimized for rapid heat transfer and low thermal mass. We expect that this method will open new avenues in the study of dynamic cellular events, such as intracellular transport, membrane trafficking, cell division, or endocytosis by correlating live stimulation, light microscopy, and EM. We also envision there will be numerous interesting applications of this new technique for studying the dynamic morphology and the cellular uptake of nanoscale drug delivery systems using a combination of light microscopy, EM, and X-ray tomography.

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Microfluidics for research on pancreatic islets

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The stimulation of insulin secretion by high glucose requires the metabolic breakdown of glucose in the pancreatic beta cell. The activated mitochondrial metabolism leads to the closure of K_{ATP} channels in the plasma membrane and the resulting depolarization leads to Ca^{2+} influx via voltage-dependent Ca^{2+} channels and to Ca^{2+}-triggered exocytosis of the insulin-containing granules. This model, however is insufficient to explain many features of insulin secretion and its deficiencies in type 2 diabetes. It is generally acknowledged that an additional pathway exists, termed “amplifying pathway” which enhances the depolarization-induced granule exocytosis and which may be of critical importance for the extent of the secretory response to a glucose stimulus. There is experimental evidence that this pathway also emerges from the beta cell mitochondria.

The beta cell is contained within a multicellular aggregate of several endocrine cells termed “Islet of Langerhans” or pancreatic islet. One pancreatic islet can be regarded as the functionally competent micro-organ with regard to signal transduction and hormone secretion. However, the isolation of pancreatic islets from the exocrine tissue is time-consuming and yields only small amount of tissue. So, it is imperative to extract as much of information as possible from one single islet. This is why efforts have been made to design microfluidic devices specifically for research on pancreatic islets. For the research on the “amplifying pathway” the properties of such a device should permit the optical measurement of parameters of mitochondrial metabolism in combination with the measurement of oxygen consumption and carbon dioxide production. A device entirely made from glass appears to best meet these requirements. Preliminary data obtained with such a microfluidic chip are presented.

“Type 2 Diabetes understanding at single cell level utilizing "Airborne Chemistry" and Surface Acoustic Wave-Mass Spectrometry"

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The development of nanoparticle-based capillary electrochromatography e (CEC) in our lab will be reviewed. The nanoparticles are suspended in the electrolyte for use as pseudostationary phase (PSP) in CEC [1-6]. In PSP-CEC, the stationary phase is used only once allowing fast column regeneration and circumventing carry-over effects. Nanoparticles possess a favorable surface-to-volume ratio allowing highly efficient separations

New insights in biomedicine and related areas require the parallel development of new analytical methods. Levitation technique is one such method that was developed by us to study intra- and extra- cellular reactions at single or few cell levevel. Created wall-less test tube technique makes it possible to handle small volumes without any adsorption to walls or contamination of container. Such microenvironments are suited for specific cell types to serve as biomimetic systems.

Drops (100-500nl) containing cell/cells are trapped in an ultrasonic field. Flow-through droplet dispensers are used to add cells and stimulators to the drop to follow the cell reaction7.

The described technique can be used to determine insulin resistance in connection with Type 2 diabetes and obesity[8]. After exposure of the cells to drugs, activators or inhibitors, the cell response (or lack of response) is monitored using fluorescence imaging detection or other non-invasive detection methods8.

The airborne system combined with MS can acquire data on single Langerhans islet and further on β-cell metabolism, at the single/few cells level, associated with stimulation using acetylcholine and increasing extra-cellular glucose concentration. Most results obtained were in good agreement with known metabolism of islets and β-cells. In response to both acetylcholine and elevated glucose concentration, rapid insulin release was observed, together with other compounds, such as C-peptide, amylin, somatostatin, glucagon[9]. The achieved low attomole detection limit shows the potential of the method.

Recently another method has been used to interface cell samples with MS, where we are delivering aerosols of sample to MALDI plates, by making use of Surface Acoustic Waves (SAW)[10] SAW has shown several advantages such as low protein degradation, high viability of cells (when used), cost and time effective, minimized sample volumes.

Small volumes of sample for analysis are applied on a paper, propagating acoustic waves causes formation of aerosols from the liquid, while cell clusters are entrapped in paper fibers easing the way of the analysis.

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Zellkulturmodelle epithelialer Barrieren für die präklinische Arzneimittelentwicklung

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In der präklinischen Entwicklung von Arzneimitteln sind pharmakokinetische Untersuchungen, insbesondere zur Wirkstoffresorption, unerlässlich. In der Regel werden solche Studien am Versuchstier oder an exzidierten Geweben durchgeführt. Dies ist mit einer Reihe von Nachteilen verbunden, wie einer schlechten Standardisierbarkeit der Tierexperimente, dem Töten der Tiere für experimentelle Zwecke, den hohen Kosten und den Schwierigkeiten bei der Übertragbarkeit der Ergebnisse auf die humane Situation. Seit mehr als zwei Jahrzehnten werden daher zunehmend Zellkulturmodelle epithelialer Barrieren wie Darm-, Lungen- oder Hautgewebe entwickelt und deren Verwendung als Alternative zum Tierversuch evaluiert.

Einfache, zweidimensionale Zellkulturmodelle sind in der Regel nicht in der Lage, die komplexen physiologischen In-vivo-Verhältnisse der Absorptionsbarrieren abzubilden. Erst durch die Co-Kultivierung von Zellsystemen und die Entwicklung dreidimensionaler Gewebekonstrukte wurden Modelle erhalten, die valide Ergebnisse in Arzneistoffabsorptionsuntersuchungen liefern. Mit der Einführung mikrofluidischer Systeme konnten zudem Kulturbedingungen realisiert werden, die angepasst an die Mikroumgebung des Ursprungsgewebes einen höheren Grad an organotypischen Eigenschaften der Absorptionsbarrieren erzielen. Dies führte in jüngster Vergangenheit zur Entwicklung von Organ on chip Technologien, die zum einen bessere Modelle hervorbringen, welche eine stärkere Vergleichbarkeit des Testsystems zum humanen In-vivo-Gewebe aufweisen, und zum anderen zu verbesserten experimentellen Bedingungen, wie dynamische Donor- und Akzeptorführung, und somit zu aussagekräftigeren Ergebnissen in Absorptionsstudien zur Bewertung von Formulierungsstrategien führen [1].

Im Vortrag wird am Beispiel der humanen Cornea die Entwicklung, Charakterisierung und Validierung von dreidimensionalen, organotypischen In-vitro-Zellkulturmodellen epithelialer Barrieren als Ersatz für exzidiertes Gewebe gezeigt [2-4]. An ausgewählten Beispielen wird die Verwendung des Modells bei der Entwicklung generischer Formulierungen von Ophthalmika, sowie der Bewertung neuartiger Permeationsenhancer (Poly-L-Cystein) und oligomerer Breitband-Konservierungsstoffe (Polyquads) dargestellt.

Neben der Vermeidung von Tierversuchen im Sinne des 3R-Prinzips bieten die standardisierten Zellkulturmodelle die Möglichkeit, unter Umgehung interindividueller Schwankungen von Spendermaterial, Daten zum transcornealen bzw. transepithelialen Permeationsverhalten von Arzneistoffen aus Drug Delivery Systemen zu erhalten.

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Small Volume Dissolution Apparatus for High Potency, Low Dose Performance Testing

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The concept of small volume dissolution arises from the need to analytically determine the rate of dissolution from low dose, high potency drugs. These new type of drugs and novel drug delivery design technologies demand adjustments of typical pharmacopoeial official dissolution and drug release apparatus. To maintain quantifiable levels of analyte during the dissolution test, a reduction in vessel volume accompanied by an alteration of the compendial dissolution apparatus may be required. Dissolution experiments in general are part of the conundrum of data collected for decision making during drug development stages but also the assurance of quality when the formulation reached full-scale production and to maintain future assurance of product quality and stability. This study will explore the use of USP apparatus and modifications to the standard USP apparatus designed to yield small volume dissolution methods with reliable, accurate results. Three case studies involving the modification of USP Apparatus 7, the Reciprocating Holder apparatus to evaluate drug release performance of drug eluting stents, vaginal rings and drug coated pellets will be discussed.

Biosensing Enhanced by DNA Nanotechnology

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Scaffolded DNA origami [1] is a simple and efficient technique to design two- and three- dimensional objects of programmed shape. The DNA origami serves as molecular breadboard to arrange objects such as bait molecules, dyes and nanoparticles [2]. We present examples of how DNA origami can enhance biosensing through novel signal enhancement mechanisms. Applications include a localized, molecular amplification cascade, the development of nanoscopic rulers for superresolution microscopy [3], as well as the measurement of ensemble and single-molecule kinetics without a change of the nano-environment [4, 5]. Furthermore, arranging nanoparticles on a three-dimensional DNA nanostructure creates a plasmonic hotspot between the nanoparticles that enables fluorescence enhancement of up to 5000fold [6, 7]. Since the DNA nanostructures offer additional docking sites for biomolecular assays, receptors or dyes, they represent a unique platform for labelling and sensing with increased sensitivity.

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