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Achim Weber, Carmen Gruber-Traub, Marc Herold, Kirsten Borchers, Günter E. M. Tovar and Herwig Brunner

Biomimesis by Nanoparticles: Concept, Design and Applications in Biotechnology and Biomedicine

Communication of living systems is done by molecular recognition. This central principle of the living world is performed at the contact sites of different objects such as single macromolecules or highly complex supramolecular assemblies as which living cells may be described. Molecular recognition capabilities can be evoked at artificial materials. Thus biomimetic materials are created which are excellently suited to communicate with the living world. When the material is scaled down to nanoscopic dimensions, insoluble solids are prepared to be efficiently contacted with biological systems, easiest by dispersing nanoparticles in a liquid. This is enabled by the NANOCYTES^{TM}-technology of the Fraunhofer IGB.

The biomimetic nanoparticles described here, possess such molecularly recognizing properties. For this purpose they carry molecularly defined binding sites at their surface. These binding sites are either composed from biologically derived macromolecules or fully synthetic receptors. When biological building blocks shall be used for their outstanding specificity, they have to be conjugated with a synthetic carrier. These carriers have to be carefully prepared by fine-tuning their surface properties. Core-shell nanoparticles are particularly suited for this purpose, e.g. to immobilise a specific protein or a protein complex at their shell surface. The demanding task for the preparative work before conjugating the biological active agent, e.g. a receptor or agonist, is to render the artificial carrier compatible for the close contact to the biological substance without denaturing the complex structure of the biomolecule. Biomacromolecules are often surrounded by a specific supramolecular environment in their natural state. This must be mimicked by the artificial surface. Also, the binding site of the macromolecule is always located at a specific steric region of the molecule. Thus a three-dimensionally defined anchoring system must be introduced to the carrier. Most elegantly, the

core material is surrounded by a soft organic shell. This shell enables for the binding of the biomacromolecule and ensures its native state. Resulting are core-shell particles to which the macromolecules are conjugated and which then possess hybrid properties of the central artificial substances and the surrounding biological macromolecules (Figure 1).

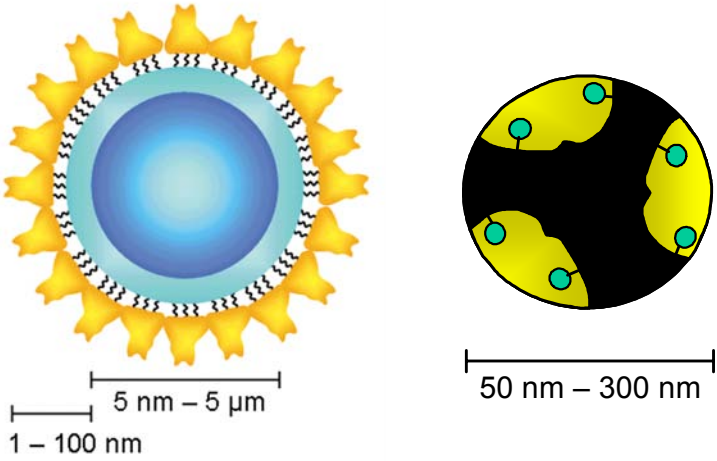

Fig. 1: Scheme of core-shell nanoparticles for molecular recognition applications. The shell consists of a supramolecular organic arrangement which either (a) ensures the steric directivity of attached biologically derived receptors and their bioactivity or (b) forms entirely synthetic molecular binding sites. The chemical design and typical applications for both concepts of biomimetic nanoparticles are described throughout the article. The core inside the particles can contain specific physical properties such as a magnetic moment or fluorescent activity.

Entirely synthetic molecularly recognising nanoparticles can also be prepared by chemical nanotechnology. A cooperative chemical reaction evokes the formation of specific molecular binding sites at the surface of copolymer nanoparticles. This reaction, although complex, is run in a single reaction chamber and in a single step chemical process. The trick in this procedure is, that nanoscopic monomer emulsions are prepared that are „imprinted" by molecular templates during their polymerisation to copolymer nanospheres. Negatives of the shape and the chemical composition of these templates are thus formed at the surface of the resulting monolithic beads. Chemical interaction between the template and the resulting synthetic receptor is composed of cooperative weak interactions, e.g. H-bonds, van-der-Waals forces and electrostatic forces. Highly specific binding sites can be created for a huge variety of

different compounds ranging from low molecular weight compounds to biomacromolecules. The composition of the copolymer is chosen such that complementary chemical groups can arrange themselves sterically ideally to form the binding sites. A straightforward application is to use the synthetic receptors as specific absorbers e.g. to remove of toxins or contraries from mixtures which may even be of complex nature. Other applications range from specific chromatography or membrane processes to diagnostic purposes and will be highlighted later in this paper.

A general advantage of small particles is their high specific surface area – with a very small mass of nanoparticles, large surfaces can be provided for specific interaction with their corresponding binding partners. Therefore nanoparticles are also used for surface functionalisation of larger two-dimensional surfaces by depositing nanoparticle layers. The surface area of the original planar device is thereby enhanced by the resulting three-dimensional arrangement of nanoparticles. Furthermore, by using biomimetic core-shell nanoparticles for surface functionalisation, the molecularly defined surfaces of the nanoparticles constitute the contact area of the resulting modified biochip surface. The nanoparticle layers can be deposited in a micro-structured way by a large variety of different lithographic or printing techniques.

The present article will highlight the design and application of biomimetic nanoparticles based on the structural concepts described above.

Core-shell nanoparticles enable new drug concepts

Future drugs will be increasingly based on proteins as the active agent. If a protein is soluble, applications might be straightforwardly based on administering them in a liquid. However, in multicellular organisms, receptor ligands are often presented at the surface of cells. Such cell membrane-bound ligands bind highly specific to their corresponding interaction partners, based on molecular recognition processes. The protein tumor necrosis factor (TNF) is mirrored by cell surface membrane receptor proteins [1]. TNF is a cytokine and capable of inducing apoptosis – the programmed cell death. This capability makes it a promising and highly attractive candidate for new strategies in cancer therapy or pathway studies in cells. In the past, studies directed towards new TNF-based cancer therapies have been performed with the soluble cytokine (sTNF). However, typical for the members of the TNF ligand family, TNF is a transmembrane protein (mTNF) and initially expressed at the cell membrane. From this membrane-bound protein sTNF is subsequently derived by an enzyme process. Both mTNF and sTNF bind to two different cell

membrane receptors, termed TNF-R1 and TNF-R2. Whereas TNF-R1 is ubiquitously expressed in all tissues, TNF-R2 is highly regulated in expression and mainly found in immune cells and endothelial cells but also in neuronal tissue. The two TNF forms possess differential capabilities for the induction of larger signalling complexes. Whereas sTNF binding enforces receptor trimerization only, cell-associated mTNF might subsequently cause the formation of larger complexes by induction of capping of the membrane-bound ligand molecules and the receptors at the respective interacting cellular sites. In light of these data, it is very likely that the physiological role of TNFR2 is largely underestimated, simply because sTNF has been used in by far the most studies. Accordingly, there is a need for a mTNF-adequate stimulus that can be easily applied in experimental systems *in vitro* but also in animal model systems *in vivo*. To overcome the lack of an appropriate mTNF-like stimulating reagent, we have constructed core-shell particles covalently coupled with mutated functional TNF derivatives, forming bioactive homotrimers at the particle surface. These novel particles are able to initiate mTNF-resembling cellular responses – the action of membrane-bound TNF was successfully mimicked by the biomimetic nanoparticles. Moreover, these tools can be used for selective and spatially restricted activation of either of the two TNF receptors and allows live imaging of receptor proximal events and apoptosis events. Future areas of application for similar but biodegradable (nano)particles might include the *in vivo* usage of particles carrying more than a single effector or targeting protein for therapeutic treatment.

Tailoring of biomimetic core-shell nanoparticles

Core-shell nanoparticles are composite materials which are composed of at least two different components. In the examples highlighted in this article, the shells consist of organic substances and provide supramolecular arrangements for the interaction with organic molecules. The core may be composed from organic matter but also from metals, metal oxides or rather ceramic materials. A widespread approach for core preparation consists in synthesising spherical silica nanoparticles. They are often prepared from organosilanes via sol-gel chemistry [3] and their diameter can be freely chosen in the range between 10 nm and 10 μm. Such particles dispose surfaces that are densely covered by silanol groups. Organic shells of silica cores can be tailored by the use of organofunctional silanes (Figure 2) [4].

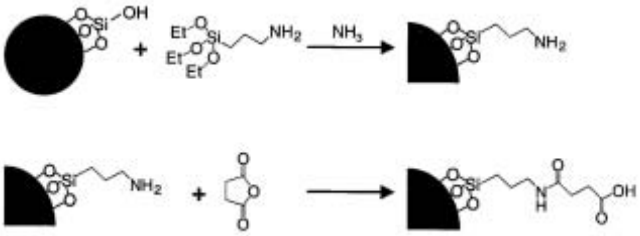

Fig. 2: Reaction scheme of the organo-chemical functionalization of the particle surface: upper: silanization of the silica surface covered by silanol groups with aminopropyltriethoxysilane (APS), which results in an amino-functionalized surface of the nanoparticles. lower: Further reaction of the amine functions with succinic anhydride by a ring opening linker elongation to receive a carboxyl-functionalized surface of the nanoparticles [4].

The shape and composition of the particles is characterised by various independent analytical methods and enables to monitor and fine-tune precisely the preparative steps used to tailor core-shell nanoparticles. E.g. the change in surface properties of the particles upon a second surface reaction using the organic modifier succinic anhydride is monitored by zeta-potential measurements as a function of the pH to decide on a useful pH regime for successive bioconjugation reactions. In a next reaction step proteins are bound to the particle shell. To bind specific proteins, often an intermediate protein shell is bound first, e.g. composed of the protein streptavidin. This protein is a prominent molecular building block with an extremely high affinity to the low molecular weigh compound biotin. Biotin, on the other hand, is then introduced to the protein to be immobilised to the shell by a biotinylation reaction. Biotin serves then as an anchor to conjugate the protein to the streptavidin shell. This procedure is one way to control the orientation of the protein as well as enhancing the protein compatibility of the shell. The resulting protein modified core-shell nanoparticles can be directly used in a vast variety of applications in biotechnology and protein analytics. Due to their high specific binding capacity and their colloidal stability they are entirely compatible with liquid handling systems, e.g., in (ultra)high-throughput screening techniques. They can be used as solid carrier systems of protein receptors to capture analytes from biological samples and present them for further analysis using powerful read-out techniques such as fluorescence spectroscopy or MALDI mass spectrometry [5].

Shell preparation in a single step – surfmer technology

An elegant way to prepare the shell of nanoparticles with full control of its composition is based on the use of polymerisable surfactants – so-called surfmers [6]. Surfmer is an abbreviation of the two terms surfactant and monomer and this describes two intrinsic functions of such a molecule: to stabilise as an emulsifier the emulsion during the particle synthesis or modification as well as the resulting nanoparticles. And to react in a polymerisation chain reaction and thereby introducing itself covalently in the particle shell. Additionally, the surfmers described here, possess a third functionality. They introduce a so-called active ester group to the nanoparticle shell [7]. This chemical functionality is stable to polymerization and storage. And it can be activated at any desired moment for gentle covalent anchoring of functional components such as biomolecules [8].

Surfmers are employed in emulsion polymerization and render nanoparticles with tailor-made surface properties in a single step. Typical particle diameters lie in the range from 80 nm–200 nm. Organic shells are prepared by this technology onto polymeric cores and just as well as to almost any other core material by a so-called seeded emulsion polymerisation. The polymerisable endgroup can be freely chosen for optimal reactivity with the desired core material. Most importantly, the surfmer technology allows replacing tedious multi-step preparation techniques employed to date in industrial practice. Additionally, the surfmer technology enables to create particles that are about a thousand times smaller than the beads currently employed in biotechnology for biomolecule immobilisation.

Nanoparticles as nanocarriers and nanocontainers for drug delivery

Polymeric nanoparticles as carrier for drugs are able to control the release of active agents (controlled release) [9]. The purposes behind the controlled drug delivery are more effective therapies as the level of the active agent within the organism can be controlled.

Biodegradable polymers which are fully metabolised by the body are of increasing interest for drug delivery. The physical and chemical properties of the particles influence the degradation process and its rate and thus define the release kinetics. These properties are strongly affected by the polymer composition. The polymer matrix material is either commercially available as is the case for the family of biodegradable polylactides or biocompatible blockcopolymers are designed and synthesized for this purpose. Biodegradable li-

near polyesters are well established and approved by the FDA but often suffer from insufficient properties. Tailor-made new polymeric matrix systems with improved features and different molecular weights can overcome the limitations.

The drug release kinetics can be fine-tuned to needed requirements by changing different parameters during the particle preparation. E.g. the molecular weight and the ratio of hydrophilic to hydrophobic monomer units influence the release kinetics drastically. A variety of emulsion techniques are employed for the formation of the nanoparticles. The particles were prepared by a double emulsion technique using an aqueous phase being emulsified in an oily phase which then is emulsifies in an aqueous phase (water-oil-water double emulsion). The particle diameter can be freely chosen between 60 nm –300 nm. The process is tailored according to the chemical nature of the compound to be released – active agents range from simply structured low molecular weight molecules to complex biomacromolecules such as proteins like cytokines and growth factors. For drug targeting, the shell of the nanoparticles is specifically functionalised for a specific interaction with the surrounding medium – another application of the general principle of molecular recognition.

Entirely synthetic receptors – molecularly imprinted nanoparticles (nanoMIPs)

Entirely synthetic receptors which combine the specificity of biological binding sites with the superior chemical stability of synthetic materials are most desired moieties. Molecularly imprinted nanoparticles come close to such an ideal. Molecular imprinting is a way to induce specific molecular binding sites in otherwise non-functional materials [10]. Wanted is a specific interaction of so-called templates with a comonomer mixture during the polymerisation process which leads to a monolithic copolymer network to which the templates are strongly adhered, e.g. by H-bonds, van-der-Waals-forces and electrostatic forces. When the template is extracted, empty binding sites are retained in the copolymer which constitute artificial molecular recognition sites. Although successful in the lab, a technological break-through of these „plastic antibodies" or „plastic enzymes" suffered from the fact that for practical use of the imprinted material, it always had to be crushed and sieved in order to obtain useful particles for desired applications. NANOCYTES^{TM}-technology overcomes this crucial limitation by scaling down the

preparation of the imprinted material to the nanoscale [11]. Monomer nanodroplets are prepared by emulsion techniques. These droplets interact with the templates in the liquid state. Then they are converted to nanoscopic polymer monoliths – all in the presence of the template. The resulting particles have only to be freed from the template by extraction and entirely synthetic receptor nanoparticles are obtained [12]. The change to an emulsion polymerisation techniques offers additionally to the benefit of size and morphology control, also superior control on the chemical composition of the resulting material – and thus it provides a key to fine-tuning the recognition properties. Molecularly imprinted nanoparticles (nanoMIPs) can be produced economically in quantitative yield. A variety of active agents ranging from low molecular weight drugs to peptides and proteins have been successfully imprinted in this way [11].

The emulsion technique for the nanoMIPs preparation is called a miniemulsion polymerisation and results particles with typical sizes between 50 nm and 300 nm. Besides classic miniemulsion polymerisation (hydrophobic phase emulsified in hydrophilic phase – here water) also a MIP technique based on inverse miniemulsion polymerisation has been established. Thus possible templates can be chosen fro the full range of hydrophilic to amphiphilic to hydrophobic molecules. A further advantage of this new technique is that the nanoparticles created can be used under physiological conditions, assuring the sustainability of the „biological key".

NanoMIPs can be used to absorb specific compounds from mixtures e.g. in order to remove toxic compounds or undesired by-products. Easiest is to employ the material as a suspension or powder. Due to their defined morphology they are also excellent coating material for sensors or membranes in a separation process [13]. NanoMIPs can be integrated in a membrane set-up, e.g. by forming the functional heart of a composite membrane. Common ultrafiltration membrane discs are used as support and cover of a nanoMIP multilayer. To work up the imprinted material to a membrane allows to integrate the principal of molecular recognition in a new fashion in biotechnical or chemical industrial processes for specific separation of molecules. Here, the aim may either be to eliminate undesired compounds or to gain effectively valuable products.

NANOCYTES^{TM}-based 3 D-biochips

Biochip-technology is a key factor in today's biotech research: To gain a comprehensive insight into complex metabolism sequences or to safeguard

diagnostic conclusion, it is essential to probe particular molecules of interest with a great variety of potential interaction partners [14]. Preparation of biochip-interfaces is done e.g. by printing small amounts of biological or synthetic capture-molecules on a solid substrate. This results in arrays of microspots, which display different specific binding-affinities. Thus, biochips enable scanning of minor biological sample volumes for a multiplicity of different analyte molecules in one go.

The reactive microspot within a biochip can be drastically enhanced by micro-structured deposition of functional nanoparticles. The resulting three-dimensional micropads provide enlarged reactive surfaces for the detection of biomolecules [15]. The whole variety of core-shell nanoparticles described above can be used for generation of microarray-surfaces. This concept of modular assembly allows for flexible tailoring of biochip-interfaces. NANOCYTES^{TM}-based 3D-biochips are compatible with fluorescence detection and MALDI mass spectrometry, the state-of-the-art read-out techniques of modern biotechnology [5].

Deposition of defined amounts of particles is accomplished by contact-printing nanoparticle suspensions using a pin-ring spotter. By tuning the amount of particles deposited per spot the binding-capacity of nanoparticle-microarrays can be increased. In contact with a sample, high receptor-density shifts the equilibrium towards the formation of the receptor-ligand complex and therefore results in a greater amount of bound analyte for a given concentration of analytes in the sample. The separation of the bioconjugate-chemistry from the actual array printing-process allows for tailoring different immobilization strategies for different capture-molecules. The concept of simultaneous detection of multiple analytes on a nanoparticle biochip-surface was proven using antigens and antibodies as particle-bound capture-molecules [16].

By NANOCYTES^{TM}-based microarray-technology, surfaces are tailored for optimised binding of biomolecules. Furthermore, the constitution of the active micropads from nanoparticles provides enlarged reactive surfaces within the biochip. The technology is compatible with multiplex analysis. It provides a flexible platform for the generation of innovative biochip-surfaces – a field of growing impact on biomedical and diagnostic issues.

Conclusion

Smart nanostructured particles that have the ability to molecularly recognise specific compounds already have found widespread use in research and tech-

nology. Such systems can be fine-tuned by a variety of technological means which are mastered by todays chemical and biochemical nanotechnology. They are and will be applied in the chemical industry, in biomedical engineering and in biotechnology. Their task is to gain valuable compounds in production lines, to enable diagnostic and sensoric processes or to enable new therapeutical approaches. Biomimetic nanoparticles mark an enthralling field of highly interdisciplinary research and development and will continue to attain importance.

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