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- Nanomedicine in Alzheimer’s Disease
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# Table of Content

## Editorial 1
**CLINICAL NANOMEDICINE, A NEW NANO DOMAIN**
Heinrich Rohrer

## Editorial 2
**Beat Löffler**

## Editorial 3
**NANOMEDICINE – SHAPING THE FUTURE OF MEDICINE IN A CONTEXT OF ACADEMIA, INDUSTRY AND POLITICS**
Patrick Hunziker

## Clinical Nanomedicine
**NANOMEDICAL APPROACHES IN ALZHEIMER’S DISEASE**
Felix Fluri

## Nanophysiology
**THE JANUS FACE OF IMMUNE STIMULATION BY NANOMEDICINES: EXAMPLES FOR THE GOOD AND THE BAD**
Janos Szebeni, Julianna Lisziewicz

## Therapeutic Nanomedicine
**CROSSSLINKED MICELLES WITH TRANSIENTLY LINKED DRUGS – A VERSATILE DRUG DELIVERY SYSTEM**
Cristianne JF Rijcken, Marina Talelli, Cornelus F van Nostrum, Gert Storm and Wim E Hennink

## Preventive Nanomedicine
**NANOMEDICINE FOR DENDRITIC CELL-TARGETED IMMUNOTHERAPY**
Agnes Gudics, Julianna Lisziewicz

## Nanomethods
**SCANNING X-RAY SCATTERING: EVALUATING THE NANOSTRUCTURE OF HUMAN TISSUES**
Bert Müller, Hans Deyhle, David A. Bradley, Michael Farquharson, Georg Schulz, Magdalena Müller-Gerbl, Oliver Bunk

## Nanopharmaceuticals
**NANOPHARMACEUTICALS**
Raj Bawa

## The Landscape of Nanomedicine
**NANOMEDICINE AND NANOTOXICOLOGY AT PHARMA–COPENHAGEN UNIVERSITY**
Clinical Nanomedicine is a newcomer in the rapidly growing family of nano science & technology domains. A newcomer, be it a scientist or a research field, is not prejudice-laden, he has little past but a promising future. That is the big chance of Nanomedicine. Progress in Science and Technology is more hampered by biased minds and narrow thinking than by lack of ideas, funds, support, and research personnel. Likewise, science does not suffer from a shortage of utmost intelligent brains, but much more of brave spirits and great hearts ready to venture into and to master the unknown. Novelty comes about by having ideas and realizing them – ideas alone are not sufficient –, by questioning general believes, by thinking the hitherto unthinkable, by doing what was believed to be impossible, by solving what was considered unsolvable and by reflecting on what others considered uninteresting, in short by going beyond generally accepted knowledge, thinking, skills, and limits. Everything is possible except it is proven to be impossible (R. Feynman); and, from my own experience, the proofs are mostly wrong. The sky for new ideas in Nanomedicine is wide open. With visions and ideas you can make tomorrow to today.

The immediate beneficiary of Nanomedicine is each individual of society. Nanomedicine is, therefore, of direct personal social interest and the expectations, demands, and pressures are correspondingly very high. Equally high are also the challenges and hurdles. The most crucial one I consider interdisciplinarity which lies at the heart of Nanomedicine, actually of everything carrying the name Nano. Awareness of the urgency, importance, and significance of “Inter” became omnipresent, be it in science in general, be it in economy, politics, or society. This awareness, however, rarely ends up in specific and successful actions and we do badly everywhere, from international politics to human interactions to interdisciplinarity in science. This is most deplorable since “Inter” is in many ways the key and salient ingredient for progress. “Inter”-disciplinarity emerges from the necessity to solve modern, very demanding problems of holistic nature. Interdisciplinarity is “in addition to”, not “instead of”. It requires learning, insight, knowledge, skills and talents in addition to that of a solid and excellent disciplinary basis. Unfortunately, this extra effort and talent receives so far little appreciation in the scientific community. Science is still stuck to a great extent in the disciplinary age where scientists think and appreciate science predominately along disciplinary excellence which leaves then little room for recognition, esteem, fund allocation, and so on, different from a top-notch disciplinary performance. It is, however, the scientific standard of the problem to be solved which is the measure of the scientific quality and impact and setting this standard and living up to them requires again both intelligent brains and brave spirits.

Nanomedicine is no doubt a tough and hot place; Nano means ultimately measuring, modifying, material removal and deposition, growing nanostructures, and initiating and controlling processes – in short performing all kinds of experiments - at a given nano-location with a given nano-precision. Indeed tough and hot, but it is the most capable scientists which reach for the cherries hanging very high. Nanomedicine has all the chances to become a prime player in the health sector and I expect that many of its new tools, methods, and techniques will also become a crucial element in personalized medicine and systems biology. And last but not least it should serve as a shiny example for the embracing power of interdisciplinarity.

Wollerau, May 1, 2010

Heinrich Rohrer
Dear readers

This issue of the European Journal of Nanomedicine appears just ahead of the 3rd European Conference for Clinical Nanomedicine the CLINAM Foundation for Clinical Nanomedicine, which welcomes 99 speakers from 25 countries and participants from 30 countries all over the world.

The goal of the conference is to advance the state of nanomedicine in clinical applications for the benefit of patients and to explore its implications in an interdisciplinary context. Presenters and participants consist of medical doctors and scientists from nanoscience, biology, chemistry, physics pharmaceutics and other fields, including a number of speakers who count among the fathers and pioneers of nanoscience and nanomedicine.

Starting from unsolved medical problems and presenting the latest developments in the nanosciences, new ways are being sought to find more effective and gentler approaches to various fields of medicine, this year with an emphasis on cancer; but also discussing other topics including, diabetes, circulatory disorders, infections and respiratory diseases. Again there will be discussions addressing the issues of the toxicity of nanoparticles, the ethical implications and also the relevance for future healthcare.

Like prior CLINAM conferences, this is a “debate conference”. Most speakers make their statement in 10 minutes. After four to five such statements they are followed by in-depth interdisciplinary discussion of 20- 45 minutes with the participants who bring in their broad knowledge of their fields, like medicine, biology, physics, chemistry and nanoscience. A particular highlight of the conferences is the “Late Breaking Clinical Trial Sessions”, where the latest clinical studies will be presented and critically examined.

The final debate of the conference is dedicated to the topic “Saving Lives versus Avoiding Risks in Developed and in Developing Countries”. The topic is most complex: The fundamental motivation of medicine is to benefit the patient without adding harm. How can benefit be defined or measured for individuals, and what is risk in science for society? How can a benefit for an individual or a group of patients be weighted in relation to potential or unknown risks? Is it ethical to treat a human individual with a lethal disease with a novel therapeutic approach of uncertain efficacy or unproven safety or is it more ethical to withheld the therapy? Is the current practice of drug development suited to individual severe disease, to orphan disease, in children, in the elderly, in the developing countries? Facing the nanomedicine paradigm of composite therapeutic systems that can be tailored to an individual patient, what new avenues in drug development and regulation need to be taken?

New is the accompanying University Village 2010. The healthcare sector is experiencing turbulent times: Open Innovation provides both opportunities and challenges for the academic sector. In a comparatively new area such as Nanomedicine, worldwide competition makes it essential that translation of Nanomedicine becomes much more efficient. Universities are being offered leadership roles – however this is a global opportunity and if European groups do not seize the opportunities others will! We must align academic research in the nanosciences to help patients; the small academic team model is unlikely to succeed and multi-disciplinary knowledgeable teams will be essential. This first CLINAM Nanomedicine University Village will present novel approaches, new research projects and initial outcomes of research and experimental results. The members of this village will discuss the approach to leadership of the Universities and the ingredients for such excellence.

Beat Löffler
The development of Nanomedicine within the past decade have led to the conviction of many that this field will shape the medicine of the future to a large degree: Basic discoveries in the nanosciences are published daily, new miniaturized tools and intelligent materials are currently applied in preclinical experiments, as well as in early clinical studies, and lend to the impression that advances in diagnosis and therapy are — technically speaking — limited only by our imagination. Diagnosis will be fast, accurate and comprehensive, even in the family doctor’s office setting, and therapy will be highly effective, still mild and with a minimized risk of side effects; all of that will be at affordable cost and in a “green tech” manner that minimizes resource utilization and ecological impact.

However, beyond the technological aspects, important questions in the “meta” domain are open to debate: What will energize, who will guide and to what extent should society channel this revolution in medicine? Historically, medicine was the art of the physician to understand health problems, to recommend a healthy lifestyle, to relieve suffering, to avert premature death, and to console in conditions where no cure was available, all of this driven by a humanitarian spirit (even now a major factor for electing medicine as a profession) or a faith-driven commitment, triggered by the societal needs, but also practiced as a profession to make an income. At the same time, development of new technology by artisans, engineers and scientists, and manufacture of medicines by pharmacist were driven primarily by the interaction of patient, physician, pharmacist and artisan, often triggered by new discoveries made at universities in a context of free academic research.

Today, the situation is vastly different. Health, defined by the World Health Organisation as “a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity” is seen as a fundamental right of the citizen which society is responsible to fulfil. The heavy utilization of the healthcare system by the average citizen to get his due right, together with the shift in the socioeconomic structure of society is expensive, calling for massive government financing. Chemical, pharmaceutical and MedTech industries are an important part of many economies. Leading in technology is seen as a strategic development goal of political entities.

Modern medicine and in particular nanoscientific-based medicine is a field where this paradigm change is evident: The historic roots of nanoscience, e.g. the invention of the raster microscopes with atomic resolution by Rohrer and Binnig were done in a context of free scientific exploration (where in particular for the raster microscopes, few actually believed in a successful outcome leading to practical utility). Today, nanoscience research has been transformed into a politically governed, industry guided process, linking predetermined technical development goals, industry-led technology platforms and consortia with research funding for academia, thus transforming significant parts of academic research into R&D enterprises of nations and supranational organizations and companies. Supranational coordinated research has, in specific domains like nuclear or space research, shown successful in other domains. In healthcare and in nanomedicine in particular, the answers are yet to be given: Will the currently dominant funding of product- and solution-oriented research promote or hinder the exploration of fundamental, unexpected, but sea changing, innovation? Will the political interests of having strong, financially successfully pharmaceutical, MedTech and biotech industries prove compatible with the aim to lower healthcare expenditures? Will the typical desire of the patients, e.g. to swallow as few pills as possible, be compatible with the industrial business necessity, to have as many patients as possible to be treated with their block-buster drug? Is the notion of less expensive healthcare compatible with the strategic vision of a flourishing healthcare industry? Will negative answers, e.g. about side effects, toxicity, or implications arising from research equally be published when they originate from industry-guided projects? Will the coupling of biomedical research and industrial business models have an impact on medical ethics? Will it facilitate or limit the benefit of nanomedicine to the parts of the world unable to generate funds to pay for it?

In Europe, the existence of various bodies embracing different positions in this field of development of nanomedicine may enhance understanding of these questions. The European Technology Platform for Nanomedicine as a EU-governed, industry-led organisation, the European Society for Nanomedicine as a registered non-profit association of professionals in nanoscience and medicine, and the CLINAM Foundation for Clinical Nanomedicine as a registered non-governmental, non-profit and non-industry led foundation with the aim to advance clinical application, but also to explore implications, will allow to reflect upon the success of the different models. I anticipate that the answer will not be to choose an either-or, but to find a mode for each of the models to accomplish their specific, unique, and complementary tasks.

Patrick Hunziker
NANOMEDICAL APPROACHES IN ALZHEIMER’S DISEASE

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ABSTRACT

Alzheimer’s disease (AD) is the most common cause of dementia and afflicts an estimated 16 million people worldwide. Clinical diagnosis can be made with only 65–90% accuracy, even when the disease progresses. Although there are some drugs that can slightly alleviate its symptoms, there is currently no treatment that can prevent this neurodegenerative disorder, delay its onset or slow its progress. In order to solve some of the AD challenges, nanotechnology may offer some promising techniques. Recently proposed applications of nanotechnology for the early diagnosis of AD include localized surface plasmon resonance (LSPR) spectroscopy, nanomaterial-based two-photon Rayleigh scattering (TPRS) assay for detecting proteins or nanofluidic biosensor using a controlled, reproducible surface enhanced Raman spectroscopy (SERS). Nanotechnological therapies of AD comprise neuroprotection against oxidative stress, lowering brain metal ions by the administration of chelator-nanoparticle systems, anti-amyloid therapeutics and drug delivery beyond the blood brain barrier using nanoparticle carriers. All of these applications may lead to an improvement of diagnosis and therapy of AD and thus are a step toward the cure of AD.

Key words: Alzheimer’s disease, nanotechnology, nanodiagnostics, neuroprotection, drug delivery system

INTRODUCTION

Alzheimer’s disease (AD) is the most common form of dementia with an average death prognosis of 9 years (1). The clinical diagnosis of AD is most often based on the criteria developed by the National Institute of Neurologic and Communicative Disorders and Stroke–Alzheimer’s Disease and Related Disorders Association (NINCDS–ADRDA) (2). The classic clinical features of AD are an amnesic type of memory impairment, (3-5), deterioration of language (6) and visuospatial deficits (7;8). Clinical diagnosis, based on a patient’s clinical history; in vivo brain imaging (9;10); and neuropsychological, cognitive, and neurological tests, is only about 85% accurate (11). A definite diagnosis of AD can only be made by neuropathology (12).

At the microscopic level, the characteristic lesions in AD are senile or neuritic plaques and neurofibrillary tangles (12) in the medial temporal lobe structures and cortical areas of the brain, together with a degeneration of the neurons and synapses.

Several pathogenic mechanisms that underlie these changes have been studied, including amyloid aggregation and deposition with plaque development, tau hyperphosphorylation with tangle formation, neurovascular dysfunction, and other mechanisms such as cell-cycle abnormalities, inflammatory processes, oxidative stress, and mitochondrial dysfunction (13).

The major plaque component is beta-amyloid (Aβ(14), a proteinaceous polymer having a beta-pleated sheet conformation that accumulates extracellularly (15;16). Aβincluding the 40 and 42 amino acid cleavage products (Aβ40 and Aβ42), is proteolytically derived from amyloid precursor protein (APP) (17). A number of hypotheses for the formation and progression of amyloid plaques have been suggested, including glial secretion of amyloid (18-20), somal and synaptic secretion of Aβ from neurons (21-23), and endosomal–lysosomal aggregation of Aβ in the cell bodies of neurons (24; 25), but none of these hypotheses fully account for the focal accumulation of amyloid in plaques (26-30). Recent data support the hypothesis that disruption of axonal transport leads to axonal swelling and dystrophic neurites in AD (31; 32). During inhibition of retrograde axonal transport, mitochondria (33) and autophagic vacuoles (34) trafficking from the distal axon to the soma, accumulate in dystrophic neurites resulting in the autophagocytosis of mitochondria (35) without normal lysosomal degradation. Recent evidence from transgenic mouse models of AD suggests that the degeneration of these autophagosomes may lead to amyloid production within dystrophic neurites (36). Aβ levels correlate with neurological status in the injured human brain. Using intracerebral microdialysis after acute brain injury of patients, Brody and co-workers (37) found a strong positive correlation between changes in brain interstitial fluid Aβ concentrations and neurological status, with Aβ concentrations increasing as neurological status improved and falling when neurological status declined.

Another neuropathological hallmark in AD are neurofibrillary tangles that were shown to be composed of abnormally hyperphosphorylated tau protein (38; 39). Tau is a normal axonal protein that binds to microtubules through its microtubule-binding domains, thereby promoting microtubule assembly and stability (40). Tau hyperphosphorylation in Alzheimer’s disease starts intracellularly and leads to sequestration of normal tau and other microtubule-associated proteins, which causes disassembly of microtubules and thus impaired axonal transport, compromising neuronal and synaptic function (41). Tau also becomes prone to aggregation into insoluble fibrils in tangles, further compromising neuronal function. Tau pathology starts early in the disease process in neurons in the transentorhinal region, spreads to the hippocampus and amygdala, and later to the neocortical association areas. Whether tau hyperphosphorylation and tangle formation is a cause or consequence of AD is unknown (42).

NANODIAGNOSTICS IN AD

There are two general approaches for detecting soluble markers for AD. One approach is based on measuring the total tau protein or amyloid-β protein (Aβ) concentration in cerebrospinal fluid (CSF) or plasma (43-46). This approach is hampered by significant overlap of such marker in healthy and unhealthy subjects and has led to inconclusive results (47-50). The other approach targets only the suspected pathogenic markers, such as cleaved tau protein, phosphorylated tau protein (51), or amyloid-β-derived diffusible ligands (ADDLs). Although this approach - detecting pathogenic markers - might lead to more definitive results, the concentrations of such markers in CSF are so low in the early stages of the disease that they cannot be identified accurately with conventional ELISA or blotting assays. In order to determine the approximate ADDL concentration in CSF, Georgenpoulou and co-workers combined ADDL-specific monoclonal antibodies with a nanoparticle-based protein detection strategy termed biobarcode-amplification (BCA) (52, 53). BCA relies on magnetic microparticle (MMP) probes functionalized with monoclonal antibodies that specifically recognize and bind ADDL. The ADDLs are then sandwiched with a gold nanoparticle probe, modified with double-stranded DNA and an anti-ADDL-polyclonal antibody. After repeated washing while using a magnetic field to immobilize the MMPs, a dehybridization step releases hundreds of barcode-DNA strands for each antigen-binding event (53). The increased sensitivity advantage derives mainly from the very effective sequestration of antigen and the amplification process that occurs as a result of the large number of barcode DNA strands released for each antigen recognition and binding event. By using this approach and adequate antibodies, as low as 30 copies of a target protein can be routinely detected, often in a complex sample such as plasma (53).
Another nanodiagnostics of AD is a nanoscale optical biosensor based on localized surface plasmon resonance (LSPR) spectroscopy to monitor the interaction between the ADDLs, and specific anti-ADDL antibodies (54). The optical biosensors consisting of triangular Ag nanoparticles (perpendicular bisectors = 90 nm, heights = 25 nm) were synthesized on mica substrates and functionalized with antibodies specific for ADDLs. Thereafter, the nanoparticle surface was exposed to varying concentrations of synthetic ADDLs and finally, the nanosensor was exposed to a fixed concentration of anti-ADDL antibody to complete the assay. In order to passivate the nanoparticles for nonspecific binding the Ag nanoparticles were functionalized with a self-assembled monolayer. The anti-ADDL antibody was covalently attached to the nanoparticles via incubation in anti-ADDL antibody solution. Samples were then incubated in varying concentrations of ADDLs. Finally, to enhance the LSPR shift response of the ADDLs, the samples were incubated in an anti-ADDL solution. Using this sandwich assay, this nanosensor provides quantitative binding information for detection of both, ADDLs and anti-ADDL antibodies that permits the determination of ADDL concentration and offers the analysis of the aggregation mechanisms of ADDL at physiologically relevant monomer concentrations. Monitoring the LSPR-induced shifts from both ADDL and anti-ADDL antibody - anti-ADDL antibody interaction of ADDL concentration revealed two ADDL epitopes that have binding constants to the specific anti-ADDL antibodies of 7.3 x 1012 M^-1 and 9.5 x 108 M^-1. In this study, the analysis of human brain extract and cerebrospinal fluid samples from control and AD patients revealed that the LSPR nanosensor provides new information that may be relevant to diagnose AD earlier (54).

Neely and co-workers developed a gold nanomaterial-based two-photon Rayleigh scattering (TPRS) assay for the ultrasensitive and selective detection of tau protein (55). Monoclonal anti-tau antibody-conjugated gold nanoparticles were synthesized. In the presence of tau protein, these nanoparticles bound to each protein, thereby producing nanoparticle aggregates. As a result, a colorimetric change has been observed from red to bluish color and a new broadband appeared around 150 nm far from their plasmon absorption band. When monoclonal anti-tau antibody-conjugated gold nanoparticles were mixed with various concentrations of tau protein, two-photon scattering intensity increases by about 16 times. These findings revealed a very distinct two-photon scattering intensity change (2.2 times) even upon the addition of 1 pg/mL tau protein. This TPRS assay showed a high sensitivity to tau protein and it was able to distinguish from bovine serum albumine (BSA), which is one of the most abundant protein components in CSF (55).

Skaat and Margel described recently (56) a novel method for selective marking of Aβ40 fibrils by non-fluorescent and fluorescent-magnetic maghemite (γ-Fe₂O₃) nanoparticles and the completely removal of the magnetized fibrils from the aqueous continuous phase by a magnetic field. Non-fluorescent γ-Fe₂O₃ nanoparticles were used to conjugate the antibody, which was then mixed with various concentrations of tau protein, two-photon scattering intensity increases by about 16 times. These findings revealed a very distinct two-photon scattering intensity change (2.2 times) even upon the addition of 1 pg/mL tau protein. This TPRS assay showed a high sensitivity to tau protein and it was able to distinguish from bovine serum albumine (BSA), which is one of the most abundant protein components in CSF (55).

Clinical Nanomedicine

An application of nanotechnology in AD is neuroprotection and drug delivery beyond the BBB. Concerning neuroprotection, nanodevices with antioxidant and anti-inflammatory properties are a therapeutic approach. Agents with such properties are water-soluble derivatives of C60 fullerenes. Some C60 derivatives reduce Aβ 1-42 induced apoptosis in neuronal cell cultures (59), blocked the Aβ 25-35 induced increase in cytosolic free calcium (60) and effectively inhibited Aβ 1-40 aggregation (61). Furthermore, the numerical density of Aβ plaques detected using monoclonal anti-tau antibody-conjugated gold nanoparticles containing the fluorescent probe rhodamine (γ-Fe₂O₃-R) were synthesized similar to the non-fluorescent γ-Fe₂O₃ nanoparticles, substituting the gelatin for gelatin-covalently bound to rhodamine isothiocyanate (RITC). γ-Fe₂O₃ nanoparticles containing the fluorescent probe Congo red (γ-Fe₂O₃-CR) were prepared by the electrostatic interaction of the CR with the surface of the γ-Fe₂O₃ nanoparticles. Aβ40 fibrils were formed by incubating the monomeric Aβ40 dissolved in aqueous continuous phase at pH 7.4 and 37°C. Magnetic Aβ40 fibrils/γ-Fe₂O₃ nanoparticle assemblies were prepared by interacting the γ-Fe₂O₃ nanoparticles with the Aβ40 fibrils during or after their formation. The nanoparticles were attached selectively to the Aβ40 fibrils in both cases, even under competitive conditions, e.g., in the presence of 4% HSA. Kinetics of the Aβ40 fibrillation process in the absence and the presence of the γ-Fe₂O₃ nanoparticles were elucidated. The formed Aβ40 fibrils/γ-Fe₂O₃ nanoparticle assemblies were then completely removed from the aqueous continuous phase by a magnetic field (56). These fluorescent-magnetic nanoparticles as multimodal imaging agents appear to have a great advantage due to the combination of the magnetic and fluorescence imaging into one nanostructured system. This hybrid system, which selectively marks Aβ40 fibrils, might enable the early detection of plaques using both MRI and fluorescence microscopy, and therefore may be applied in vivo AD diagnosis studies. These fluorescent-magnetic nanoparticles may also be useful as selective biomarkers to detect the location and the removal of other amyloid plaques derived from different amyloidogenic proteins (56).

In order to observe β-amyloid peptide (Aβ) in different conformational states during the Aβ self-assembly process, Chou and co-workers (57) created a nanofluidic biosensor using a controlled, reproducible surface enhanced Raman spectroscopy (SERS). Aβ SERS samples were prepared by mixing Aβ with gold nanoparticles in solution (volume ratio 1:10). After mixing, 120 μL of the gold colloid-protein mixture with an average diameter of 60 nm and concentration of 0.01% in water was then loaded immediately into a reservoir connected with a microchannel of a nanofluidic channel device. This device consisted of a microchannel (2μm), connected with a nanochannel (40 nm). Because of the capillary force of the channel, the nanoparticles in solution were transported down the microchannel. Since the diameter of the gold nanoparticle is larger (60 nm) than the depth of shallow nanochannel (40 nm), the nanoparticles were trapped at the nanochannel entrance, creating a high density of gold nanoparticle clusters. Raman spectra were taken of the nanoparticle clusters using a Renishaw System 1000 Raman spectrometer. Using this SERS technique, the bioactivity of Aβ is preserved, allowing the protein to undergo conformational changes. Therefore, proteins may be probed in solution without modifying the protein, implying direct detection of Aβ in CSF. Furthermore, sensitivity of this system to distinguishing between α-helices and β-sheets as well as other protein conformational changes facilitates the discrimination between harmless monomeric forms of Aβ and more toxic β-sheet oligomeric or protofibril/fibril forms. This technique may be valuable in the detection and structure determination of Aβ associated with the progression of Alzheimer’s disease (57).

Wadghiri and co-workers (58) presented a method based on nanotechnology to detect Aβ plaques in the brains of transgenic mice by magnetic resonance microimaging. This method uses Aβ 1-40 peptide, known for its high binding affinity to Aβ magnetically labelled with monocrystalline iron oxide nanoparticles (MION). Intraarterial injection of MION labelled Aβ 1-40, with mannitol to transiently open the blood–brain barrier, enabled the detection of Aβ plaques. Furthermore, the numerical density of Aβ plaques detected by magnetic resonance microimaging and by immunohistochemistry showed excellent correlation. This approach provides an in vivo method to detect magnetic resonance microimaging in AD transgenic mice, and suggests that diagnostic MRI methods to detect magnetic resonance microimaging in AD patients may be feasible (58).

NANOTHERAPEUTICS IN AD

An application of nanotechnology in AD is neuroprotection and drug delivery beyond the BBB. Concerning neuroprotection, nanodevices with antioxidant and anti-inflammatory properties are a therapeutic approach. Agents with such properties are water-soluble derivatives of C60 fullerenes. Some C60 derivatives reduce Aβ 1-42 induced apoptosis in neuronal cell cultures (59), blocked the Aβ 25-35 induced increase in cytosolic free calcium (60) and effectively inhibited Aβ 1-40 aggregation (61). Furthermore, C60 derivatives have been shown by transmission electron microscopy to inhibit the fibrillation of Aβ 25-35 (62). In the same study, an intraventricular injection of C60 derivatives to male Wistar rats significantly improved the performance of the cognitive task in control rats (62).
A dyshomeostasis of metal ions is suggested in AD with abnormally high levels of redox-active metals, particularly iron, but also copper, aluminium and zinc (63-65). Although it is unclear whether metal excesses are the sole cause of oxidative stress and neurodegeneration or a by-product of neuronal loss, the finding that metal chelators can partially solubilize Aβ deposits in AD suggests a promising therapeutic role for chelating agents (66; 67). However, the blood–brain barrier (BBB) and toxicity of known chelators limit their utility (68). Nanoparticles have been shown to penetrate the BBB probably by mimicking low density lipoprotein (LDL), thus permitting them to interact with the LDL receptor and resulting in their uptake by brain endothelial cells (69). Nanoparticles also have the potential to re-cross the BBB into the bloodstream. They may behave like lipoprotein particles by preferentially adsorbing apolipoprotein A-I (Apo-A-I), known to facilitate removal of nanoparticles from the brain through the same transport systems (70). Liu and co-workers (71) developed a chelator-nanoparticle system (CNPS) that was complexed with iron (ICNPS), using 2-methyl-N-(2'-aminoethyl)-3-hydroxy-4-pyridinone (MAEHP) and desferrioxamine (DFO) as chelators. CNPS has been shown not only to be effective at chelating iron from human tissue but also to bind to apolipoprotein E (ApoE) and Apo-A-I (71). Moreover, it was suggested that the inherent toxicity of the chelators is obviously decreased after conjugation with nanoparticles (71). Another chelator, D-penicillamine combined with a nanoparticle was investigated in AD by Cui et al (72). The Cu(I) chelator D-penicillamine was covalently conjugated to nanoparticles via a disulphide bond or a thioether bond. nanoparticle-chelator conjugates were stable between pH 6–8 in aqueous suspension, and did not aggregate when challenged with salts and serum. Release of D-penicillamine from the nanoparticles was achieved using reducing agents such as dithiothreitol (as a model for glutathione). Nanoparticles treated only under reducing conditions that released the conjugated D-penicillamine were able to effectively resolubilize copper–Aβ (1–42) aggregates (72).

The accumulation and aggregation of Aβ in the brain leads to amyloidogenesis process through which the toxic amyloid species are formed. Klajnert and co-workers (73) investigated the impact of dendrimers on aggregation properties of Aβ 1–28. Dendrimers are repeatedly branched molecules and thus possess high endgroup density at their “surface” (74-76). Three different types of dendrimers were selected in the study of Klajnert and co-workers: poly(amideamine) (PAMAM) dendrimers (5th and 6th generations), poly(propyleneimine) (PPI) dendrimers (3rd generation) and phosphorous dendrimers (P-dendrimers, 4th generation). To monitor interactions between dendrimers and Aβ, electron paramagnetic resonance (ER) technique was used. Both the spin probe (the neutral TOH and the positively charged CAT1 spin probes were used) and the spin label (the PAMAM dendrimers were labelled with a nitroxide radical) techniques were used to monitor the peptide-dendrimer interactions. The computer aided analysis of the EPR spectra at 255 K provided the information on the kind and strength of interactions occurring in the different systems. ER analysis showed that dendrimers possess an anti-assembly effect: Aβ 1–28 dipolarly interacted with the dendrimers preventing the separation of the probes in form of aggregates. Part of the probes interacted with the peptide, but this interaction was perturbed by the addition of the dendrimers. Another part of the probes were free, captured in the hydration layer at the dendrimer/peptide interface. The mobility and the relative amount of the interacting and the free component, obtained from spectral computation, change as a function of the dendrimer type, indicating a stronger interaction with PAMAM dendrimers, which are therefore suspected to work better as peptide-aggregation scavengers with respect to the other dendrimers (73).

Nanodevices play also a role in target drug delivery in AD. A recent study (77) evaluated brain up-take of rivastigmine, a reversible cholinesterase inhibitor by using poly(n-butylcyanoacrylate) nanoparticles. This study has been shown that the brain concentration of intravenously injected rivastigmine can be enhanced over 3.82 fold by binding to poly(n-butylcyanoacrylate) nanoparticles coated with 1% nonionic surfactant polysorbate 80 (77). Several mechanisms have been proposed for the transport of nanoparticles coated with polysorbate 80 across the BBB (78; 69; 79). Among those, the mechanism of endocytosis was supported by several studies (78; 69; 79). Poly(butylcyanoacrylate) nanoparticles coated with polysorbate 80 adsorbed apolipoprotein B and/or E after injection into the blood stream. The polysorbate acts mainly as an anchor for the apolipoprotein-overcoated nanoparticles, thus would mimic lipoprotein particles and could interact with and then be taken up by the brain capillary endothelial cells via receptor-mediated endocytosis (80).

However, selective targeting of nanoparticles to cerebral blood Aβ deposits remains still a challenge. A possible solution may be smart nanoparticles (SNVs) as they were developed by Agyre and co-workers (81). These SNV consisted of a diagnostic agent coated on the surface and/or a drug entrapped in their biocompatible polymeric core. In order to synthesize the polymeric core, chitosan was chosen in this study (81), because it is biocompatible and does not cause allergic reactions. Furthermore, chitosan is bioadhesive and improves drug absorption at cellular barriers due to its high positive charge density (82). Another advantage of chitosan nanoparticles (CPCs) is that they can be prepared under exceptionally mild conditions (83), which helps ensure the integrity of delicate compounds such as peptides, proteins, DNA, and siRNA. In Argyre and co-workers’ study, the polymeric core was coated with a Fab ‘2 fragment of Aβ antibody (IgG4.1) modified with paterns, referred to as pFab(“)24.1. A recent study has shown (84) that upon intravenous administration (IV) in AD transgenic mice, pFab(“)24.1 can cross the BBB, locate and bind to the cerebrovascular Aβ deposits as well as the amyloid plaques in brain parenchyma. A similar polymeric core coated with bovine serum albumin (BSA) served as a control nanovehicle (CNV). The BBB uptake of 125I-SNVs and 125I-CNVs was evaluated in mice. The uptake and transcytosis of SNVs and CNVs across bovine brain microvascular endothelial cells was evaluated using flow cytometry and confocal microscopy. This study demonstrated that the uptake of 125I-SNVs in various brain regions was about 8 to 11 times higher than that of 125I-CNVs. Furthermore, confocal micrographs revealed the uptake and transcytosis of SNVs, but not CNVs across the bovine brain microvascular endothelial cell monolayer.

A further therapeutic approach in AD is the administration of acetylcholine (ACh) that can improve the dementia, but free ACh cannot enter the brain because of its strong polarities and ease of decomposition in blood. Therefore, currently brain ACh can only be increased by clinical administration of certain mild inhibitors of acetylcholinesterases that hydrolyze ACh (85). As a possible drug carrier of ACh, carbon nanotubes (CNTs) have been investigated by Yang and associates (86). CNTs are nanoparticles with great promise in biomedicine as drug carriers, although their biosafety is of great concern. CNTs can interact with mammalian cells and enter cells via cytoplasmic translocation (87-90); they therefore can deliver a range of therapeutic reagents into the cell such as plasmid DNA (91), single stranded DNA (92), or polymers (93). Because single-walled CNTs (SWCNTs) have the ability to absorb inorganic (94; 95) and organic chemicals,(96-100) in addition to the ability to enter the brain via nerve axons, they are ideal for the delivery of ACh molecules. In addition, ACh is composed of an acetyl group and a quaternary ammonium group, which allows its easy absorption by SWCNTs. The curative effects on experimental AD indicated that SWCNTs successfully delivered ACh into the brain. Once inside the brain, they enter the lysosomes of the neurons – the pharmacological target organelles - and then release the drug ACh as a result of the increased polarity and hydrophilicity of ACh at the low lysosomal pH. Whereas SWCNTs did not induce reactive oxygen species (ROS) production in lysosomes, they did so in mitochondria. SWCNTs significantly decreased mitochondrial membrane potential (MMP), which was fol-
lowed by the increase of ROS demonstrating that mitochondria are the direct-target organelles of SWCNT damage (86). This toxicity could be avoided by maintaining the SWCNT doses under 300 mg/kg; at this dose, SWCNTs entered only lysosomes, the pharmacological target organelles, whereas few or none entered mitochondria (86). Thus, to evaluate the ability of nanomaterials to deliver drugs, it is important to assess their pharmacological and toxicological effects, to determine whether their negative effects can be avoided by any method.

CONCLUSION

Nanotechnology has a potentially revolutionary impact on the basic understanding, diagnostic and therapeutic approaches of AD. Promising multifunctional nanosensor for simultaneous detection of ultra low concentrations of different biomarkers has been developed: By using bio-barcode assay and adequate antibodies for example, one can routinely detect as low as 30 copies of a target protein, often in a complex sample such as plasma. This approach of using pathogenic markers of AD combined with the barcode assay or other nanosensors points toward potential reliable detection methods for diagnosing AD that are faster, higher throughput, and less expensive than current diagnostic techniques. This approach promises an earlier treatment of AD.

The current therapeutic approach in AD is to lower the symptoms. In order to design further useful means of treatment in AD, nanotechnology may play a crucial role. Targeted drug delivery systems beyond the BBB, chelator-nanoparticle systems and anti-amyloid therapeutics are three potential benefits of nanotechnological application in developing more potent therapeutics for AD. Although AD lacks definite diagnostic approaches and effective cure up to now, advances in nanotechnology may change this in future.

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THE JANUS FACE OF IMMUNE STIMULATION BY NANOMEDICINES: EXAMPLES FOR THE GOOD AND THE BAD

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Abstract

Nanomedicines are pharmaceutical agents consisting of ~20-400 nm size particles that overlap with the size of viruses and, hence, are recognized by the immune system. The resulting stimulation of the innate and antigen-specific immune responses can be either beneficial or harmful. This review focuses on two examples of the Janus faced interaction between the nanomedicines and the immune system; the beneficial stimulation of the complement (C) system leading to hypersensitivity reactions, called C activation related pseudoallergy (CARPA). In addition to the illustrated survey of the molecular-cellular interactions underlying these phenomena, this review provides an update on the clinical development of DermaVir and the state of CARPA prevention - anti-CARPA therapy.

Immunoreactivity of Nanoparticles

A major trend in the upcoming „age of nanotechnology” is the increasing medicinal use of complex, multi-modal vaccine or other drug delivery systems that provide superior protective or therapeutic effects compared to all previous approaches. However, these benefits are not without a price; the complex, multimodal character of „nanomedicines” generally involves an increase of the size over 50 nm, the approximate threshold of immune recognition (Fig. 1). In fact, the 50–400 nm size range of nanomedicines corresponds to the size of pathogenic viruses against which nature developed sensitive immune surveillance. Triggering the immune system by nanomedicines is a Janus faced phenomenon: its beneficial side is the efficacy of some nanomedicinal vaccines, while its harmful side is the adverse immune effects of cutting-edge nanomedicinal drugs. The contrasting characteristic of immune responses is originating from the size and the structure of nanomedicines since the body considers them as harmful viruses that needs to be eliminated. The goal of this review is to present examples of these good and bad faces of the immune activity of nanomedicines; a promising utilization of immune activation in a state-of-the art HIV therapeutic vaccine, DermaVir, and the self-destructive side effects of certain nano-drugs via complement (C) activation-related pseudoallergy (CARPA). The double focus of this review is further subdivided into: essentials in a nutshell and updates on clinical progress in terms of use or prevention.

Utilization of Immune Stimulation: DermaVir, A Therapeutic HIV Vaccine

DermaVir, a therapeutic vaccine candidate against HIV, represents a prototype pDNA/PEIm vaccine nanosystem consisting of plasmid DNA (pDNA), the active pharmaceutical ingredient (API), and mannosylated polyethylenimine (PEIm), acting as a pharmacologically active nanocarrier(1). These components assemble in 80–400 nm „soft” particles which, according to a recent proposal for unified nanoparticle classification (2), are S-3 category polymeric micelles. The pDNA expresses HIV antigens for presentation to T cells via Class I and II MHC molecules on the surface of dendritic cells (DC) (Fig. 2), as well as for whole proteins that form replication defective virus-like particles (VLP+) that are lacking, among other activities, integrase and reverse transcriptase. These particles are also taken up by antigen presenting cells (APC) that process them via the traditional antigen presentation route. The pharmacological activity of pDNA/PEIm nanoparticles resides in immune stimulation, both as opsonin for phagocytic uptake by Langerhans cells (LC), which are precursors of DC, and as a trigger for proinflammatory stimuli activating all immune cells that participate in the immune responses.

DermaVir is topically administered in a patch, „DermaPrep”, which assures that the vaccine nanoparticles penetrate through the epidermis and reach LC. The 80–400 nm size and repetitive mannose residues on the outside surface make DermaVir resemble immunogenic viruses. Following topical DermaPrep administration, LC take up the antigenic particles via endocytosis, mature to DC and migrate to the lymph nodes where the antigens are presented to CD4+ and CD8+ T cells via Class I and II MHC molecules. After endocytosis, the nanoparticles enter the endosomes where PEIm protects the pDNA from the endosomal degradation promoting the transport of pDNA to the nucleus for antigen expression (Fig. 2). Therefore, the pDNA/PEIm nanoparticles mimic entry, gene expression and antigen presentation of viruses that are potent inducers of functional CD4+ helper and CD8+ CTLs.

Although the exact mechanism of induction of immune responses by DermaVir has not been fully explored, it is known that PEI complexes formed with pDNA are potent activators of the complement (C) system (3,4). Byproducts of C activation, on the other hand, most importantly C3a and C5a anaphylatoxins, are potent activators of DC (5). Thus, as soon as DermaVir reaches the blood or other C-containing tissue fluids, the mechanism of C activation may become significant. In addition to the classical antigen expression and presentation on the surface of DC via Class I and II MHC molecules (Fig 2), gene expression after DermaVir immunization leads to the assembly of non-infectious, defective virus-like particles (VLP+), which bud off these cells just like HIV. Uptake and processing of these VLP+ by APC represents a further amplification step in the immunogenicity of DermaVir.

The novel mechanism of action of DermaVir is based on the expression of antigens in DCs present in lymph nodes, the organ where immune responses are induced (Fig. 3a). Therefore, DermaVir immunization is different from other pDNA or viral vector-based vaccines that express antigens at the injection sites. Incubation of DermaVir with cultured human immature DC resulted maturation, IL12 production and antigen-specific activation of naïve T cells (6). DermaVir-primed DCs induced potent Th1 type antigen-specific CD4+ T helper and CD8+ cytotoxic T cells (Fig 3b). In comparative studies DermaVir immunization was superior to that obtained using DNA vaccines in the induction of antigen-specific memory T cells (7) (Fig. 3c).

It is possible that DC maturation and IL12 production induced by the vaccine itself is responsible for the potent priming of Th1 type memory T cells. IL12 adjuvants that significantly improve the immunogenicity of DNA vaccines does not influence DermaVir-induced immune responses, because IL12 production by DC is already induced by DermaVir (8). These nanoparticle -primed DC are
very immunocompetent since immunizations of naïve macaques with autologous DC cultured with DermaVir, induce functional memory CTLs (6). These results encouraged us to develop pDNA/PEIm polymeric micelles as immunotherapeutic nanomedicine products for the induction of antigen-specific memory T cell responses.

**CLINICAL PROGRESS WITH DERMAVIR**

We have investigated in non-human primates the potential clinical indications of DermaVir for the treatment of HIV-infected individuals. First, we have immunized chronically infected monkeys, some of them with AIDS, with DermaVir in combination with antiretroviral therapy (ART+DermaVir) (Fig 4a). Following 6 weeks of DermaVir+ART treatment ART was interrupted for six weeks. We were surprised to see in these immunodeficient macaques a significant decrease of viral load rebound that was further improved by repeating the combination of ART+DermaVir therapy (9). These results suggest that DermaVir immunization in combination with ART might improve the potency and durability of the ART regimen. Next, we investigated DermaVir monotherapy in chronically infected macaques since there is a significant unmet medical need to provide a treatment option for the majority of HIV-infected individuals who are presently not receiving ART. We found that repeated DermaVir immunizations result in transient decrease of viral load that might be responsible for the improved survival (Fig 4b). These results suggest that DermaVir immunizations might be able to slow disease progression and maintain the health of HIV-infected people prior to ART.

pDNA/PEIm-based nanomedicine represent a platform technology for the induction of antigen-specific memory T cell responses against different antigens encoded by the pDNA. Changing the encoded antigens in the pDNA generates new immunotherapeutic nanomedicines. The first such pDNA/PEIm nanomedicine candidate, DermaVir Patch, recently completed three Phase II clinical trials in HIV-infected individuals. The phase I clinical trial previously demonstrated that a single dose DermaVir Patch immunization is safe, well-tolerated and immunogenic in HIV+ human subjects on ART. Phase II clinical trials have investigated repeated DermaVir immunizations in HIV+ ART naïve patients as well as in HIV+ individuals on ART (10). These trials are expected to demonstrate the safety, immunogenicity and preliminary efficacy of repeated DermaVir immunizations. Based on the encouraging non-human primate data we have been investigating the hypothesis that DermaVir immunizations used as a first line therapy prior to ART, in contrast to conventional vaccinations, does not induce additional immune activation that increases viral load facilitating disease progression. Subtype-specific DermaVir nanomedicine product candidates are under development for personalized HIV-specific immunotherapy that take into account the antigenic variability of the virus. We envision that a subtype-specific DermaVir product portfolio will be available for immunization of patients during regular visits at the doctor’s office. For the induction the optimal antigen-specific immune responses the HIV subtype replicating in the individuals will be diagnosed and the closest matching DermaVir product variant will be selected for treatment.

DermaVir immunotherapy does not induce drug resistance that could reduce the success of ART; in contrast, it might improve the response of patients to ART. The second pDNA/PEIm nanomedicine product candidate is HupaDerm which has been developed for the treatment of human papillomavirus (HPV)-associated diseases. The nanomedicine pipeline (Fig. 5) includes ChlamyDerm for the treatment of Chlamydia infections and DermAll for the treatment of allergies caused by house dust mites. Encoding further pathogen-, allergen- and cancer-associated antigens in single pDNAs can rapidly expand the pDNA/PEIm immunotherapeutic nanomedicine product portfolio. Since only the pDNA encoded antigen sequences differentiates these products from each other, the toxicity and bio-distribution, manufacturing and quality control methods are expected to be the same that significantly reduce the development cost and timeline.

**IMMUNE STIMULATION AS A BARRIER TO THERAPY WITH NANOMEDICINES**

C-mediated immunogenicity and immune toxicity of nanomedicines

Unlike with transcutaneous exposure, when the first encounter of a nanoparticle with the immune system occurs in the blood, the triggered immune response may be toxic. The phenomenon is best illustrated by the hypersensitivity reactions (HSRs) to many antitumor drugs that are formulated in either liposomes or in micellar solvent systems whose component particles activate the C system. Complement activation (Fig. 6A) entails the formation of C3a, C3b, C5a and C5b-9 split products, which attack the nanodrug in a highly organized multistep fashion (Fig. 6B). In one attack, the C5b-9 terminal C complex (TCC) damages the particle, for example the bilayer membrane of liposomes; a phenomenon whose seminal studies in the late sixties (11,12,13,14) led to the foundation of “liposomal immunology” (15). In another attack, opsonins, mainly C3b, flag the nanocarriers for phagocytic uptake (16), stimulated and coordinated by the inflammatory peptides C3a and C5a (Fig. 6B) (17). The consequences of nanodrug-induced C activation are numerous (list in Fig 6B) and fall under 2 categories; immunogenicity and immune toxicity.

**COMPLEMENT-MEDIATED IMMUNOGENICITY**

The significance of a drug’s immunogenicity lies in its potential loss for repeated or chronic human therapy. This is because antibody binding may influence the pharmacokinetics (PK) of the drug, and, hence its efficacy or toxicity. One example of such nanomedicine-induced immunogenicity is the accelerated blood clearance (ABC) of a second i.v. dose of PEGylated liposome 3-4 days after the first “immunizing” low dose (18,19). It has been established that the ABC phenomenon is mediated by anti-PEG IgM and is liposome size-dependent, with vesicles <40 nm not causing ABC (19). Among the underlying causes of PK and tissue distribution changes, C-mediated immunogenicity can cause structural damage and size enlargement of nanoparticles due C5b-9 buildup and C3b binding (Fig 6b). In addition, immunogenicity can give rise to adverse reactions, such as deficiencies in essential biomolecules due to cross-reactions with anti-drug antibodies and (late) hypersensitivity reactions (HSRs).

Although immunogenicity is a widely recognized barrier in protein and nanodrug therapies, it should be pointed out that studies have focused mainly on the identification of epitopes by T and B cells and other structural features of the drugs with little or no consideration of the likely role of C activation. In particular, C3 fragments and anaphylatoxins are known to bridge innate and adaptive immunity via numerous pathways, including facilitation of the cooperation between APC and T lymphocytes (20); stimulation of B cells and follicular dendritic cells via ligation to CD21-CD19/Tapa-1 intracellular signaling (23-26). Thus, C activation directly by the biological agent or nanodrug, or in a second step, after the binding of IgM, may represent a positive feedback mechanism for the activation of specific immunity.

**COMPLEMENT-MEDIATED HYPERSENSITIVITY**

A special form of C-mediated nanodrug immune reactivity, manifested in non-IgE-mediated HSRs, or infusion reactions, termed C activation-related pseudoallergy (CARPA) has been studied for the past 10 years (27-31). Although the symptoms of classical, IgE-mediated (Type 1) allergy and CARPA are identical or very similar (Table 1), there are major differences. Most importantly, CARPA arises at the first encounter of the drug and, unlike IgE-mediated allergy, the reaction decreases or entirely disappears at later exposures. The symptoms vary from light to severe, or even lethal, depending on the strength of the attack, species and individual sensitivity. CARPA is more frequent than classical allergy (frequency up to 40% (32)) despite the fact that the available preventive methods, discussed below,
substantially decrease its risk. Table 2 lists the groups and specific drugs for which CARPA-like reactions have been reported. Of note, 6 of the top 10 brand name drugs today can trigger such reactions (Table 3), highlighting the everyday relevance of the problem. As for its pathological mechanism, the primary trigger, C activation, launches a cascade of molecular-and cellular interactions with at least 3 relay/control/amplification steps: 1) formation of anaphylatoxins, 2) C3a/C5a triggering of mast cells (and basophils) and 3) activity of anaphylatoxins at the level of autonomic effector cells (smooth muscle and glandular secretory tissues) (Fig. 7).

The mentioned rationalization for C activation by nanomedicines, i.e., their similarity to viruses in terms of shape and size, represents only partial explanation. A further factor is the lack of anti-complementary proteins on the surface of reactogenic nanoparticles, which are present in host cells and prevent their autoimmune destruction. These include CR1, decay accelerating factor (DAF), membrane cofactor protein (MCP) and CD59 (33). In fact, the lack of an anti-complementary shield on the nanoparticle membrane surface explains that almost all activation pathways have been identified as being activated by various nanoparticles, even the lectin pathway, via ficolin binding (34). Fig. 8 shows that the most successful nanomedicines tested to date, the liposomes, can activate C both via the classical and the alternative pathways. Within the classical path, C1q can be activated by at least 3 different ways, directly, via antibodies and via C reactive protein. In short, C activation appears to be an omnipresent characteristic of liposomes and other nanoparticles above 50-60 nm. The exact size threshold for C activation most likely varies depending on other factors, such as shape and charge.

PREVENTION OF CARPA

Slow infusion, application of steroids, antihistamines, non-steroidal anti-inflammatory agents all have been successfully used against CARPA, but none of these procedures provide reliable, specific interventions (35). Other possible approaches to block the CARPA cascade at its various relay points (Fig 10), with C or C5ARI or COX inhibition, have not been clinically proven and, hence, are not accepted as standards of care. Based on the fact that CARPA often manifests itself as a self-limiting phenomenon, desensitization protocols should in theory work.

CLOSING REMARKS

Immune stimulation by nanomedicines can be useful and harmful, as illustrated by the two examples of this review. The HIV vaccine approach presented here as a nanomedicine with useful immune reactivity, is considered as nanomedicine because of the pDNA/PEIm particles therein are in the nanometer size range. However, most, if not all vaccines contain nanoparticles: polymers or aggregates of either the immunogens or adjuvants, or both. Thus, our closer scrutiny at how DermaVir stimulates immunity might teach us how vaccines might work in general. CARPA, on the other hand, provides a glimpse into the complex word of allergy and anaphylaxis. Complement activation by nanomedicines is usually intertwined with other direct and indirect activation mechanisms of mast cells and macrophages, and may be only a contributor mechanism, or a paraphenomenon, part of many redundant danger signals that foreign particle-like medicines activate. Further studies in these areas will allow us to gain deeper insights into the exact relationship between medicinal nanoparticle features, immunogenicity and immune toxicity.

LEGENDS TO THE FIGURES

Fig. 1. Sight of the immune system. Shading is the field of recognition, or “sight” of the immune system, illustrating a proposition that the minimum size and molecular weight of nanosystems recognized by the immune system is around 50 nm and 5 x 103 D, respectively. Some known drug carrier nanoparticles are positioned on the chart according to their size and MW range to illustrate their immune visibility. Abbreviations: MWCNT, multiwall carbon nanotube, MLV, multilamellar vesicles, SUV, small unilamellar vesicles.

Fig. 2. Schematic structure of DermaVir and key steps in its immunogenicity up to antigen presentation.

Fig. 3. Unique properties of DermaVir. (a) Antigen expression by lymph node DC after topical DermaVir administration. Upper panel: immunohistochemistry demonstrating pDNA-encoded antigen expression in the lymph node of a mouse. Lower panel: in situ hybridization demonstrates DC in the draining lymph node of a macaque expressing DermaVir pDNA. (b) Activation of naïve CD4+ and CD8+ T cells by cultured human DC primed with DermaVir nanomedicine. (c) Induction of memory T cell responses in mice by DNA vaccines (upper panel) and DermaVir immunization (lower panel).

Fig. 4. Non-human primate trials to determine the potential indication of DermaVir immunotherapy for HIV infection. (a) Median viral load of late stage SIV-infected macaques repeatedly treated with ART+DermaVir. (b) Survival of chronically SIV-infected macaques treated with DermaVir in the presence or absence of ART (9).
Fig. 5. pDNA/PEIm nanomedicine platform technology and pipeline.

Fig. 6. A and B. Scheme of the complement cascade and its various activation pathways (A), and pathways of complement attack on nanoparticles (B).

Fig. 7. The CARPA cascade. C5a liberated upon complement activation triggers mast cells for release of pre-formed inflammatory mediators, the effector molecules acting on smooth muscle and glandular secretory cells.

Fig. 8. Pathways by which complement can be activated by liposomes.

Fig. 9. Potential approaches for the inhibition of CARPA.
Table 1. Symptoms of classical (type I) and complement activation-related pseudoallergy

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<th>Neuro-</th>
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Table 2. Nano- and other drugs causing pseudoallergic symptoms (infusion reactions)

Table 3. Top 10 global biotech brands. Names outlined in red have been shown to cause infusion reactions corresponding to CARPA

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ABSTRACT

The holy grail in the treatment of various diseases is to deliver high doses of bioactive compounds with a high specificity to their target sites for maximum treatment efficacy, while minimising side effects to normal organs. Nanocarriers can accumulate extensively in solid tumour masses, inflammatory and infectious sites because of their size by virtue of the enhanced permeability and retention (EPR) effect of the vasculature. To date, various (macro)molecular assemblies with nanoscale dimensions such as liposomes, polymer-aggregates, carbon nanotubes and polymeric micelles have been or are currently evaluated as drug delivery systems. However, traditional polymeric micelles have thus far not shown their real potential benefits in the clinic. The dominant origin is the instability of a (drug-loaded) micellar structure in the dynamic environment of the blood stream. In recent years, a novel micellar platform technology has been developed in our laboratories and is based on an innovative, highly tuneable class of polymers designed to improve the risk-benefit ratio of a broad range of therapeutic agents. The superior blood circulation, fully controlled drug release profile, good tumour accumulation and excellent biocompatibility are proven features. Since it is a custom-made system, we have all tools available to develop this broadly applicable drug delivery technology towards actual medicinal products with an evident added value in the field of disease management.

1. NANOSIZED DRUG DELIVERY SYSTEMS

The holy grail in the treatment of various diseases is to deliver high doses of bioactive compounds with a high specificity to their target sites for maximum treatment efficacy, while minimising side effects to normal organs. Advanced drug delivery systems can contribute to this unmet need of “targeted therapies”. In recent years, particularly nanosized drug delivery systems are attracting increasing attention. Nanocarriers can accumulate extensively in solid tumour masses, inflammatory and infectious sites because of their size by virtue of the enhanced permeability and retention (EPR) effect of the vasculature (Figure 1) [1-3]. Characteristics of this EPR-effect include extensive angiogenesis, defective vasculature architecture, impaired lymphatic drainage, and increased production of permeability mediators. These characteristics are not (widely) present in healthy tissues and consequently provide a unique opportunity for selective targeting with nanosized drug delivery systems. Especially in oncology, this passive way of targeting has received great interest for the site-selective delivery of chemotherapeutic agents, that otherwise have low therapeutic indices, high systemic toxicity and/or a short blood half-life.

Figure 1 The unique enhanced permeability and retention (EPR) effect of the vasculature in tumour tissue allows preferential extravasation of circulating nanoparticles and accumulation in the tumour [1]. Reprinted with permission from Clinical Cancer Research

An ideal drug delivery system should fulfil the following requirements: • is synthesised conveniently and reproducibly with high yield and purity. • (self-)assembles into well defined and stable drug-load- ed structures. • allows encapsulation of diverse bioactive agents with high loading capacity. • increases the half-life of the entrapped compound. • masks bioactive compounds, which prevents occurrence of high peak levels in the blood, thus reducing toxic side effects (i.e. improving the so-called risk-benefit ratio). • displays selective tissue targeting. • releases the drug with controllable and tuneable kinetics (e.g. triggered by pH, ion, temperature and/or other internal or external stimuli). • increases the therapeutic efficacy. • degrades into non-toxic fragments to be cleared from the body without adverse effects. • offers clear medical advantages and differentiation. • improves patient compliance

To date, various (macro)molecular assemblies with nanoscale dimensions such as liposomes, polymer-aggregates, carbon nanotubes and polymeric micelles have been or are currently evaluated as drug delivery systems [4-6]. Polymeric micelles have thereby received considerable attention because of their marked advantages over other systems [7]. Polymeric micelles are nanosized particles that are formed by amphiphilic block copolymers (see Figure 2). In water, these copolymers self-assemble into core-shell structures above the critical micelle concentration (CMC) due to intermolecular hydrophobic interactions. The CMC as well as various other micellar properties are mainly a function of the type of amphiphiles, e.g. the chemical composition, the overall chain length, and the ratio between the hydrophilic and hydrophobic blocks [8].

Conceptually, the dense hydrophilic shell of a micelle prevents fast clearance by the immune system. Their hydrophobic core encapsulates poorly water soluble compounds, thus protecting the body against toxic peak levels while simultaneously the agent itself is protected against degradation. Their small size enables prolonged circulation in the blood stream and favour preferential accumulation in tumour tissue or inflammatory sites by virtue of the above described EPR-effect. In sum, the combination of micellar features is anticipated to facilitate selective drug accumulation at target sites and decreased toxicity of the entrapped cargo. From a producer’s perspective, polymeric systems are tailor-made and can be used with a
Therapeutic Nanomedicine

broad variety of hydrophobic agents. For the patient, ultimately, all beneficial advantages should result in a higher therapeutic efficacy, less side effects, enhancement of survival rate and improved quality of live.

However, traditional polymeric micelles have thus far not shown their real potential benefits in the clinic [7, 9-14]. The dominant origin is the instability of a (drug-loaded) micellar structure in the dynamic environment of the blood stream [6, 15]. Consequently, entrapped compounds are too quickly released from the micelle (instead of gradual, controlled release), resulting in high, toxic peak levels and circulation times as well as therapeutic responses that are at best equal to freely administered drugs. In the last years, we have developed a novel micellar platform technology that overcomes this intrinsic instability as well as other hurdles (e.g. ease of formation, freeze dried formulation etc.).

2. BIODEGRADABLE THERMOSENSITIVE MICELLES

2.1. THE CONCEPT

In the continuing search for the ideal drug carrier system, a novel class of polymeric micelles was developed in our laboratories and is based on thermosensitive polymers. Typically, an aqueous solution of a thermosensitive polymer is characterized by a so-called lower critical solution temperature (LCST) or cloud point (CP). Below this CP, water is bound to the polymer and prevents intra- and interpolymer interactions thus rendering the polymer water-soluble. Once the polymer solution is heated above the CP, the hydrogen bonds between the water molecules and the polymer chain are disrupted and water is expelled from the polymer chains. Interactions between the hydrophobic moieties of the polymer chain can now take place, which is associated with the collapse of the polymer and finally results in phase separation and aggregation/precipitation of the polymer. Poly(N-isopropylacrylamide) (PNIPAAm), which has a reversible and sharp phase transition at 32 °C in water, has been most extensively investigated as thermosensitive polymer [16-18]. Our former colleague, Osamu Soga, found that also oligolactate derivatised poly(N-(2-hydroxypropyl)methacrylamide) (poly(HPMAm-Lacn)) demonstrates a very nice thermosensitive behaviour [19]. Interestingly, the CP of this new class of polymers can be fine-tuned by the number and length of the lactate side chains. For example, the CP of a copolymer composed of HPMAm-Lac1 and HPMAm-Lac2 varies between 13 °C and 65 °C in water, depending on the ratio of the two monomers present in the copolymer (see Figure 3).

Interestingly, poly(HPMAm-Lac2), having a CP of 13 °C, is converted in time into the more hydrophilic and biocompatible poly(HPMAm) by hydrolysis of the lactate ester side chains at physiological conditions. Such a “hydrophobic-to-hydrophilic” conversion of the core of polymeric micelles is an interesting strategy to destabilise polymeric micelles and release their payload in a controlled way [20]. With this idea, we developed novel thermosensitive block copolymer micelles of PEG and poly(HPMAm-Lac2) [21]. Owing to their unique degradation properties, polymeric micelles (~ 60 nm) formed with these block copolymers showed controlled instability at body temperature. In other words, the block copolymers self assembled in polymeric micelles with a core of poly(HPMAm-Lac2), since the CP of this block is below 37 °C. Then, hydrolysis of the lactic acid side chains gave rise to hydrophilisation of the whole polymer in time and consequently increased the CP (see Figure 4). Next, when the CP of the polymer increases from below to above body temperature, the copolymer converts from the collapsed to the dissolved state in time and dissolution of the micelles occurs.

![Figure 4 Hydrolysis of mPEG-b-poly(HPMAm-Lac2)](Image)

It was shown that the dissolution time of empty micelles of mPEG-b-poly(HPMAm-Lac2) was approximately 1 week at physiological conditions (37 °C, pH 7.4) [22]. This may be too long for drug delivery in vivo, and therefore we designed similar block copolymeric micelles with a much shorter destabilisation time based on poly(N-(2-hydroxyethyl)methacrylamide-oligolactates) (mPEG-b-poly(HEMAm-Lacn)) [23]. The characteristics of these micelles were comparable with the mPEG-b-poly(HPMAm-Lac2), i.e. the critical micelle temperature was 6 °C and the average particle size was 80 nm. The hydrolysis of HEMAm-Lac was however much faster than HPMAm-Lac, which resulted in complete micelle disintegration within only eight hours at pH 7.4 and 37°C.

Safety of the micelles is ascertained as they are composed of building blocks and degrade into fragments that are already used in FDA approved products and/or reported to be safe in several clinical trials.

2.2. DRUG LOADING AND CHARACTERISATIONS

The mPEG-b-poly(HPMAm-Lacn) based micelles were loaded with paclitaxel (PTX) taking advantage of the thermosensitivity of poly(HPMAm-Lac2). The loading method was very straightforward: heating an ice-cold polymer-drug mixture till above the CMT resulted in micelles of 60 nm with a maximum loading capacity of 22% w/w. Incubation of the drug-loaded micelles showed a stable incorporation of PTX in the micelles at non-degrading conditions (i.e. pH 5), whereas precipitation of PTX occurred at elevated pH coincident with the time point at which the dissolution of the micelles started [22]. In vitro cytotoxicity studies showed that PTX loaded micelles were comparably toxic with respect to the commercial paclitaxel delivery vehicle (Taxol®). The advantage was however that the empty micelles were not toxic as compared to the Cremophor EL vehicle.
used in Taxol (Figure 5). Similarly, the safety of empty micelles was also demonstrated upon incubation with 14C cells [24].

![Graph](image)

**Figure 5** In vitro cytotoxicity of PTX-loaded mPEG-b-poly(HPMAm-Lac2) micelles (a) and Taxol (b) on B16F10 cells after 72 h of incubation. Left (gray) bars: formulation with PTX. Right (white) bars: control formulation without PTX. Data represent the mean and standard deviation of three independent experiments [22]. Reprinted with permission from Elsevier.

The easy method of micelle formation has been applied to encapsulate a large variety of other hydrophobic drugs (almost) quantitatively within the micellar cores (see depicted in Table 1) [22, 24]. Very recently, also hydrophobic iron oxide nanoparticles were entrapped within the micelles resulting in particles with a size of approximately 250 nm and with a relatively monodisperse size distribution (polydispersity of 0.2) [25].

![Graph](image)

**Figure 6** Modification of the terminal OH groups of mPEG-b-poly(HEMAm-Lac1)-co-(HEMAm-Lac2) with methacryloylchloride (n = 1, 2; y is % HEMAm-Lac1, z is % HEMAm-Lac2; m varies between 3 and 12 %).

Micelles were as usual formed by rapidly heating an ice-cold polymer solution to above the CP of the hydrophobic block. Subsequently, micelles were photopolymerised using Irgacure® 2959 as initiator. No change in particle size was found, e.g. non crosslinked (NCL) micelles had a diameter of 67 ± 8 nm (PD 0.10 ± 0.05) while after crosslinking the size was 68 ± 7 with a PD of 0.07 ± 0.05 (n=5). The physical stability of the core crosslinked (CCL) micelles was demonstrated by the preservation of small and monodisperse micelles when cooled below the CP as well as upon the addition of a surfactant (sodium dodecyl sulphate) that normally introduces rapid particle disintegration [31, 36, 37]. The degradation of core crosslinked micelles occurs in two steps. First, the non modified lactate side chains are rapidly hydrolysed and generate a hydrophilic core that swells in size depending on the crosslinking density. Secondly, after the slower hydroysis of the ester bonds in the crosslinks, the micelles disintegrate.

The circulation kinetics and biodistribution of 3H-labelled CCL and NCL micelles with the same size (~57 nm - PD of 0.1) was investigated in 14C-tumour bearing mice [38]. The NCL micelles were rapidly eliminated from the circulation and only 6 % of the injected dose (ID) was present in the blood after 4 hours. Low amounts (<1 % of the ID) of micelles resided in the spleen while the liver uptake was high (28 % ID after 4 hours). Although NCL micelles showed some initial tumour localisation (2.5 % ID 4 hours post injection), only 1.1 % ID/g was recovered in the tumours after 24 hours, indicating the absence of significant extravasation and tumour retention of the NCL micelles. Contrary to NCL assemblies, CCL micelles displayed significantly prolonged circulation times and more than 50 % ID was still present in the systemic circulation after 6 hours (Figure 7). Consequently, the AUC (0-24h) of CCL was substantially higher than that of NCL micelles (990 versus 136 % ID h/mL blood). After 24 hours, the liver accumulation of CCL was more than 2-fold lower than that of NCL micelles (10 % versus 24 % ID). The % ID of CCL micelles in the tumour was already fairly high after 1 hour (5 % ID/g) but this can probably be attributed to the high blood levels. More importantly, a considerable tumour accumulation was observed after 48 hours (5.8 % ID/g) despite the negligible blood levels of 3H-labelled CCL micelles by then. This clearly indicates that the small CCL micelles extravasated from the circulation and were retained into the tumour tissue, likely as a result of their small size and high AUC similarly as observed for long-circulating liposomes [39], which display blood half-lives of 5 - 20 hours [40, 41].

**Table 1** Overview of bioactive compound-loaded micelles based on mPEG-b-poly(HPMAm-Lacn) block copolymers (* final concentration achieved upon concentration by ultrafiltration)

<table>
<thead>
<tr>
<th>compound</th>
<th>micellar size (nm)</th>
<th>micellar polydispersity</th>
<th>loading capacity (w/w drug/polymer)</th>
<th>max. drug conc. mg/mL</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>corticosteroids</td>
<td>77</td>
<td>0.11</td>
<td>10%</td>
<td>2</td>
<td>manuscript in preparation</td>
</tr>
<tr>
<td>iron oxide</td>
<td>250</td>
<td>0.2</td>
<td>20%</td>
<td>5 *</td>
<td>[25]</td>
</tr>
<tr>
<td>paclitaxel</td>
<td>60</td>
<td>0.04</td>
<td>22%</td>
<td>2</td>
<td>[22]</td>
</tr>
<tr>
<td>photosensitiser</td>
<td>66</td>
<td>0.05</td>
<td>10%</td>
<td>2.2 *</td>
<td>[24]</td>
</tr>
<tr>
<td>vitamin K</td>
<td>120</td>
<td>0.3</td>
<td>10%</td>
<td>1</td>
<td>[48]</td>
</tr>
</tbody>
</table>

**2.3. CROSSLINKING TO IMPROVE THE STABILITY**

Ideally, the hydrophobic micellar core serves as a (protected) reservoir and delivery vehicle for hydrophobic drugs, whereas its hydrophilic shell is responsible for a favourable pharmacokinetic behaviour of the micelle. However, in vitro studies showed that the addition of either human albumin or serum to drug-loaded micelles substantially decreased their stability, thereby inducing rapid release of the encapsulated drug [26-29]. This is caused by two effects: first, the dilution of the micelles in serum causes a shift in the association equilibrium and consequently a decrease of the micelle concentration even if the total polymer concentration remains above the CMC; second, a further adverse shift of the micelle equilibrium can be expected upon adsorption of the polymers to plasma proteins (such as albumin or lipoproteins) that possess affinity for the hydrophobic blocks. Both aspects result in unwanted early disintegration of the micelles with a concomitant premature (burst) release of the loaded compounds and unfavourable biodistribution [30-33]. Apparently, the non-covalent hydrophobic forces that hold the micelles together are not strong enough to maintain the micellar integrity. Consequently, for in vivo applications, micelles require stronger interactions to be able to resist the physiological destabilisation forces and to prolong the circulation in the blood stream. Increasing the micellar stability can be established by either physical or chemical crosslinking of the shell, intermediate layer or core of the micelles [8, 34, 35]. Derivatising the hydroxyl groups of the lactate side chains of mPEG-b-p(HEMAm-Lacn) polymers with methacryloylchloride introduced polymerisable groups onto the thermosensitive block to allow core crosslinking (Figure 6).

![Graph](image)
Therapeutic Nanomedicine

The blood circulation time of 3H-DMSC co-crosslinked in 14C-labeled micelles was evaluated in B16F10-tumour-bearing mice and compared to 3H-DMS that was non-covalently loaded in core-crosslinked micelles and free 3H-DMS (Figure 9) [43]. The non-covalent encapsulation of 3H-DMSC in micellar core marginally improved blood circulation and tumour accumulation when compared to free 3H-DMS. However, the co-crosslinking resulted in 3H-DMSC tumour levels of approximately 25-fold higher than free 3H-DMS. This clearly indicates that non-covalently encapsulated 3H-DMS, despite the stability and prolonged circulation of the crosslinked micelles themselves, is rapidly released from the carrier after intravenous injection. Only upon covalent co-crosslinking of 3H-DMSC inside the micellar core, the drug levels exactly matched high 14C-polymer levels in all tissues. This unambiguously proves that the DMS-loaded core-crosslinked micelles circulated as an intact entity throughout the body.

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The latter study clearly demonstrated that the transient conjugation of dexamethasone to the crosslinked micellar core prevented premature burst release in the blood stream. Consequently, these drug-loaded micelles travelled together through the blood circulation like a loaded “bullet”. Next, this stable complex displayed a long blood circulation and as a result, the drug-loaded micelles accumulated significantly in tumour tissue. The type of linker used determines the release rate of the native DMS, which can then exert its therapeutic (anticancer) effect. The biodegradable character of the crosslinked micelles themselves assures disintegration into small fragments that can be eliminated via renal clearance [38].

Liposomes, which can be regarded as the current golden standard of nanoparticulate drug carriers, display prolonged blood circulation and effective tumour accumulation [39, 44]. In comparison with dexamethasone-loaded liposomes, our optimised micellar drug delivery system showed higher AUC values leading to higher tumour accumulation [43]. This is an important feature as the AUC is the major factor for allowing passive accumulation at sites of increased capillary permeability. Next, the micelles display almost quantitative encapsulation of a broad variety of hydrophobic compounds. In contrast, liposomes can mainly entrap hydrophilic compounds with a loading efficacy of approximately 10 – 15%, thus requiring laborious methods for the removal of non-encapsulated drugs.

Another well known type of drug delivery vehicles are polymer-drug conjugates [2, 45, 46]. These have several disadvantages: each drug necessitates the synthesis of a novel polymer-drug conjugate (with a corresponding risk of batch-to-batch variation); they have a low loading capacity, require a high molecular weight (to have a sufficiently prolonged blood circulation), and a drug connected to a water-soluble polymer remains exposed to its environment, whereas drugs encapsulated in micelles are hidden and cannot exert any (side) effects.

Traditional micellar drug delivery systems do not create stable nanoparticles in vivo, do not posses a really controllable release profile, and are consequently merely solubilisers instead of an actual drug delivery system. Our novel micellar technology combines the best features of known nanosized drug delivery systems into one single, transiently stable nanoparticle structure with tuneable release of entrapped agents.
The novel micellar platform technology is based on an innovative, highly tuneable class of polymers designed to improve the risk-benefit ratio of a broad range of therapeutic agents. The superior blood circulation, fully controlled drug release profile, good tumour accumulation and excellent biocompatibility are proven features. Their ease of formation, quantitative drug encapsulation and long term stability are additional advantageous properties. Its exclusive profile is anticipated to improve the therapeutic value of various bioactive agents significantly.

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REFERENCES
NANOMEDICINE FOR DENDRITIC CELL-TARGETED IMMUNOTHERAPY

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Active immunotherapies against tumors and viruses have to induce functional memory CTLs that requires the activation of naïve T cells by dendritic cells (DC). Sipuleucel-T, the first cancer immunotherapeutic product expected to create new treatment paradigm, employs transplantation of ex vivo cultured DCs. To improve the clinical benefits and reduce the costs of ex vivo therapies, in vivo DC loading nanomedicine technologies are under development. These DC-based nanomedicine products must overcome the complex problems of receptor targeting and uptake of the antigen, maturation and migration of DCs to the lymphoid organs, antigen processing and presentation to naïve T cells. The combination of two new technologies, antigen nanoformulation and topical DermaPrep administration, has shown to target antigens in vivo to lymph nodes by a subset of epidermal DC leading to the induction of functional memory T cells. This DC-targeting nanomedicine platform technology has been developed for the treatment of infectious diseases and cancers, including HIV/AIDS.

1. INTRODUCTION

The immune system resembles a performing orchestra: a conductor leads all the musicians. Until the discovery of dendritic cells (DC), we did not know the conductor. In 1973 Ralph Steinman and Zanvil Cohn first described the function, cellular and physiological characteristics, and tissue distribution of DCs in mice [1]. However, extensive tests could not be performed until the nineties, when the appropriate technique for isolating DCs in large quantities became available.

Unfolding the central role of DCs altered the science of immunology. These cells harbor innate and adaptive immune responses and are involved in the induction of both humoral and cellular immunities. Due to their ability to recognize “pathogen associated molecular patterns” (PAMP) they can rapidly trigger innate immunity and in parallel they function as professional antigen presenting cells (APC) of the adaptive immune branch. As APC, antigen loaded DCs undergo maturation and migrate from the periphery to the lymph nodes where they reside within the T cell regions presenting antigenic epitopes to -specific T lymphocytes and thus initiating a specific immune reaction. DCs are the only APCs capable of priming naïve T cells and inducing new immune responses. Such antigen-primed lymphocytes undergo proliferation, leave the lymph nodes and circulate throughout the body to find and selectively eliminate infected and cancerous cells [2].

The potential of DCs to induce functional memory T cell responses has triggered their use in translational research in the field of immunotherapy. Since the immune system represents the first line of defense against infections and tumors, immunotherapy might become the key approach to transform lethal diseases into chronic diseases. Clinical trials of cancer immunotherapy indicate that immunization with antigen-primed DC gives the highest immune response [3]. To date, clinical benefits are still moderate, but technology improvement in this field is expected to result in the development of new biological products defined as “first in class” because of their novel targets or new mechanism of action.

Various antigens have been used for the ex vivo loading of DCs including peptides [4, 5], proteins [6], RNA [7, 8] and tumor cell lysates [9, 10]. Ex vivo loading of DCs is performed on cultured autologous DCs isolated from patients in a well-controlled laboratory environment. Technological improvement aims to overcome burdens of ex vivo immunization and increase clinical benefits.

Here we outline successes and challenges of a state-of-the-art DC targeting immunotherapy and present how nanomedicines might optimize in vivo DC targeting in the field of infectious disease and cancer therapy.

2. THE FIRST DC-BASED CANCER IMMUNOTHERAPY TO TREAT PATIENTS WITH PROSTATE CANCER

Clinical trials conducted before the millennium demonstrated the excellent safety and tolerability profile of ex vivo DC-based immunotherapies [11, 12]. These trials employed immature rather than mature DC, which later turned out to be much less immunogenic [11]. Since then, the field has developed rapidly and the first DC-based cancer immunotherapy, called Sipuleucel-T, may soon be available for the treatment of prostate cancer. Two Phase III clinical trials on cancer patients treated with Sipuleucel-T for 36 months evidenced a 4.5 months average survival benefit compared to placebo-treated patients [13]. Sipuleucel-T induces functional new immune responses against a self-antigen and has the potential to create a new treatment paradigm in the oncology field.

Sipuleucel-T is an ex vivo immunotherapy for late-phase prostate cancer patients. It stimulates prostate-specific T cell immunity by ex vivo antigen-primed autologous DCs. Patients undergo a mononuclear cell leukapheresis and these specimens are transported to the vaccine manufacturing facility. Autologous DCs are pulsed with a recombinant antigen consisting of prostatic acid phosphatase (PAP) linked to granulocyte-macrophage colony-stimulating factor (GM-CSF) in order to facilitate DC maturation. Then antigen-loaded cells are transported back to the clinic and infused intravenously into the patient [14]. PAP-loaded mature DCs reach the lymph nodes and present antigens to naïve T cells. PAP-primed T cells then proliferate and become PAP-specific CTLs. Unlike most other tissues of the body, the vast majority of prostate cancer cells express PAP, leading to the killing by PAP-specific CTLs of prostate cancer cells.

Ex vivo DC-targeting immunotherapeutic products have tremendous manufacturing, logistic and cost challenges, and patients need to undergo leukapheresis that is not always possible because of their poor physical conditions. Regulatory agencies consider ex vivo DC-based therapies as a transplantation process, accordingly the quality of the product differs from patient to patient. Therefore, Sipuleucel-T and similar ex vivo products are not suitable for the treatment of millions of people suffering from cancer. Nanotechnology could offer an improvement, employing novel approaches for in vivo DC loading of antigens. DC-targeting nanomedicines might be manufactured and administered in vivo at the doctor’s office similar to conventional vaccines. The lower costs and the standard logistics could make such nanomedicine treatments accessible for millions of people including patients living in resource poor countries (Figure 1).

3. NANOTECHNOLOGY FOR IN VIVO ANTIGEN TARGETING TO DCs

A possible key for the next generation of immunotherapy is to deliver antigens in vivo distinctively to DCs. This kind of nanotechnology must find a solution for both DC-targeted antigen delivery and DC activation and address complex problems such as receptor targeting, uptake of the antigens, maturation and migration of DCs antigen processing and presentation to naïve T cells.
Preventive Nanomedicine

Encapsulation of antigens into liposomes or polymer nanoparticles has been exploited to achieve targeted in vivo delivery to DCs, on the one hand to protect the antigens from degradation and on the other to facilitate endocytosis by changing their size and surface charge [15]. Receptor ligands can also be incorporated e.g. pathogen associated molecular patterns (PAMPs) that are recognized by the surface pattern recognition receptors (PRRs) of DCs. Natural DC ligands and receptor-specific antibodies attached to nanoparticles can target PRRs including Toll-like receptors, C-type lectins and Fc receptors.

Receptor DEC-205 is a C-type lectin family mannose receptor responsible for antigen uptake. This receptor was targeted with anti-DEC-205 antibodies – either conjugated to HIV p24 protein or incorporated into liposomes containing the protein antigen [16, 17]. C-type lectin receptor DC-SIGN targeted by its antibody (anti-DC-SIGN) improved DC-transducing capability of an adenovirus vector [18]. Selection of the target is essential for efficacy: for example targeting Fc receptors induced Th2 type immune response that is unfavorable for infectious disease or tumor immunotherapy [19]. Maturation of antigen-loaded DCs is essential for their migration to the lymphoid tissues and presentation of the antigen-derived epitopes to naïve T cells. For the induction of maturation IFN-γ and anti-CD40 are used, as well as Toll-like receptor ligands like polyI:C, CpG, and LPS [17, 20, 21].

Anti-DEC-205 as a targeting molecule in combination with IFN-γ or LPS as a maturation signal has been incorporated into liposome and injected into mice to improve tumor immunotherapy [17]. A similar study has used anti-DEC-205 conjugated to HIV p24 protein in combination with anti-CD40 and polyI:C as maturation signals to induce protective immunity in mice [21]. It remains to be determined whether these intravenously infused in vivo DC-targeted nanomedicines will provide a significant improvement in commercial product development.

Due to the central role of DCs in the induction of immune responses, it is a challenge to find the optimal combination for immunostimulation. DCs in the blood are the precursors of different subsets of tissue DCs that reside in the epidermis, intestine, liver, etc. [22]. These subsets differ both in surface markers and immune response stimulation characteristics and determine T cell homing independently from the type of the immune responses [23]. Therefore, it is extremely important to target the antigens to the appropriate DC subset according to the desired tissue specific immune response.

4. CLINICAL STAGE DERMAVIR NANOMEDICINE FOR IN VIVO DC-TARGETING IMMUNOTHERAPY

We have developed two new technologies for in vivo DC targeting, an antigen nanoformulation and a topical administration that need to be used in combination for effective antigen loading to lymph node DCs and for the induction of functional memory T cells (Table 1). In DermaVir nanomedicine the plasmid DNA-encoded antigens are encapsulated in a mannansylated polymer to form a pathogen-like nanoparticle [24]. This nanomedicine is administered topically to DCs by DermaPrep medical device. DermaPrep activates the epidermal subset of DCs, called Langerhans cells (LCs), by a slight epidermal injury for uptake of the nanomedicine and consequent maturation and migration to the lymph nodes. DermaPrep administration of the pathogen-like nanomedicine mimics the natural way of DC activation and antigen loading by pathogens after a skin injury. Based on these technologies we have been developing the first in vivo DC-targeting nanomedicine for the treatment of HIV-infected individuals. DermaVir Patch is a Phase II clinical stage combination product of DermaVir, an HIV-specific pDNA-based nanomedicine and DermaPrep, an in vivo DC targeting medical device [25].

We have designed the pathogen-like nanomedicine to target DCs, facilitate gene expression and antigen presentation [26]. It consists of three ingredients in a liquid formulation: (1) the active pharmaceutical ingredient (API) is a plasmid DNA (pDNA); (2) a novel polymer, polyethylenimine mannose (PEIm); and (3) a water based solvent. PEIm encapsulates pDNA resulting in nanoparticles of 80-400 nm size. Since PEIm is the nano-carrier of the pDNA, the nanoparticles have mannose residues on their surface. For DC and Langerhans cells (LC) these nanoparticles appear to be pathogen-like particles due to their size, charge and mannansylated surface. These nanoparticles enter cells via endocytosis and the polymer protects the DNA from the endosomal degradation and delivers it to the nucleus for antigen expression. We have demonstrated that incubation of this nanomedicine with cultured human DC results in the potent activation of naïve T cells to Th1 type antigen-specific T cells and in the induction of functional memory CTLs in vitro and ex vivo after injection in macaques [27]. The greatest advantage of this pathogen-like nanomedicine is that nanoparticles mimic the entry, endosomal release, gene expression and antigen presentation of a pathogen, due to the smart activity of the polymer nano-carrier.

To overcome the challenges associated with the ex vivo DC-targeting immunizations we have developed DermaPrep medical device to target the nanomedicine in vivo to lymph node DCs where new immune responses are generated (Figure 2). DermaPrep consists of three components: a body sponge, a medical tape and an empty patch (Table 1). The body sponge and medical tape are used for the exfoliation and cleansing of the skin surface. These two components ensure a reproducible skin preparation method that adequately interrupts the barrier function of the stratum corneum and causes a minor skin injury (erythema) to mimic the natural danger signal that activates LCs. After preparation of the skin the empty patch is applied to the skin surface. The adhesive is laminated only to the periphery of the patch, thus after application, a pocket is formed between the patch and the prepared skin site. The administration of the liquid nanomedicine into the patch is performed with a needle free syringe by inserting it into the pocket area through a triangular opening. The patch is suitable for keeping the nanomedicine in contact with the activated LCs for several hours.

For the induction of functional memory CTL we had to employ LCs and not dermal DC, because dermal DC prefer to induce humoral immunity [28]. Therefore, horizontal targeting of the epidermal LCs is preferable to vertical targeting in order to avoid the activation of dermal DCs that would push the balance toward Th2 type immune responses. Furthermore, it has been recently demonstrated that antigen delivery to the injured epidermis is essential for the induction of protective T cell responses against infectious diseases and cancer compared to intradermal or subcutaneous administration [29]. We have developed a patch-based horizontal administration technology covering larger surface than skin scarification because the LC network locates horizontally in the epidermis under the stratum corneum. The presently used prototype DermaPrep device covers 80 cm² of skin surface which ensures that approximately 7-10 million epidermal LCs are involved in the uptake of the nanomedicine [30].

The topical DermaPrep delivery of the pathogen-like nanoparticles mimics a natural skin infection resulting in induction of functional CTLs for the elimination of the intracellular pathogens (Table 1, Figure 2). The skin preparation procedure generates a „danger signal” similar to a mild skin injury and then pathogen-like nanoparticles penetrate the skin mimicking pathogen entry during an infection. The pathogen-like nanoparticles are endocytosed selectively by the active matured LCs that mature to DCs and migrate to the draining lymph nodes. Meanwhile, PEIm protects the pDNA from degradation and facilitates the expression of pDNA-encoded antigens [31]. These antigens are processed by the DCs like the antigens of intracellular pathogens. Once the DCs reach the lymph nodes they present the expressed and processed epitopes of the pDNA-encoded antigens to naïve T lymphocytes, in the same way they would in a case of an intracellular pathogen.

We have demonstrated that topical DermaPrep immunization with the pDNA/PEIm nanomedicine results in potent HIV-gag-specific memory T cell response. In contrast, intramuscular injection of
the same naked pDNA was inefficient at inducing memory T cells (Figure 3) [34]. The relevant HIV-specific DermaVir nanomedicine improved survival and reduced viral load in chronically-infected macaques including three with late-stage AIDS [32, 33, 34, 35]. A Phase I study was conducted on nine HIV-infected subjects treated with fully suppressive HAART. A single topical immunization with 0.1, 0.4 and 0.8mg DNA doses suggested that DermaVir is safe and well-tolerated [36, 37]. This human study confirmed the preclinical animal studies with the immunogenicity results that suggested the induction HIV Gag-specific memory T cells (Figure 4).

Combination of antigen nanoformulation and topical administration technologies to target antigens in vivo to DCs form a platform technology. These technologies have been developed to induce functional antigen-specific memory CTL responses against different antigens encoded in the pDNA. By changing the nucleotide sequence of the pDNA in the nanomedicine, novel immunotherapeutic nanomedicines can be rapidly developed against different infectious diseases and cancers (Figure 5). Regulatory pathways of such pDNA-based nanomedicines are expected to be similar. Furthermore, the nanomedicine toxicity and biodistribution will be the same in animal models, because all products consist of the same chemical components, namely pDNA, PEIm and solvent. The large-scale manufacturing and quality control of nanomedicines products and components will also be comparable. These products might represent an improvement to the ex vivo DC immunization technologies and could be accessible for patients living outside the US and Europe. The scientific community has to face this key issue because in case of e.g. HIV/AIDS and cervical cancer most of the affected patients live in developing countries.

5. Conclusions

DCs are capable of inducing clinically effective and long lasting memory CTLs against cancer and infectious diseases [3, 39]. Ex vivo DC-targeting immunotherapy technology will hopefully soon deliver the world’s first cancer vaccine for the treatment of late-stage prostate cancer. The unmet need to improve clinical benefits and to reduce treatment costs drives the product development of DC-based therapies towards in vivo targeting nanomedicines. The development of in vivo DC-based nanomedicines is challenging because it requires innovative and sophisticated targeting technologies to minimize the antigen uptake by bystander cells that cannot induce functional CTLs. It also has to ensure the appropriate activation and the maturation of the targeted DCs. DermaVir Patch nanomedicine unites both biological and physical targeting of epidermal LCs that deliver antigens to the lymph node and induce functional memory CTLs with comparable efficiency to ex vivo approaches. DermaVir Patch is the next generation innovative nanomedicine candidate suitable for the treatment of individuals living with HIV/AIDS.

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References

Preventive Nanomedicine


FIGURE LEGEND
Figure 1: Treatment approaches with ex vivo and in vivo DC-based nanomedicine products. Grey arrows indicate how many times a patient has to attend the clinic for one treatment.

Figure 2:
Mechanism of action: In vivo antigen targeting of an epidermal DC subset, Langerhans cells (LCs) using nanomedicine formulation and DermaPrep administration
(a): Potential administration sites on the skin and cross-section of the epidermis with immature LCs (b): DermaPrep skin preparation method interrupts the stratum corneum and activates LCs; danger signal: mild, transient erythema (c): The patch is placed on the prepared skin surface and liquid nanomedicine is filled under the patch (d): Activated LCs take up the pathogen-like nanoparticles (e): Antigen-loaded LCs mature to DCs and migrate to the draining lymph nodes while pDNA encoded antigens are expressed and processed (f): DCs activate antigen-specific CD4+ and CD8+ T cells which start to proliferate (g): Th1 type functional immune response is induced: antigen-specific CD4+ and CD8+ T lymphocytes circulate in the body of the treated patient.

Figure 3:
Comparison of topically administered pDNA/PEIm nanomedicine with intramuscular injection of naked pDNA
pDNA (CMV-gag expression plasmid) was administered to mice intramuscularly and topically in pDNA/PEIm nanoformulation. Memory CTL activity was measured by caspase activation assay. Caspase positive cells were detected after 7 days peptide stimulation. pDNA administered i.m. (●) and pDNA/PEIm administered topically (■) [34].

Figure 4: DermaVir-induced Gag-specific T memory cells in patients treated by fully suppressive HAART

Memory T cells were quantified by a PHPC assay (Precursors with High Proliferative Capacity) [38]. A single DermaVir immunization of three different doses (n=3 in each cohort) significantly increased Gag-specific memory T cell pool compared to baseline (PHPC counts prior to immunization). Bars show mean and standard deviation. (* data shown after 24 weeks, no samples were available one year after vaccination).

Figure 5: Development of DC-targeting nanomedicine products for immunotherapy of infectious diseases and cancers

Combination of two technologies to target antigens in vivo to DCs, an antigen nanoformulation and a topical administration, supports the rapid development of new products for the induction of functional memory CTLs.

Table 1. A multi-level in vivo DC targeting system

<table>
<thead>
<tr>
<th>Components:</th>
<th>Function:</th>
<th>Result:</th>
</tr>
</thead>
<tbody>
<tr>
<td>DermaPrep</td>
<td>For in vivo nanomedicine delivery to DCs</td>
<td>Body Sponge  Improves penetration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medical Tape  Mimics epidermal injury to activate LCs</td>
</tr>
<tr>
<td>Patch</td>
<td>Topical horizontal administration</td>
<td>Targets 900-1800 epidermal LC/mm2</td>
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<tr>
<td>pDNA</td>
<td>Encode disease-specific antigens</td>
<td>Expression of pDNA encoded antigens</td>
</tr>
<tr>
<td>PEIm</td>
<td>Encapsulating the pDNA into mannosylated nanoparticles</td>
<td>Endocytosis of the nanoparticles by LCs</td>
</tr>
<tr>
<td>Pathogen-like nanomedicine</td>
<td>Endocytosis, endosomal escape of pDNA and its transfer to the nucleus</td>
<td>LC maturation and migration to the draining lymph nodes</td>
</tr>
<tr>
<td>For antigen delivery to DCs</td>
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Nanomethods

SCANNING X-RAY SCATTERING: EVALUATING THE NANOSTRUCTURE OF HUMAN TISSUES

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INTRODUCTION

In general, human tissue is organized in three-dimensional fashion and exhibits anisotropic properties that rely on its structure ranging from the atomic to the macroscopic level. While the anatomy describes the morphology and function of entire organs, the underlying processes on the cellular level are the focus of cell biology. Biochemistry concentrates on the molecular interactions.

Physicists are also fascinated by the human being but can only hardly contribute to the understanding of medical sciences. The exact description of complex structures comprising of a huge number of components is simply impossible. Note, the average human being in Switzerland is composed of \(2.5 \times 10^{27}\) atoms. Even the number of cells within such a person \((10^{14})\) is much larger than the number of stars within the Milky Way \((3 \times 10^3)\). Consequently, physicists are always impressed by the work of medical doctors. The medical doctors often come to decisions within seconds just looking at the patient asking a very few well-defined questions. Some of these medical doctors, however, need feedback from imaging facilities. Therefore, powerful radiology departments have been built up. Computed tomography, the facility with the best spatial resolution available for radiologists, reaches sub-millimeter resolution and therefore cannot provide images of individual cells within the human body. Only recently, micro computed tomography images obtained at synchrotron radiation facilities show individual cells of human tissue.

Images of cells are usually obtained by means of optical or sometimes electron microscopy. These data show a wealth of micro- and nanostructures, but are restricted to transparent tissues and surfaces. Surfaces, however, require expensive preparation procedures, which often result in substantial artifacts. Therefore, X-rays may be considered as the probe of choice to make visible morphological features within human tissues in a rather non-destructive way.

Since the real-space imaging using X-rays hardly reaches the nanometer scale, diffraction or scattering techniques are much better suited for the quantification of nanostructures. They yield exact mean values of the illuminated volume, which can be regarded as advantage. For the anisotropic and inhomogeneous human tissue, however, direct spatial resolution is highly desired. As a consequence, scanning along the specimen has been combined with the X-ray scattering methods. One distinguishes between wide-angle X-ray scattering (WAXS) for the analysis of the atomic structure and small-angle X-ray scattering for uncovering the nanostructure. Their combination with focused X-ray beams for scanning with true micrometer resolution requires high photon fluxes provided by synchrotron radiation sources and highly efficient and fast detection systems, which only became recently available.

The review demonstrates the power of scanning X-ray scattering for the visualization and quantification of the nanostructure in selected biological hard and soft tissues: tooth, bone, brain, and urethra, which goes beyond published work often related to bone and breast tissues in healthy and diseased states. The technique yields the abundance and orientation of all nanometer-sized organic and inorganic components within the X-ray beam focused to diameters in the micrometer range. The computer code for scattering data analysis is constantly developed and already reached a sophisticated level, which enables the suitable representation of X-ray absorption, abundance and orientation of nanometer-sized components with predefined length scale. These representations are easily readable for clinicians from different areas including dentistry and clearly reflect the tissue anisotropies usually related to the direction of growth and mechanical stimulation.

EXPERIMENTAL

X-rays that interact with matter are scattered and form patterns, which reflect the structure including the texture of the specimen. Experimentally, the scattered X-rays are recorded at the distance \(D_{sd}\), whereby the through beam is covered by the beam stop (see Figure 1). This is necessary because the intensity of the through beam is much higher than the scattered portion of the X-rays. The pattern to be detected represents exact average values of the illuminated volume of the specimen. In order to obtain spatially resolved data, the specimen or the beam has to be moved in x-y-directions and a scattering pattern from each point has to be acquired for further analysis. To obtain the desired spatial resolution in real space, the monochromatic X-ray beam is often focused to micrometer size.

Figure 1: The X-ray beam of micrometer size hits a tissue slice - such as a histological section of the human thalamus - and forms a scattering pattern on the detection unit in distance \(D_{sd}\). Scanning the tissue slice in micrometer steps in x-y-diretions, the patterns are recorded for subsequent evaluation.

The sizes of the periodic nanostructures within the specimen are inversely proportional to the scattering angle \(2\theta\), given by the distance \(r\) from the through beam in the scattering pattern (see Figure 1). Therefore, two methods were introduced: small angle X-ray scattering (SAXS) with \(D_{sd}\) of several meters and wide angle X-ray scattering with rather short \(D_{sd}\). SAXS serves for detecting nanometer-sized features between about 1 nm and 1 µm, whereas WAXS covers the scales below until the atomic distances are reached.

The total acquisition time for the scanning X-ray scattering measurements depends on the number of spots in the raster scan and the exposure time used. The latter in turn will depend on the scattering contrast in the specimen and the signal-to-noise ratio of the data desired. To give an example, one may raster scan a tissue specimen less than 100 µm thin in steps of 50 µm between adjacent spots and record thereby 40’000 patterns per square centimeter. At a data rate of 20 scattering patterns per second less than one hour is needed for
the data recording. In general, several samples can be mounted on the motorized holder to measure the specimens one-by-one in automatic way.

Solid specimens such as resin or paraffin embedded histological slices can be directly measured. Specimens in a wet environment (phosphate buffer saline or formalin solution) are held in a polyimide sachet.

**EVALUATION OF X-RAY SCATTERING PATTERN**

Scanning SAXS and WAXS are raster scanning rather than full-field imaging techniques. This means, that each image is the result of a computerized analysis of typically several ten thousands of individual measurements.

Figure 2 shows a scattering pattern with many details obtained at the cSAXS beamline of the Swiss Light Source (Paul Scherrer Institut, Villigen, Switzerland). The ring structures are interrupted by the periphery of each module of the detection unit. Because of the symmetries in the pattern, however, the rings can be easily interpolated.

To quantify the acquired information, each of the thousands scattering patterns is divided in 16 radial segments around its center (see Figure 2 on the right). The integrated intensity of each segment at the pre-defined radius range is plotted as the function of the angular position (see red-colored data points in the graph of Figure 2). The symmetry-equivalent intensities from opposite segments are averaged. This means, one obtains eight values for the 16 segments. Approximating these eight values by means of a sine yields three parameters of interest. First, the abundance of nanostructures on a length scale range given by the radius range over which the intensity is integrated is derived from the offset \( I_{\text{sym}} \). Second, the anisotropy of these nanostructures relates to the amplitude \( I_{\text{sym}} \). Third, the phase shift of the sine determines the preferential orientation of the nanostructures. The three parameters are finally presented in the x-y-plane to be interpreted by the specimen suppliers. For the soft and hard human tissues, detailed anatomical knowledge is required to describe the features the medical experts are looking for.

![Figure 2: The scattered intensity is integrated along a ring around the central beam, divided in 16 segments. The intensity in each segment is plotted as angular position \( \phi \). The abundance of nanostructures is derived from the mean intensity of the ring \( I_{\text{sym}} \). The anisotropy of the nanostructures relates to the amplitude \( I_{\text{sym}} \). The phase shift of the sine determines the preferential orientation of the nanostructures along the X-ray beam.](image)

**EXAMPLE 1: BONY TISSUE OF THE FEMORAL HEAD**

Scattering patterns of a section from the femoral head (see Figure 3) were acquired at a photon energy of 12.4 keV generated at the cSAXS beamline (Swiss Light Source, Paul Scherrer Institute, Villigen, Switzerland) with a sample-detector distance \( D_s \) of 2.15 m. The specimen was scanned across the highly intense X-ray beam in 15 \( \mu \)m steps in x- and y-directions.

The representations of SAXS pattern of selected length scales properly demonstrate abundance and orientation of nanostructures at the periphery of the femoral head. The features on the selected length scales are predominately related to the cartilage anchored to the bone. Note, that the specimen originates from a decalcified, diseased human femoral head and thus mainly the collagen fiber orientations and abundances became to light. More detailed information on the nanostructures associated with the collagen fibrils of collagen-II at characteristic spacings of around 60 and 90 nm were published recently.

It became clear that from the surface of the articular cartilage towards the bone the orientation of the collagen-II fibers gradually changes from parallel to normal with respect to the joint surface, on the one hand, accommodating the gliding motion required of opposing articulating joint surfaces and, on the other hand, to accommodate the forces delivered onto the bone. A similar behavior of orientation changes for collagen-I prevalent in bone can be found below the cement line that is located at the cartilage-bone interface. Such orientational information from SAXS patterns illustrates loss of collagen organization as the result of articular cartilage degradation.

![Figure 3: The image on the left shows a thin slice of a human femoral head, where the area for scanning X-ray scattering is marked. The image series on the right are representations of the SAXS patterns related to ranges from 1.6 to 1.7 nm (top, left), from 3.9 to 4.5 nm (top, right), from 12.5 to 13.7 nm (bottom, left) and from 20.2 to 23.1 nm (bottom, right), respectively. The colors are chosen according to the nanostructure’s orientations, see color wheel, the brightness relates to the nanostructure’s abundance. The bar corresponds to 1 mm.](image)

**EXAMPLE 2: HARD TISSUES OF THE HUMAN TOOTH**

Human teeth are known as anisotropic hard tissues with strong preferential orientations on the micro- and nanometer scale. The directional ordering of the dentin and enamel nanostructures with respect to the tooth morphology, however, requires spatially resolved scattering data, which became available only recently. The high-resolution imaging facilities with several ten thousand megapixel-images, however, is only reached very recently.

Teeth are composed of the anisotropic natural materials dentin and enamel. Enamel is hard and brittle, composed mainly of densely packed calcium phosphates organized in prisms or rods with very few organic components. Dentin, also composed mainly of inorganic calcium phosphates, is interwoven by collagen-I, making it tougher and allowing it to absorb or redistribute mechanical stress. The combination of these two hard tissues forms a highly durable structure, which lasts a lifetime under extreme mechanical and chemical conditions in the oral cavity of the human being. Especially the dentin-enamel junction, the interface between dentin and enamel acts as a crack barrier, preventing cracks formed in the brittle enamel to propagate through the entire tooth.

SAXS experiments on human teeth slices were performed using a photon energy of 18.58 keV and a sample-detector distance of 7.14 m. The 500 \( \mu \)m-thin tooth slice, which was held between ap-
appropriate polymer membranes to prevent dehydration, was scanned across the beam in 50 µm steps in x- and y-directions. This specimen was also measured in WAXS geometry with identical photon energy and step sizes, but with a sample-detector-distance of only 40.4 cm.

In the SAXS data, enamel yields scattering signals mainly parallel to the dentin-enamel junction, suggesting a perpendicular alignment of these nanostructures. The nanostructures in the dentin, however, lead to scattering signals normal to the dentin-enamel junction. The SAXS signals in enamel decrease with decreasing length ranges, almost disappearing below 10 nm (see Figures 4 and 5), which can be explained from the 15 nm-long rods in the enamel. At the length of a few nanometers and the sub-nanometer range, the differences in the chemical composition and structure between enamel and dentin become visible. Enamel maintains a highly oriented texture, while dentin structures can only be recognized for specific components (see Figure 5).

Figure 4: The hard tissue components of the human teeth with nanometer extensions show well-defined orientations. The nanostructures range from 185 to 231 nm (image top, left), from 53 to 71 nm (top right), 40 to 52 nm (bottom left), and 14 to 24 nm (bottom right). The colors are chosen according to the nanostructures orientations, see color wheel, their brightness relates to their abundance. The bar corresponds to 2 mm. The nanostructures in the dentin are nearly perpendicular to the dentin-enamel junction, whereas the ones in enamel are almost parallel to the dentin-enamel junction.

Figure 5: The scanning X-ray scattering to larger angles, represented by scanning WAXS, provides data on the molecular scales, which contain the structural and chemical composition of the tooth section. One clearly recognizes differences between the two kinds of hard tissue, i.e. dentin and enamel. Selecting certain scattering angles or lattice parameters, here 0.92 to 1.74 nm (image top, left), from 0.71 to 0.92 nm (top right), 0.40 to 0.53 nm (bottom left), and 0.22 to 0.24 nm (bottom right), the spatial distribution of the different components is uncovered. The colors are chosen according to the nanostructures orientations, see color wheel, their brightness relates to their abundance and the bar corresponds to 2 mm.

EXAMPLE 3: THALAMUS AS PART OF HUMAN BRAIN

The human brain is a prominent example for anisotropic and oriented soft tissue. SAXS pattern give rise to many details, which complement histology, since the periodicity of the nanostructures can be tracked deeply into the nanometer range as shown in the left image of Figure 6. Sophisticated analysis tools have to be developed to obtain an overview about all kinds of drawbacks and opportunities of SAXS pattern from differently stained and non-stained histological slices. Note, the potential influence of staining and embedding procedures can be studied, as the slices can even be investigated in wet state before the application of any preparation step.

The scanning X-ray scattering data shown were recorded at a photon energy of 11.2 keV and a specimen-detector distance of 7.1 m. The unstained thalamus slice, which was placed in a polyimide sachet, was scanned through the beam in steps of 100 µm in x- and y-directions with an acquisition rate of 5 images per second. It should be emphasized that the thalamus section scanned has centimeter extensions.

Figure 6: The human thalamus contains well-oriented nanostructures as shown by the two selected scanning SAXS images of a non-stained section prepared for histology. The left image represents the nanostructures from 4.6 to 6.3 nm and the right image shows the features of sizes from 161 to 185 nm. The orientation-dependent colors are according to the color wheel, their brightness is associated with their abundance. The bar corresponds to 5 mm.

EXAMPLE 4: URETHRA

The tissue of the human urethra resembles the porcine urethra. Figure 7 shows the scattering intensities of a 380 µm-thin section of an embedded porcine male urethra perpendicular to the symmetry axis. The images were generated with an 11.2 keV X-ray beam, scanned along the specimen in 50 µm and 75 µm steps in y- and x-direction, respectively.

The two main parts of the urethral tissue can also be distinguished in these SAXS images, which cover the entire nanometer range: the tunica mucosa in the central part surrounded by the tunica muscularis. The tunica mucosa consists of epithelium and the lamina propria. Epithelium is the innermost layer. The lamina propria is a loose tissue with abundant elastic networks. The tunica muscularis is composed of inner longitudinal and outer circular smooth muscles and an outermost ring of skeletal muscle fibers.
REFERENCES


The high risk, high payoff global nanotechnology phenomenon is in full swing. Innovations at the intersection of engineering, biotechnology, medicine, physical sciences and information technology are spurring new directions in research, patenting, commercialization, business development and technology transfer [1-5]. In fact, the future of nanotechnology is likely to continue along this interdisciplinary path with significant technologic advances cutting across a wide landscape of scientific disciplines. Estimates place the worldwide market potential for nanotechnology goods to be $2.6 trillion in 2014, up from $50 billion in 2006 [6]. A report from the US National Science Foundation (NSF) has proposed a more conservative figure: the worldwide market size for nanoproducts could reach $1 trillion between 2010 and 2015 [7].

The term nanotechnology is very much in vogue. But what does it mean? A nanometer (Greek, nanos, dwarf) is one billionth of a meter, or 1/75,000th the size of a human hair. An atom is about one third of a nanometer in width. Nanotechnology is an umbrella term used to define the products, processes and properties at the nano/micro scale that have resulted from the convergence of the physical, chemical and life sciences.

Miniaturization of materials often imparts novel mechanical, electrical and/or optical properties. Specifically, as a particle’s size decreases, a greater proportion of its atoms are located on the surface relative to its core, often rendering the particle more reactive (over its conventional “bulk” counterparts). In addition, as the particle size decreases, its total surface area increases exponentially. This reduction in particle size increases its dissolution rate and saturation solubility and, if the particle is a drug, it frequently correlates to improved in vivo drug performance.

However, one of the major problems regulators and lawyers face regarding nanotechnology is the confusion and disagreement about its definition [8,9]. One often used – yet clearly wrong – definition of nanotechnology was proposed by the US National Nanotechnology Initiative (NNI). It simply limits nanotechnology to “dimensions of roughly 1 to 100 nanometers” [10]. Various government agencies, including the US FDA and the US Patent and Trademark Office (PTO) continue to use this vague definition based on a sub-100nm size. Clearly, a definition based on physical limits is an unorthodox way of defining a technology field. Other technologies are defined by a key technology or breakthrough. For instance, genetic engineering technology is based upon recombinant DNA, while the Internet is a collection of “bulletin boards” networked in a World Wide Web.

The NNI nanotechnology definition presents numerous difficulties. For example, although the sub-100nm size range may be important to a nanophotonic company (e.g., a quantum dot’s size dictates the colour of light emitted therefrom), this size limitation is not critical to a drug company from a formulation, delivery or efficacy perspective because the desired property (e.g., improved bioavailability, reduced toxicity, lower dose, enhanced solubility, etc.) may be achieved in a size range greater than 100nm. Moreover, this NNI definition excludes numerous devices and materials of micrometer dimensions (or of dimensions less than 1 nanometer), a scale that is included within the definition of nanotechnology by many nanoscientists. Therefore, experts have cautioned against an overly rigid definition based on a sub-100nm size, emphasizing instead the continuum of scale from the “nano” to “micro.”

Add to this confusion the fact that nanotechnology is nothing new. For example, nanoscale carbon particles (“high-tech soot nanoparticles”) have been used as a reinforcing additive in tires for over a century. Another example is that of protein vaccines – they squarely fall within the definition of nanotechnology. In fact, many biomolecules are in the nanoscale. Peptides are similar in size to quantum dots and some viruses are in the size range of nanoparticles. Hence, most of
molecular medicine and biotechnology can be classified as nanotechnology. Technically speaking, biologists have been studying all these nanoscale biomolecules long before the term “nanotechnology” became fashionable. Even though the US National Institutes of Health (NIH) concurs that while much of biology is grounded in nanoscale phenomena, it has not reclassified most of its basic research portfolio as nanotechnology. In this regard, NIH identifies three broad areas that it considers nanotechnology:

(i) studies that use nanotechnology tools and concepts to study biology;
(ii) the engineering of biological molecules toward functions very different from those they have in nature; or
(iii) manipulation of biological systems by methods more precise than can be done by standard molecular biological, synthetic chemical or biochemical approaches.

In light of this confusion, the following definition of nanotechnology, unconstrained by an arbitrary size limitation, has been developed [8,9]:

“The design, characterization, production, and application of structures, devices, and systems by controlled manipulation of size and shape at the nanometer scale (atomic, molecular, and macromolecular scale) that produces structures, devices, and systems with at least one novel/superior characteristic or property.”

Naturally, disagreements over the definition of nanotechnology carry over to the definition of nanomedicine. At present, there is no uniform, internationally accepted definition for nanomedicine either. One definition, not constrained by size, yet correctly emphasizing that controlled manipulation at the nanoscale results in medical improvements and/or significant medical changes, comes from the European Science Foundation [11]:

“…the science and technology of diagnosing, treating and preventing disease and traumatic injury, of relieving pain, and of preserving and improving human health, using molecular tools and molecular knowledge of the human body.

Hence, the size limitation imposed in NNI’s definition must be abandoned, especially when discussing nanopharmaceuticals or nanomedicine. The phrase “small technology” may be more appropriate to accurately encompass both nanotechnologies and microtechnologies.

An internationally acceptable definition and nomenclature of nanotechnology should be promptly developed.

DRUG COMPANIES FOCUS ON NANOTECHNOLOGY

Pharmaceutical companies face enormous challenges ranging from revenue losses due to patent expirations on blockbusters, to greater regulatory oversight, to an ever-increasing challenge from generic manufacturers.

Drug revenues worth $70–$80 billion will potentially be lost by 2011 as various drugs go off-patent [12]. The cost (often $800+ million) and time (frequently spanning 10–15 years) of developing and launching a new drug to market are daunting. Annual research and development (R&D) investment by drug companies has risen from $1 billion in 1975 to $40 billion today, while annual new drug approvals in the past few years have remained flat at between 20–30 drugs [12].

Some argue that pharmaceutical companies are more focused on shareholder profits and defending their patents via costly litigation than innovative therapies. Others point to the fact that big pharma loses more on marketing campaigns and promotional advertising than on R&D. All agree that in today’s global economy, big pharma faces enormous pressure to deliver high-quality products to patients while maintaining profitability. Therefore, it is not surprising that pharmaceutical companies are turning to miniaturization and nanotechnology to enhance or supplement drug target discovery and drug formulation. In theory, nanotechnology should reduce the cost of drug discovery, design and development. It should enhance the drug discovery process itself through miniaturization, automation, speed, massive parallelism and reliability of assays. The resulting improved R&D success rate should enable a faster introduction of new, cost-effective products to the marketplace. For example, nanotechnology can be applied to current microarray technologies, exponentially increasing the hit rate for promising candidates/targets that can be screened. Inexpensive and higher throughput DNA sequencers based on nanotechnology can reduce the time for both drug discovery and diagnostics.

NANOPHARMACEUTICALS IN DRUG DELIVERY

A long-standing issue in the drug industry is the difficulty to deliver the correct dose of a particular active agent to a specific disease site. Since this is generally unachievable, active agents have to be administered in excessively high doses, thereby increasing the odds of toxic side effects. The concept of site-specific delivery of a therapeutic arises from this classic drawback of traditional therapeutics. Nanopharmaceuticals have enormous potential in addressing this failure of traditional therapeutics – they offer site-specific targeting of active agents [13]. Such precision targeting via nanopharmaceuticals will reduce toxic systemic side effects, resulting in better patient compliance. As a result, nanopharmaceuticals present novel opportunities for reformulation of active agents whose previous versions were unsuitable for traditional oral or injectable delivery.

In this paper, nanopharmaceuticals will be defined as colloidal nanoparticles (or nanometer scale complex systems) of 10 to 1,000 nanometers (1 micron). Furthermore, in the absence of a universal convention or nomenclature for nanopharmaceuticals, various nanoscale structures of different sizes, shapes and chemical composition have been included by this author within this broad definition (Figure 1). Some of the common shapes include spheres (hollow or solid), tubules, particles (solid or porous) and tree-like branched macromolecules.

![Figure 1: Nanopharmaceuticals for Drug Delivery](Image)

Nanopharmaceuticals often offer an advantage as compared to their “bulk” counterparts primarily because of their reduced size (i.e., an enormously increased surface area relative to volume). As mentioned earlier (See section titled “What is Nanotechnology?”), as a particle’s size decreases, a greater proportion of its atoms are located on the surface relative to its core, often rendering the particle more reactive and more water soluble. Nanopharmaceuticals are selected for characteristics such as biodegradability, biocompatibility, conjugation, complexation or encapsulation and their ability to be functionalized. For simplicity, they can be divided into two groups [13]:

(1) those where the active agent possesses intrinsic therapeutic properties and acts as its own polymeric carrier (examples include multivalent dendrimers, cerium oxide and platinum nanoparticles); and

(2) those where the active agent is encapsulated within a polymeric carrier (examples include dendrimer, polymer, silica and carbon nanotubes).
Nanopharmaceuticals

(2) those where the active agent is directly coupled (functionalized, entrapped or coated) to a distinct polymeric carrier. In the ideal futuristic situation, these polymeric (or lipid) carriers will be able to transport the active agent to a specific desired target site (ligand, receptor, active site, etc.) to impart maximum therapeutic activity with maximum safety (i.e., protecting body tissues from adverse reactions while preventing the degradation/denaturation/inactivation of the active agent during delivery/transit).

Nanopharmaceuticals are synthesized by various methods (self-assembly, vapour or electrostatic deposition, aggregation, nanomanipulation, imprinting, etc.) where the protocol is dictated by factors such as the specific therapeutic used and the desired delivery route.

The functional complexity and application potential of nanopharmaceuticals is the result of:

(a) enormous compositional range due to the large variety of polymeric nanomaterials they are composed of (e.g., liposomes, carbon nanotubes, dendrimers, colloidal gold, nanocrystals, fullerenes, etc.);
(b) variety of therapeutics that are incorporated with these nanomaterials (e.g., small molecule drugs, proteins, nucleic acids, etc.);
(c) biodistribution and targeting capabilities due to specific targeting moieties that can be surface-functionalized thereto (e.g., antibodies, ligands, etc.);
(d) various available routes of delivery (oral, topical, IV, etc.);
(e) their shape/geometry;
(f) their nanoscale dimensions (large surface area to volume ratio);
(g) their surface charge and
(h) their tunable or controlled release properties.

Nanopharmaceuticals typically accumulate non-uniformly within the body and their ultimate location is determined by their size distribution, surface charge and surface properties (discussed ahead). In fact, these properties can be tuned to provide long or short circulation times. Furthermore, their release kinetics can be adjusted to match the mechanism of action of the active agent making up the nanopharmaceuticals. For example, if a prolonged exposure to the active agent is desired, then a slow release is preferred [14]. Targeting to specific tissue sites (e.g., hepatocytes versus Kupffer cells in the liver [15]) can be achieved via

(a) linking specific ligands or molecules (e.g., antibodies, glycoproteins, etc.) to the polymeric carrier, or
(b) by altering the surface characteristics of the polymeric carrier so that it evades the mononuclear phagocytic system as present in the liver and spleen, i.e., the reticuloendothelial (RES) system.

Although there many FDA-approved marketed nanopharmaceuticals, numerous others are under development or nearing commercialization (Table 1). Obviously the elongated time-line is a consequence of the extremely complex and demanding requirements of clinical trials by the FDA. In future, nanopharmaceuticals will greatly influence medical practice and healthcare because of their ability, in many cases, to shorten the time-to-market for active agents, extend the economic life of proprietary drugs and create additional revenue streams. However, if this is to happen effectively, there are a few key biological requirements for nanopharmaceuticals to fulfill or to improve upon the current generation of nanopharmaceuticals. These include:

(i) they must exhibit “stealth” qualities to evade macrophage attack and the immune response;
(ii) be nontoxic and traceable within the body;
(iii) display effective pharmacokinetic properties;
(iv) be long-lived yet biodegradable following systemic administration through any route (but the polymer must protect the embedded active agent);
(v) they must selectively and effectively target specific tissue sites, i.e., an enhanced delivery to, or uptake by, target tissue sites; and
(vi) reduced toxicity to non-target tissue sites.

SIZE DOES MATTER IN DRUG DELIVERY

As explained in the previous section, the size and surface properties of nanopharmaceuticals (including the presence of targeting moieties) largely dictate their in vivo behaviour. Specifically, these properties permit systemic circulation and determine their biodistribution within the human body. Therefore, an understanding of these properties can aid in designing nanopharmaceuticals that can be localized to specific tissue/body sites.

The small size of nanopharmaceuticals imparts them with unique properties in contrast to larger particles – it is this small size that allows them access to places in the human body where larger particles cannot reach. It is generally accepted that for systemic applications, the diameter of nanopharmaceuticals should be in the range of 10-100 nanometers, with minimum surface charge [16]. Nanopharmaceuticals have a high surface-to-volume ratio when compared to their larger counterparts. Therefore, their surface properties are critical to their in vivo performance. In fact, their interaction with the local environment (which, again, is the end-result of a combination of size and surface properties) determines if they will be lost to undesired locations within the body. A large number of approaches focus on minimizing nonspecific binding of nanopharmaceuticals to undesired tissue surfaces as well as reducing interactions with each other. The endothelial surfaces, as well as cell membranes, are typically negatively charged – which repel negatively charged nanopharmaceuticals. Also, as the surface charge on the nanopharmaceuticals becomes larger (either positive or negative), a greater clearance by the macrophage-mediated RES is generally observed. In this context, synthesis of sterically stabilized nanopharmaceuticals is the subject of active R&D. For example, incorporation of polyethylene glycol (PEG) polymers on the surface of nanopharmaceuticals (i.e., PEGylation) provides a means to increase solubility, reduce immunogenicity, prolong half-life and prevent a rapid renal clearance via the RES (due to larger particle size resulting from PEGylation) [17]. In addition to this, it may also be necessary to design nanopharmaceuticals that can undergo efficient intracellular uptake and arrival at specific organelles [18].

Numerous active agents can be delivered in the form of nanopharmaceuticals via a variety of routes (Table 1). Nanopharmaceuticals are better suited than their microparticle counterparts for intravenous (IV) delivery because the tiniest capillaries are in the 5 to 6 micron range, a size that impedes most microparticles (or aggregations thereof) from distributing into the bloodstream.

The blood-brain barrier (BBB) and the blood-retinal barrier (BRB) protect the brain and eyes respectively due to their unique anatomical features, including the presence of tight junctions that seal adjacent cells. The BBB has strict size and surface property limitations for entrance. For gene delivery, both viral vectors as well as nonviral vectors have been generally unsuccessful – the former are unable to penetrate the BBB or the BRB while the latter lack sufficient efficiency. On the other hand, nanopharmaceuticals have been shown to cross biological barriers and may be able to cross both the intact BBB [19] as well as the BRB [20]. Often, nanopharmaceuticals can be delivered directly to the nervous system (NS) without prior need for drug modification or functionalization (which can affect function). Moreover, both hydrophilic and hydrophobic therapeutics can be delivered without first opening the BBB. However, in this context systemic delivery for non-NS diseases is of general concern because these agents may cross the BBB and cause brain damage or psychoactive effects. Nanopharmaceuticals can also permeate the epithelial junctions of the skin that normally impede delivery of active agents to the desired target [21]. Topical emulsion systems incorporating nanoparticles are being developed which rapidly permeate tissue to delivery actives or remove lethal toxins from the bloodstream.

Nanopharmaceuticals of specific size (generally greater than 10nm) can be designed that are able to penetrate tumours due to the “leaky” nature of their microvasculature. This classic effect, referred to as the “enhanced permeability and retention (EPR) effect,” results
in prolonged circulation and accumulation within the tumour [22]. It is generally accepted that nanoparticles in the 10–100nm size range and with a slightly positive or slightly negative surface charge should be able to disseminate within tumours when delivered to the circulatory system.

By controlling the particle size and architecture of nanopharmaceuticals, a particular pharmacokinetic release profile of the drug may be generated. Often, a near zero-order kinetic drug release profile is desired since it maintains a steadier therapeutic concentration at the site of delivery. Such a profile is more likely to be achieved by nanopharmaceuticals where a drug has been functionalized onto or encapsulated within a polymeric carrier matrix. For oral applications, research has focused on lymphatic uptake of nanopharmaceuticals by the Peyer’s patches of the gut associated lymphoid tissue (GALT). It has been shown that during oral delivery nanopharmaceuticals are disseminated systemically while their microparticle counterparts remain in the Peyer’s patches [23].

Particle size has an impact in another way also. The efficiency of drug distribution within various body cavities is influenced, in part, by the size of the drug particles. As the particle size of a drug decreases, its total surface area increases exponentially. This reduction in particle size increases its dissolution rate and saturation solubility, which frequently correlates to improved in vivo drug performance [24, 25]. In some cases, the pharmacokinetic behaviour of nanopharmaceuticals may help minimize peak plasma levels (which may be toxic) as well as prevent a drop below the targeted therapeutic range (which may lower efficacy).

It is known that drugs with poor bioavailability often result in higher cost to the consumer, not to mention the inefficient treatment and increased risk of toxicity. Ironically, due to the high-throughput technologies available today, there has also been an increase in the number of potential new chemical entities (NCEs) that are poorly water soluble [26, 27]. In recent years, various nanoparticle technologies have been successfully employed to tackle drugs with this low water (or lipid) solubility [27-29]. In fact, numerous pharmaceutical companies are revisiting shelved drugs that are “difficult” from a formulation point of view and relying more on nanotechnology to address these formulation challenges.

Because consumers prefer oral drugs over implantables or injectables, nano-engineering traditional or shelved compounds could greatly enhance oral bioavailability in some cases. A classic example of improving the bioavailability of poorly soluble drugs is Ireland-based Elan Corporation’s NanoCrystal® technology [30]. This technology is:

(a) an enabling technology for evaluating NCEs that exhibit poor water solubility and/or
(b) a valuable tool for optimizing the performance of current drugs.

NanoCrystal® technology can be incorporated into both parenteral and oral dosage forms. In this case, drug particles are produced by proprietary attrition-based wet-milling techniques that reduce their size to less than one micron [29, 31]. This reduction in size substantially increases the surface area, and hence, increases the solubility. The nano-sized drug particles are then stabilized against agglomeration by surface adsorption of selected GRAS (Generally Regarded As Safe) stabilizers [30]. This results in a final product that behaves like a solution (a colloidal dispersion). Studies have shown that reformulating old drugs by this technology can enhance bioavailability compared to commercial products [32], reduce the time to achieve maximum concentration, as well as increase in the area under the curve during the first hour [32, 33]. This technology may enable an increase in drug loading, thereby enhancing the maximum tolerated dose compared to commercial products [34]. The solid dosage tablet formulation of the immunosuppressant Rapamune® (sirolimus) is the first marketed drug developed with NanoCrystal® Technology and the first commercial launch of a nanopharmaceuticals (Table 1). Other examples of reformulated, FDA-approved drugs (Table 1) that employ this technology are Tricor® (fenofibrate), Emend® (aprepitant) and Megase® ES (megestrol acetate). It is interesting to note that the variability observed in the fasted and fed patients upon administration of micronized Tricor® was not observed upon administration of the reformulated nanopharmaceutical.

Reformulation is a classic lifecycle management option practiced by drug companies. The goal of reformulation is to alter the original NCE to enhance its bioavailability, solubility, delivery, stability, convenience, safety, etc. thereby allowing the drug company to gain additional marketing exclusivity following patent expiry of the original NCE. This critical strategy enables drug companies to realize the full value of their original NCE while providing protection from generics. Note that reformulation of an existing drug into a “nanoversion” often results in a novel NCE because it generally displays an altered pharmacokinetic profile (altered AUC and Cmax) as compared to its parent (larger) counterpart [9, 12]. In other words, nanopharmaceuticals are usually not bioequivalent to their parent (larger) counterparts, and hence, cannot apply for FDA approval via an Abbreviated New Drug Application (ANDA) route. Clearly, if the nanopharmaceutical is bioequivalent to its parent (larger) version, an ANDA can be filed to seek regulatory approval. However, the FDA approval process for NCEs generates two benefits for the innovator:

(i) the new drug (novel NCE) enjoys up to five years of non-patent exclusivity period that prevents/delays generics from entering the marketplace; and

(ii) under the Hatch Waxman Act, the owner can recover some of the patent term lost due to delay caused by the FDA regulatory review process.

**COMMERCIAL POTENTIAL OF NANOPHARMACEUTICALS**

Commercial nanotechnology, although at a nascent stage of development, is already a reality. Most agree that its full potential is years or decades away. Obviously, development is progressing more rapidly in certain sectors. The most active areas of product development are drug delivery and in vivo imaging. Although many sought-after innovations are decades away, there are hundreds, possibly thousands, of nanotech-based consumer products in the marketplace today. However, it is impossible to gauge an accurate picture of the exact potential for nanotechnology within the medical space. This is partly due to the extremely rapid development of healthcare products, fragmented marketplace, rapid rate of patent growth and unpredictable nature of the R&D process itself. However, according to most experts the market potential for medically oriented new nanotechnologies will become increasingly significant in the future.

Several variables will determine whether advances in nanopharmaceutical research in the laboratory will translate into a wide range of opportunities for the consumer. Although early forecasts for commercialization are encouraging, currently there are several challenges and risks that beset the commercialization of nanopharmaceuticals (Table 2). Some formidable challenges include legal, environmental, safety, ethical and regulatory questions as well as emerging thickets of overlapping patent claims [35, 36]. The emerging thicket of nanopharmaceutical patent claims has primarily resulted from patent protection but also because of continuous issuance of surprisingly broad [37] and/or overlapping [38] patents by the PTO. In fact, patent systems in general are under greater scrutiny and strain, with patent offices around the world continuing to struggle with evaluating the swarm of nanotechnology-related patent applications [8, 9]. Add to this confusion the fact that the NNI’s widely cited definition of nanotechnology is inaccurate and irrelevant in relation to nanopharmaceuticals (see previous section titled “Defining Nanotechnology and Nanomedicine”).

Given this backdrop, it is hard to predict the exact course nanomedicine and nanopharmaceuticals will take in the future. Will these relatively nascent areas make small yet valuable contributions to medicine, or will they become driving forces that catalyze a vast technological and healthcare revolution? The answer to this question may lie in the complex set of factors (Table 2) currently facing these two areas. This author believes that “nano” is here to stay, and
Nanopharmaceuticals

in the future it will generate both evolutionary as well as revolution-
ary drug and medical products. As evidence, one can look beyond
to these current challenges and point to governments around the world
that continue to be impressed by nanotechnology’s potential by stak-
ing their claims and doling out billions of dollars, euros and yen for
“nano” R&D.

CONCLUSION – CHALLENGES AND FUTURE PROSPECTS

Industry and commercial interest in the design, formulation and
delivery of nanopharmaceuticals have been building steadily. Drug
candidates that failed previously because of unacceptable toxicity
profiles, poor bioavailability, solubility issues or the inability to be
delivered via conventional forms/routes may be reformulated as na-
opharmaceuticals.

Additionally, nanopharmaceuticals containing targeting ligands
enhance cellular uptake into tissues of interest. In essence, they offer
the ability to control biodistribution of active agents, whether they
are small molecule drugs, proteins or nucleic acids. From a busi-
ness point-of-view, nanopharmaceuticals offer the ability to extend
the economic life of proprietary drugs and create additional revenue
streams, thereby significantly affecting the drug commercialization
landscape.

Nanopharmaceuticals often go hand-in-hand with novel drug
delivery methods and technologies. This in turn may result in more
efficacious treatments that generate new niche markets to provide
greater patent protection to already existing drug formulations [39].
As discussed earlier, nanopharmaceuticals will provide faster drug
absorption, controlled dosage releases, and effective shielding from
the body’s immune system – enhancing the effectiveness of preexist-
ing drugs.

As nanopharmaceuticals move out of the laboratory and into the
clinic, federal agencies like the FDA and the PTO will continue to
struggle to encourage their development while imposing some sort
of order. At present, both these critical agencies are in flux, and their
credibility has sunk to an all-time low. It is hoped that desperately
needed reforms to overhaul the PTO and the decades old US patent
system [40–42] along with clearer regulatory/safety guidelines from
the FDA regarding nanopharmaceuticals [12] will be forthcoming.

Given this backdrop, investors have been cautious as to what
route, if any, the FDA will take in regulating nanopharmaceuticals in
the future. Undoubtedly, regulating nanopharmaceuticals will require
greater cooperation between drug companies, policymakers and drug
regulators at the FDA. Although the FDA has previously downplayed
safety issues of nanoscale products [43], it is starting to recognize
that there are knowledge gaps in this area. In light of these challeng-
es, a multidisciplinary team of experienced drug regulators from the
drug, biologic and device areas of the FDA (working with a scientific
panel of experts), should:

(a) identify unique safety issues associated with nanopharmaceuti-
cals;
(b) adapt existing methodologies as well as develop new paradigms
for evaluating data pertaining to their safety and efficacy; and
(c) assist in developing unique tools and techniques to characterize
nanoscale materials (with an eye on quality, safety and effective-
ness).

As nanotechnology begins to appear in a wide variety of products,
safety and effectiveness of these nanoscale products will warrant a
careful review because size changes within the nanoscale are likely
to add additional complexity to the FDA product review process.

Nanopharmaceuticals may be viewed by the FDA as technologi-
cally overlapping from a review perspective, and therefore, consid-
ered as “combination products,” for which established examination
guidelines are already in place.

In the future, novel “multifunctional” nanopharmaceuticals will
be designed and delivered to the human body via a variety of routes.
It will be imperative that each of these be evaluated and characterized
on a case-by-case basis in an effort to correlate nanopharmaceuticals
physicochemical property with in vivo biological behavior and therap-
etic outcome. In this regard, any research strategy must involve
adsorption, distribution, metabolism and excretion (ADME) testing,
toxicoology tests and physicochemical characterization. Eventually,
all these undertakings will certainly expand the burgeoning field of
nanopharmaceuticals. Big pharma and biotech will further embrace
nanopharmaceuticals and this pace of adoption will enhance as they
offer novel properties that address unmet medical needs with low
development costs and risks.

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monkey eye following intravenous non-viral gene transfer. Mol
**TABLE 1: SELECTED FDA-APPROVED NANOPHARMACEUTICALS **

<table>
<thead>
<tr>
<th>Drug Product/ Brand Name</th>
<th>Nanoparticle Drug Component/ Active Ingredient(s)</th>
<th>Delivery Route</th>
<th>Manufacturer/ Alliance</th>
<th>Indication(s)</th>
<th>FDA Approval Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxil Caelyx (outside the US)</td>
<td>pegylated doxorubicin (Adriamycin)HCl liposomes (80-90 nm)</td>
<td>IV</td>
<td>OrthoBiotech Schering-Plough</td>
<td>metastatic ovarian cancer and AIDS-related Kaposi’s sarcoma</td>
<td>November 1995</td>
</tr>
<tr>
<td>Abraxane</td>
<td>paclitaxel (taxol) bound albumin nanoparticles (~120 nm)</td>
<td>IV</td>
<td>Abraxis BioScience AstraZeneca</td>
<td>various cancers</td>
<td>January 2005</td>
</tr>
<tr>
<td>AmBisome</td>
<td>amphotericin B liposomes (~45-80 nm)</td>
<td>IV</td>
<td>Gilead Sciences</td>
<td>fungal infections</td>
<td>August 1997</td>
</tr>
<tr>
<td>Rapamune</td>
<td>nanocrystalline sirolimus</td>
<td>oral solution oral tablet</td>
<td>Wyeth, Elan</td>
<td>immunosuppressant for kidney transplants</td>
<td>September 1999</td>
</tr>
<tr>
<td>Tricor</td>
<td>nanocrystal fenofibrate</td>
<td>oral tablet</td>
<td>Abbot</td>
<td>primary hypercholesteremia, mixed lipidemia, hypertriglyceridemia</td>
<td>November 2004</td>
</tr>
<tr>
<td>Emend</td>
<td>nanocrystal aprepitant</td>
<td>oral capsule IV</td>
<td>Merck, Elan</td>
<td>nausea in chemotherapy patients</td>
<td>March 2003</td>
</tr>
<tr>
<td>Diprivan</td>
<td>propofol liposomes</td>
<td>IV</td>
<td>Zeneca Pharmaceuticals</td>
<td>anesthetic</td>
<td>October 1989</td>
</tr>
<tr>
<td>Renagel</td>
<td>cross-linked poly(allylamine) resin (sevelamer hydrochloride)</td>
<td>oral tablet (capsule discontinued)</td>
<td>Genzyme</td>
<td>control of serum phosphorus in patients with chronic kidney disease on dialysis</td>
<td>October 1998</td>
</tr>
<tr>
<td>Triglide</td>
<td>nanocrystalline fenofibrate</td>
<td>oral tablets</td>
<td>SkyPharma First Horizon</td>
<td>lipid disorders; markedly reduces elevated plasma concentrations of triglycerides, LDL and total cholesterol and raises abnormally low levels of HDL</td>
<td>May 2005</td>
</tr>
<tr>
<td>Myocet</td>
<td>liposome-encapsulated doxorubicin-citrate complex</td>
<td>IV</td>
<td>Zeneus Pharma Sopherion Therapeutics</td>
<td>cardio-protective formulation of doxorubicin used in late stage metastatic breast cancer</td>
<td>Approved in Europe and Canada</td>
</tr>
<tr>
<td>DepoCyt</td>
<td>sustained release cytarabine liposomes</td>
<td>IV</td>
<td>SkyPharma Enzon</td>
<td>lymphomatous meningitis</td>
<td>April 1999</td>
</tr>
<tr>
<td>DaunoXome</td>
<td>encapsulated-daunorubicin citrate liposomes</td>
<td>IV</td>
<td>Gilead Sciences</td>
<td>advanced HIV-related Kaposi’s sarcoma</td>
<td>April 1996</td>
</tr>
<tr>
<td>Estrasorb</td>
<td>estradiol hemihydrate micellar nanoparticles (emulsion)</td>
<td>transdermal</td>
<td>Novavax</td>
<td>reduction of vasomotor symptoms, such as hot flushes and night sweats, in menopausal women</td>
<td>October 2003</td>
</tr>
<tr>
<td>Macugen</td>
<td>pegylated anti-VEGF aptamer</td>
<td>intravitreal</td>
<td>OSI Pharmaceuticals Pfizer</td>
<td>neovascular age-related macular degeneration</td>
<td>December 2004</td>
</tr>
<tr>
<td>Abelcet</td>
<td>amphotericin B phospholipid complex</td>
<td>IV</td>
<td>Enzon</td>
<td>invasive fungal infections in patients who are refractory to or intolerant of conventional amphotericin B therapy</td>
<td>November 1995</td>
</tr>
<tr>
<td>Adagen</td>
<td>pegylated adenosine deaminase</td>
<td>IV</td>
<td>Enzon</td>
<td>enzyme replacement therapy for patients with severe combined immunodeficiency disease</td>
<td>March 1990</td>
</tr>
<tr>
<td>Pegasys</td>
<td>peginterferon alfa-2a</td>
<td>subcutaneous</td>
<td>Nektar Hoffmann-La Roche</td>
<td>chronic hepatitis C virus infection</td>
<td>October 2002</td>
</tr>
<tr>
<td>Somavert</td>
<td>pegvisomant (PEG-hGH)</td>
<td>subcutaneous</td>
<td>Nektar Pfizer</td>
<td>acromegaly</td>
<td>March 2003</td>
</tr>
<tr>
<td>Neulasta</td>
<td>PEG-G-CSF or pegfilgrastim (covalent conjugate of recombinant methionyl human G-CSF (Filgrastim) and monomethoxypolyethylene glycol)</td>
<td>subcutaneous</td>
<td>Amgen</td>
<td>febrile neutropenia</td>
<td>January 2002</td>
</tr>
<tr>
<td>Copaxone</td>
<td>glatiramer acetate (copolymer of L-glutamic acid, L-alanine, L-tyrosine, and L-lysine)</td>
<td>subcutaneous</td>
<td>TEVA</td>
<td>relapsing-remitting multiple sclerosis</td>
<td>December 1996</td>
</tr>
<tr>
<td>Amphotec</td>
<td>colloidal suspension of lipid-based amphotericin B (~115 nm)</td>
<td>subcutaneous</td>
<td>Sequus</td>
<td>invasive aspergillosis patients who are refractory to or intolerant of conventional amphotericin B</td>
<td>November 1996</td>
</tr>
<tr>
<td>PEGIntron</td>
<td>peginterferon alfa-2b</td>
<td>subcutaneous</td>
<td>Enzon Schering-Plough</td>
<td>chronic hepatitis C virus infection in patients with compensated liver disease</td>
<td>January 2001</td>
</tr>
<tr>
<td>Oncaspar</td>
<td>pegasparaginase</td>
<td>subcutaneous</td>
<td>Enzon</td>
<td>leukemia</td>
<td>February 1994</td>
</tr>
<tr>
<td>Epaxal</td>
<td>hepatitis A vaccine adjuvanted with immunopotentiating reconstituted influenza virosomes (IRIV) Intramuscular (in the deltoid muscle)</td>
<td>subcutaneous</td>
<td>Berna Biotech</td>
<td>active immunization against hepatitis A for adult and children &gt;12 months (age may vary and depend upon the country)</td>
<td>available in Canada and elsewhere</td>
</tr>
<tr>
<td>Elestrin</td>
<td>estradiol gel (0.06%) incorporating calcium phosphate nanoparticles</td>
<td>transdermal</td>
<td>BioSanté</td>
<td>treatment of moderate to severe hot flashes in menopausal women</td>
<td>December 2006</td>
</tr>
</tbody>
</table>

**Note that therapeutic approval by FDA does not necessarily indicate that the therapeutic is available to consumers. Myocet and Epaxal have not been approved by the FDA.

Abbreviations Used in Table: IV, intravenous; PEG-hGH, pegylated human growth hormone; PEG-G-CSF, pegylated granulocyte colony-stimulating factor; PEG, polyethylene glycol; VEGF, vascular endothelial growth factor; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AIDS, acquired immunodeficiency syndrome.
1. THE NANOMEDICINE RESEARCH GROUP (NRG) AT PHARMA

Advances in design and engineering of nanoscale delivery systems with distinct physical and biochemical properties are beginning to positively impact clinical practice at many levels. These include detection of molecular changes responsible for disease pathogenesis and site-specific targeting of therapeutic agents with biochemically triggered-release mechanisms. Global research into targeting of drugs, biologics and diagnostic agents via intravenous and interstitial routes of administration with multifunctional nanoparticulate entities and nanoconstructs is accelerating dramatically. However, the biological performance of nanocarriers still requires optimization (in terms of targetability and controlled content release) as well as reducing their toxicity (which are related to particle dose, size, shape, surface reactivity and inherent material properties) at and off target sites. Indeed, the underlying processes of toxicity are both complex and multifaceted, and in need of urgent detailed cell and molecular investigation. We address these issues at the Nanomedicine Research Group (NRG) and strongly believe on rational nanomaterial design and precision surface-engineering of particulate carrier systems with well-defined polymers and biological ligands such as antibodies, nanoparticles and peptides based on detailed understanding of integrated biological processes at molecular level, rather than forcing applications for some materials currently in vogue. For instance, we have exploited organ-specific microcirculatory pathways (as in the spleen) and the concept of macrophage heterogeneity to target different subpopulations of vascular macrophages with engineered nanoparticles and to differentiate between quiescent and activated phagocytes. Likewise, we are mapping inter-related physicochemical and physiopathological principles that control nanoparticles drainage from the connective tissue into dermal lymphatic capillaries and their subsequent targeting to different elements of the lymphatic system and lymph nodes. Parallel programmes are being initiated with colleagues in the Copenhagen region to exploit advances in nanodevice technologies, polymer chemistry and 3D hydrogel platforms to create a synthetic follicular module of the lymph node, thus aiding effective monitoring of cellular communications between dendritic cell and effector/regulatory T cells as well as their modulation. The Group further assesses modes of nanoparticles interaction with plasma membrane and monitors related dynamic events by FRET and TIRF for optimized targeting and unraveling key mechanistic aspects. Nanoparticle and nanoconstruct tracking in cells and intracellular mechanistic events in relation to adverse events (as in apoptotic mechanisms) are also studied in depth and particularly at single cell level. We also target pathological molecules such beta amyloid with different multifunctional constructs (for its detection and elimination).

2. NRG AT THE CENTRE FOR PHARMACEUTICAL NANOTECHNOLOGY AND NANOTOXICOLOGY (CPNN)

Recently we have expanded our programme following inauguration of the Centre for Pharmaceutical Nanotechnology and Nanotoxicology (CPNN) in April 2009. The Centre is supported by a multi-million Euro funded initiative from the Danish Agency for Strategic Research (Det Strategiske Forskningsråd). CPNN is a strategic alliance between the NRG, the NanoScience Centre, Department of Public Health and the Department of Veterinary Disease Biology, all at Copenhagen University, as well as the Department of Micro- and Nanotechnology based at the Danish Technical University. Industry forms an exclusive ‘shell’ surrounding the academic core of CPNN with Lundbeck A/S and DHI as active key players; 4 other international pharmaceutical/biotechnology establishments act as members of the advisory board.

At CPNN we are generating fluorescent-labelled libraries of well characterized novel responsive and functional lipid- and polymeric-based constructs for site-specific targeting and simultaneously assessing their toxicity at detailed cell and molecular levels through “structure-activity” assessments (an integration between molecular toxicology and early discovery/development phase), Figure 1. These approaches comprise complex bio-nanotechnology, biophysical and biochemical studies to differentiate between bulk equilibrium and single-molecule/single-molecule interaction, to quantify molecular interactions between nanocarriers and biologics (allowing for better stabilization and formulation strategies), to assess nanocarrier interaction (or fusion processes) with isolated organelles, to follow real-time kinetics and particle tracking at single cell level as well as in vivo approaches in animal models of human diseases. At CPNN some partners continuously improve and optimize the performance of the state-of-the-art bio-nanotechnology tools and platforms for rapid and precision assessment of the abovementioned tasks. Therefore, on the basis of mechanistic investigations we propose and conduct new strategies for rational design and engineering of safer and clinically acceptable biodegradable nanomaterials and nanocarrier systems for parenteral administration and particularly for controlled delivery to and release of nucleic acids and other biologics at appropriate extra- and intracellular targets. Pertinent to realizing these goals is exploitation of key opportunities offered by disease states as well as molecular discoveries arising from genomics and proteomics for nanoparticles engineering. Inherently, our ultimate carrier design and targeting strategies vary in relation to the type, developmental stage and location of the disease. Such integrated and multidisciplinary approaches is expected to improve therapeutic benefit-to-risk ratio and applicable to a wide range of clinical situations to include cancer, cardiovascular diseases, diabetes, inflammatory conditions and immune disorders.
and precision of the standard toxicological procedures are of arguable value in nanomedicine research and development as it is limited to spotting extreme toxicity. For instance, we have designed and validated state-of-the-art assays to monitor complement activation products in undiluted human serum following exposure to a wide range of nanomaterials and nanomedicines and study their mechanistic aspects. Through comprehensive profiling and fingerprinting, we are identifying structural determinants of PEGylated liposomes, long-circulating polymeric nanoparticles, and pristine and functionalized carbon nanotubes that trigger complement activation, which is linked to initiation of pseudoallergic responses. We are continuously optimizing these approaches (including assays in whole blood and assessing population variations) and further engaged in designing in vitro methodologies that could be employed to predict individuals at risk of anaphylaxis following nanomedicine infusion. Such approaches could ultimately provide guidelines for immunologically safe nanomedicine dosing regimens for different diseases and patient populations. Indeed, it is of highest importance to map out key nanomaterial characteristics as small changes in surface chemistry and morphology may affect immunological responses differently. Poor understanding of material characteristics is often related to opposing reports of the safety of many nanomaterials, as exemplified with carbon nanotubes.

Cytotoxicity is also a delicate issue in experimental and clinical protocols that utilizes polycationic vectors for nucleic acid delivery and release. Our recent cell and molecular studies have confirmed that the underlying mechanisms in polyplex-induced cytotoxicity are multifaceted and involve both necrotic and mitochondrially-mediated apoptotic pathways. Through a comprehensive and integrated approach we are examining and mapping sequential molecular events pertaining cell death (in different cell types and cell cycle stage) with fully characterized and labeled blocks of polycationic vectors. Our efforts are particularly focused in relation to polyplex uptake mechanisms, real-time kinetics and tracking at single cell level coupled with biochemical monitoring of appropriate markers, and toxicogenomics. Indeed, the toxicity of polycationic vectors appears to depend strongly on a myriad of complex membrane perturbation processes (Figure 2) and associated biochemical events that triggers the activation of proapoptotic molecules. A clear understanding of these events will surely pave the way for rational design and production of safer polycations that provide duration period-specific gene expression (or silencing), which is absolutely essential for improving the benefit-to-risk ratio in clinical settings.

Photo of 20,000 nm and 40,000 nm sized nanoparticles

Today the Centre has a critical mass of academic and industrial expertise as well as world-class facilities, thus reinforcing competitiveness at international level. CPNN is truly international; this is further reflected through collaborations with centres of excellence world-wide including those in USA, Switzerland, EU and China. Our multi-collaborative, interdisciplinary and innovative nanoscience and molecular toxicology approaches to nanomedicine research will advance broad societal goals, from improved understanding of the material behaviour at molecular level to increased productivity through rational design and responsible nanomanufacturing. We envisage that our success would help the transformation of National and European biotechnology and pharmaceutical industries towards a knowledge-intensive, globally competitive one. In addition, the safe use of nanoparticulate delivery systems will also give Denmark and EU significant savings in pharmaceutical spending, which continue to grow. Ultimately, this will expand the market for many biologics and forming the basis for a highly profitable niche for such industries.

3. NRG AND THE EUROPEAN UNION’S 7TH FRAMEWORK PROGRAMME

The Nanomedicine Research group is also a partner in an EU-funded large-scale multi-million Euro research consortium (http://www.nadproject.eu/), which aims to tackle Alzheimer’s disease (AD), the most common form of dementia, through nanotechnology initiatives “(Nanoparticles for Therapy and Diagnosis of Alzheimer’s Disease, NAD)”. The consortium includes leading researchers from 18 European academic centres of excellence as well as medium and large enterprises in Italy, Spain, UK, France, Slovakia, The Netherlands, Hungary, Finland, Greece, Belgium, Portugal, Sweden and Finland.

Of 5 million cases of dementia in Europe 3 million are classified as AD, which is the forth leading cause of death in adults after heart disease, cancer and stroke. AD usually occur in the old age and is marked with a decline in memory, reasoning and planning. Affected individuals are also likely to develop seizures, hypertonicity (increase muscle movements) and incontinence (loss of normal control of the bowel or bladder). Given the continuing increase in life expectancy, an aging population is likely to fuel a steady rise in new cases and in fact these numbers are estimated to double during the next 30 years in Europe. Although substantial progress has been made in the scientific understanding of AD, there remains an urgent need to identify early detection strategies and effective therapies, in order to avert a financially overwhelming public health problem.

Hallmarks of AD include accumulation of clumps of proteins called b-amyloid plaques outside brain cells and accumulation of altered proteins inside the cells called neurofibrillary tangles (Figure 3). These plaques are target of the NAD project. Here in collaboration with our EU partners we design and engineer a wide arsenal of immunologically safe nanoscale particles capable of targeting b-amyloid simultaneously in the blood and in the brain, with the aim of plaque destruction (e.g., through the 'sink effect') as well as for diagnostic measures.

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The Landscape of Nanomedicine

REFERENCES

LEGEND TO FIGURES
Fig. 1. Structure-activity assessment cascade at CPNN.
Fig. 2. Electron micrographs showing accumulation of polyethyleneimine at the outer mitochondrial membrane.
Fig. 3. The role of BBB in pathogenesis of Alzheimer’s disease.
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