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Cover page:
Polymer Nanocarriers targeting a cell
Nadav Ben-Haim, Pavel Broz, Patrick Hunziker

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Life is Nano
Nanoscience has opened our eyes to the fact that all biological “Life is Nano”. When looking at any living cell in detail, we see that it is composed of a myriad of “nanomachines”: A prominent example is the ATP synthase nanomachine, found in almost all living organisms: this biochemical nanomachine exploits the physical chemistry of proton gradients to drive the physics of mechanical rotation, using the organic chemistry of enzymatic catalysis to produce the fuel of life, adenosine triphosphate, which then drives developmental biology and physiology of the organism. Inherited disease and microbiological toxins may impair the function of this nanomachine and lead to suffering and death while the disease course might benefit from pharmaceutics and targeted delivery.

Nanomedicine: natural, rational, interdisciplinary, and “green”
These observations highlight the importance of “Nano” for medicine. The exploration of life through nanoscience and the application of Nanomedicine to manage diseases is fundamental exploration of nature; it is rational, it is highly interdisciplinary (a practical difficulty) and it is possibly the best example of a “green technology” as many of its novel approaches are bio-inspired or associated with large reductions in drug doses or reagents and consumables. Nanoscience is also indispensable in medicine as an enabling technology to explore and combat dangers arising from natural and technological nano-size objects like LDL particles (natural nano-objects causing arteriosclerosis and a leading cause of death), viral nanoparticles (including influenza and HIV), and ultrafine particles arising from the burning of fossil fuels, from open fires, and from industrial manufacture.

Translating nanomedical research into a benefit for individuals and society
In the past decade, we have observed how the nanosciences have developed a toolbox of nanoscience tools, materials and methods of potential medical benefit. Currently, we see how these approaches have matured to the point where preclinical studies yield exciting results and technologies start to be applied in clinical trials. However, Nanomedicine still falls short of its name (derived from ‘ars medicina’, the ‘the art of the physician’) because few individual patients already benefit from these developments, and because the knowledge about these developments has not significantly permeated the medical community. To transform medicine to the benefit of both, individuals and society, nanomedical research needs to develop strong interaction with clinical medicine. The direction of specific research efforts requires fine tuning to the clinical reality and practical difficulties of important disease. Pivotal, large scale clinical trials need to be designed hand in hand with clinical physicians and leading hospitals, as well as in the outpatient setting. This also means thorough exploration of the implications for individuals, society, environment, politics, philosophy and ethics. Thus, the strong interdisciplinary, a hallmark of nanoscience since its beginnings but mainly centering around the natural sciences, needs to be expanded even further to encompass also clinical physicians and the humanities, as well as the industries that will manufacture drugs and medical devices in the future. This can be done through bridge-building platforms such as this journal, through interdisciplinary conferences and professional societies that encourage such interaction, and also through political support of the integration of the new technologies into clinical practice. The potential of Nanomedicine to break new ground in clinical fields where current medicine is of very limited efficacy, e.g. malaria, orphan diseases and the many incurable diseases of today, will inspire new hope to the suffering, and thus respond to the ultimate calling of medicine, namely to work to the benefit of individuals and society.

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Welcome to the second issue of the European Journal of Nanomedicine. It appears in time to highlight an upcoming event of the CLINAM-Foundation for Clinical Nanomedicine: the 2nd European Conference for Clinical Nanomedicine will be held in Basel, Switzerland from April 27–29 2009. Once again the starting point of the meeting will be what clinical issues wait for nanomedical solutions.

Clinicians will speak about unsolved problems in Cardiology, Oncology, Haematology, Neurology, Inflammatory and Infectious Diseases, Eye Diseases, Ear Diseases, Orthopaedics, Diabetes and Heredity Disease. Presentations by nanoscientists will be made in each of these fields and will illustrate how emerging nanoscience technologies shall and can already be applied to solve medical problems. An important aspect of the Conference will be sessions to late breaking new and ongoing clinical trials that apply Nanomedicine for therapy, diagnostics, immunization and vaccine development.

As in the first conference, the “Debate Method” will be applied. All speaker presentations are restricted to 10 minutes statements and a short question and answer period will follow. At the end of a few statements there will be ample time for questions and debates between all those participating in the CLINAM-Conference.

The Basel event will be a lively meeting for the expected 300 scientists, technologists, developers and experts. Nanomedicine is such a complex discipline that the overlapping interaction of Biologists, Pharmacologists, Chemists, Physicists, Materials Scientists, Medical Doctors and experts from many disciplines is pivotal. As such this meeting lays great emphasis on interdisciplinary debate. We hope that this will lead to further developments in the field of clinical Nanomedicine and ultimately lead to improved patient treatment.

There is no doubt that the CLINAM-Conference is also of interest to people investigating the potential of future emerging technologies in Life Sciences, policy makers and industrial experts and political health authorities.

Three Nobel-Prize laureates have agreed to come to Basel, to give keynote lectures and to participate in the debates. Since the non clinical implications of Nanomedicine are of crucial importance, a large panel and 3 workshops are entirely dedicated to the topics of Toxicology, Safety, Regulatory Processes, the role of Nanomedicine in developing Countries and as well as to questions of Ethics and Philosophy. At the end of the meeting there will be an outlook on targeted drug delivery and how to handle drug development in the future.

Come to Basel and meet speakers from 21 countries to develop your European and International relations. This is a unique opportunity to interact with colleagues in the field of applied Nanomedicine; we are putting special emphasis on the interdisciplinary approach and offering a unique opportunity to bring together developers, scientists and experts from many disciplines. We hope that you will download the Programme of this venue at www.clinam.org/conference

If you are or become a member of the European Society for Nanomedicine (www.esnam.org) you will receive a reduction on the conference fee that equals the Society’s annual membership fee.

We hope to welcome you in Basel soon. On the Sunday afternoon preceding the conference, the meeting of the European and the International Society for Nanomedicine will take place and all delegates being already in Basel on Sunday are most welcome to attend.

Löffler, B Eur J Nanomed 2009; 2:5
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Impressum
Unresolved Problems in Cardiovascular Medicine waiting for Nanomedical Solutions

Peter Christian Burger and Matthias Pfisterer *

Abstract

Despite remarkable advances in medical knowledge, new technologies, and modern drug developments, cardiovascular diseases remain the leading cause of death and disability in developed countries. Nanomedicine, by targeting specific molecular structures, has the potential to provide innovative new and powerful tools for early accurate diagnosis and highly effective treatments. This review will focus on molecular mechanisms of cardiovascular diseases, and in particular, of atherosclerosis, that might become potential targets for nanomedical approaches.

Keywords: Atherosclerosis, vulnerable plaque, cardiomyopathy, Nanomedicine

Introduction

Coronary artery disease is the leading cause of morbidity and mortality in the western hemisphere. Access to good medical systems have led to a steady increase in life expectancy and to a growing population of elderly patients. In addition, western type diet and reduced physical activity contribute to obesity, hypertension, and diabetes mellitus, which are well known risk factors for atherosclerosis. Therefore, despite the introduction of new drugs that may help to stabilize atherosclerotic lesions (e.g. statins, angiotensin converting enzyme inhibitors), the population of patients suffering from serious complications of atherosclerosis such as myocardial infarction or stroke, will continue to grow steadily. Therefore atherosclerosis is not only of relevance to every patient suffering of the disease but also contributes significantly to the ever increasing health care costs. Despite considerable advances in diagnosis and treatment of cardiovascular diseases, many problems still remain unsolved. Nanomedicine has the potential to offer completely new solutions as well in diagnostic as in therapeutic ways and at many different stages in the disease process of atherosclerosis. In addition, Nanomedicine might also be applicable to other cardiovascular diseases such as valvular heart disease, myocarditis, dilative and hypertrophic cardiomyopathies and in arrhythmias. Nanomedicine is still in its very early phase and for the majority of pending problems a lot of work remains to be done. After proof of concept there is still a long way to go (phase I - III clinical studies) until such systems may make it into clinical practice. Nevertheless, in recent years, research has advanced rapidly into the “nanoworld” and tools like atomic force microscopy or electron emission microscopy became available and revolutionized the development in the field. Consequently, new tools, such as “intelligent” targeted nanocontainers or specifically designed “nanomaterials” might become available in the near future. This review will summarize where nanomedicine might offer new options for diagnosis and treatment in cardiovascular medicine, and in particular in atherosclerosis.

Atherosclerosis and cardiovascular disease

Early stages in coronary artery disease

Atherosclerosis is generally considered a chronic inflammatory process and is characterized by the infiltration of leukocytes into foci of inflammation (for review see 1). Excess cholesterol, in particular low density lipoprotein (LDL) cholesterol, enters from the blood stream into the arterial wall, particularly at sites with turbulent shear stress. In the arterial intima, oxidative and enzymatic modification of the lipids occur. Oxidized LDL (oxLDL) is a strong activator of endothelial cells and initiates the inflammatory process. Activation leads from an antithrombotic to a prothrombotic state of the endothelium, which then mediates the cell adhesion cascade of inflammation and atherosclerosis (Figure 1A).

Figure 1A. Cell adhesion cascade: Leukocyte rolling and firm adhesion.

Upon activation with oxidized LDL, endothelial cells (Ec) start upregulation of cell adhesion molecules. P- and E-selectin mediate rolling of leukocytes, in particular monocytes (Mo). Additional cell adhesion molecules expressed by activated endothelial cells, such as ICAM-1 and VCAM-1 together with molecules released by other cell types (e.g. platelets) and deposited on activated endothelium such as RANTES, then mediate firm adhesion of monocytes on the endothelium. ICAM-1: Intercellular cell adhesion molecule-1. VCAM-1: vascular cell adhesion molecule-1. RANTES: Regulated upon Activation Normal T cell Expressed and Secreted.

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Special sets of adhesion molecules are expressed and presented on activated but not resting endothelial cells. These molecules mediate rolling (P- and E-selectin) and firm adhesion (RANTES, ICAM-1, VCAM-1) of leukocytes, in particular monocytes and T-lymphocytes (Figure 1A). These cells thereby are activated and attracted by chemokines (monocyte chemoattractant protein-1, interleukin-8, platelet derived growth factor, osteopontin), transmigrate through the endothelial cell layer into the intima, where upon further stimulation with additional cytokotnes, e.g. M-CSF, II-1, TNF-α, invaded monocytes become macrophages (MF) and replicate.


Smooth muscle cells, similar to monocytes / macrophages, change phenotype in the intima, replicate and become phagocytic. Through scavenger receptors, expressed on the cell surface, a broad range of molecules, apoptotic cell fragments and oxidized LDL are taken up and destroyed by the macrophages. Since in general not enough of the internalized cholesterol can be utilized or re-mobilized, lipid droplets will be filling the cells, which are then called “foam cells”. These are the prototypical cells of atherosclerotic plaques (Figure 1C). Atherosclerotic lesions that consist mainly of foam cells are called fatty streaks and are potentially reversible lesions.

At this stage, despite the fact that the disease is already in progress, patients are completely asymptomatic. Several of the above-mentioned molecules are potential targets for diagnostic or therapeutic interventions. In a variety of animal models, it has been shown that by abrogation of one or several of the cell adhesion molecules, the atherosclerotic process can be dramatically slowed, indicating that these molecules play indeed a crucial role in the disease process (e.g. 3). Nanomedicine might provide tools to visualize activated endothelium (4) (Figure 2) or to demonstrate early atherosclerotic lesions by imaging deposited lipids, leukocytes or foam cells (5). Using cell adhesion molecules as targets for cell specific drug delivery, Nanomedicine could influence the atherosclerotic process in a crucial early phase. Such site-specific drug therapy is likely to reduce unwanted side effects.

Figure 1B. Cell adhesion cascade: Leukocyte / Monocyte transmigration
Monocytes (Mo) get activated (yellow flash) and, attracted by chemokines such as MCP-1, II-8, PDGF, osteopontin, start transmigration through the endothelial cell layer into the intima, where upon further stimulation with additional cytokotnes, e.g. M-CSF, II-1, TNF-α, invaded monocytes become macrophages (MF) and replicate.


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Figure 1C. Cell adhesion cascade: Development of fatty streak
Platelets (Plt), attracted from the blood stream by integrins, P-selectin, fibrin, TxA2, TF, as well as smooth muscle cells (SMC), attracted by PDGF, FGf-2, TGF β, invading from the underlying tunica media, are also accumulating in atherosclerotic plaques. Smooth muscle cells may change phenotype and become phagocytic. Oxidized LDL promotes scavenger receptor expression (CD36, CD68, SR-A) on macrophages. By these receptors oxLDL can be taken up uncontrolled. Macrophages (MF) thereby become foam cells and secrete additional cytokotnes such as MCP-1, IP-10, Mig, II-8, that may further promote the inflammatory process.


The vulnerable plaque
Macrophages present fragments of internalized material to CD4+ T-lymphocytes, which initiate an inflammatory response within the arterial wall. Locally released inflammatory cytokotnes (interferon-γ, interleukin-1, tumor necrosis factor) induce the local production of interleukin-6, which in turn stimulates the release of acute

Figure 2. Atherosclerosis can be visualized
In an atherosclerotic mouse model (ApoE-deficient mouse) contrast enhanced ultrasound was used to detect in vivo microbubbles that are targeted to vascular cell adhesion molecule (VCAM-1) which is up regulated on activated endothelium. With special permission: Kaufmann BA et al Molecular imaging of inflammation in atherosclerosis with targeted ultrasound detection of vascular cell adhesion molecule-1. Circulation. 2007 Jul 17;116(3):270-84.

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Phase reactants (C-reactive protein, serum amyloid A, fibrinogen) in the liver, thus initiating a mild systemic reaction (for review see 6). In return, systemic inflammatory reactions, such as infections, may themselves promote atherosclerotic lesion formation (7). It had been noticed that atherosclerotic lesions are in general not steadily growing, but often grow in “flairs”, which are probably mediated by circulating cytokines that stimulate the local environment. Over the years, fatty streaks therefore often progress to more advanced atherosclerotic lesions such as fibrous or fibro-fatty plaques. Foam cells may become overloaded with lipids, undergo apoptosis or necrosis and die. Lipid and cell debris is deposited in the core of the atherosclerotic plaque. Calcifications within the plaques may ensue. By the initiation of a healing response, fibroblasts start forming a fibrous cap that walls off the lesion from the vessel lumen (1). Two types of atherosclerotic lesions are known, stable and unstable atherosclerotic plaques (Figure 3). Stable plaques, characterized by a thick fibrous cap, may cause clinically relevant stenoses of arteries but are not prone to rupture. Patients may complain about angina pectoris while exercising, however, acute cardiovascular events rarely occur with this type of lesion.

In contrast, unstable plaques, characterized by a lipid rich core, an abundance of inflammatory leukocytes and in particular a thin fibrous cap, are at risk of rupture (8). In these plaques, stimulated by some of the above mentioned cytokines, matrix degrading proteases (e.g. matrix metalloproteinases) are released and activated and lead to the degradation of the fibrous cap (9). Such weakened caps, can erode or rupture. The lipid rich core of a plaque whereby is exposed to the blood and activates the clotting system within the affected artery. Such Plaque rupture with subsequent intravascular clot formation and vascular obstruction is the mechanism that provokes acute cardiovascular events, such as myocardial infarctions or strokes. Unfortunately however, potentially dangerous, unstable plaques must not cause relevant stenoses and therefore might go completely unnoticed until they rupture.

A pending problem in cardiovascular medicine is the fact that there are no good means to differentiate stable from unstable plaques. Since the consequences of such a differentiation would be quite important for patient management, there have already been several efforts to characterize atherosclerotic lesions in vivo. Coronary angiography, which is the gold standard to evaluate patients with symptomatic atherosclerosis, visualizes the vascular lumen but provides no information about the pathologic process that takes place within the arterial wall. Therefore, unstable plaques, prone to rupture but not necessarily stenotic, might not be recognized by this method. Intravascular ultrasound, a technology that has recently been introduced into clinical practice, allows a rough characterization of the arterial wall and may recognize plaques with lipid rich cores and thin fibrous caps (10). Several other technologies (optical coherence tomography, near-infrared spectroscopy, thermography, angiography) have also been used for this purpose. Unfortunately, all these tools are catheter based and therefore need an invasive strategy. Computer tomography (CT) as well as magnetic resonance tomography (MRT) nowadays allows analyzing coronary arteries non-invasively. The differentiation between lipid rich soft plaques and calcified atherosclerotic lesions sometimes is possible with new generation CT scanners, but unstable atherosclerotic plaques still cannot be differentiated by these methods. However, as outlined above, activated endothelium and unstable atherosclerotic plaques have some particular molecular features, which are not present in stable conditions. Targeting one or several of the above-mentioned molecules with nanocontainers, containing substances that are able to provoke bright signals in CT or MRT, could be of great interest in diagnosing vulnerable plaques. In addition, nanocontainers with substances that block unstable plaque formation, induce plaque stabilization or promote plaque regression would be highly desired therapeutic tools.

Healing after coronary stenting
Coronary angiography with coronary angioplasty is a highly effective treatment in acute myocardial infarction as well as in situations where patients suffer from angina pectoris due to hemodynamic relevant stenoses. In addition to balloon dilatation, nowadays in most cases stents are implanted in these lesions in order to prevent early reclosure. Such stents have two major drawbacks, first, re-occlusion due to acute thrombosis is a rare but eventually fatal event, and second, in-stent restenosis, which became less of a problem with newer stent materials, remains a concern (11, 12). Drug eluting stents that are already widely used in clinical practice are quite effective in prevention of in-stent restenosis. The substances released by these stents however not only prevent restenosis formation but also prolong re-endothelialization of the stents that makes these stents more prone to acute thrombotic occlusions, even months after implantation. Nanomedicine might offer new opportunities to coat such stents in order to prevent such undesired sequelae of coronary stent implantation.

Healing after myocardial infarction
If during a myocardial infarction the oc-

Figure 3. Stable versus unstable atherosclerotic plaques
A) In stable atherosclerotic plaques a thick fibrous cap (FC) walls of the lipid core (L).
B) Unstable atherosclerotic plaques are characterized by variable lipid content (L) a necrotic core (NC), an abundance of leukocytes (Lc) and a thin fibrous cap (FC). These lesions are prone to rupture and may cause myocardial infarctions.

With special permission from PathoPic / Pathorama, Institute of Pathology, University Hospital Basel.
Valvular heart disease

Due to the growing population of elderly patients, the incidence of degenerative valvular heart disease, in particular aortic stenosis and mitral regurgitation, is increasing too. Heart valves are exposed to relevant shear forces over many years. This, in conjunction with traditional risk factors (hyperlipidemia, diabetes, hypertension) may lead to valve fibrosis and calcification and result in clinically relevant dysfunction (14). Since valvular dysfunction is a pure mechanical problem, no good medical treatment is available and in general surgery remains the only effective option. However, elderly patients often suffer from additional diseases and heart surgery therefore often is associated with a high perioperative risk. Nanomedicine might eventually offer possibilities to deliver potent drugs locally in order to stop valve degradation already early on in the disease process.

Myocarditis

Myocarditis is probably underestimated in its prevalence due to difficulties in its diagnosis. The spectrum of clinical manifestations ranges from transient discrete flu-like symptoms to a fulminant lethal course (15). In addition, the etiology of the disease is highly variable, besides viral, bacterial and fungal infections also autoimmune reactions, metabolic abnormalities and toxic substances can cause myocarditis. The disease is characterized by lymphocytic infiltrates in the heart muscle and eventually destruction of myocardial tissue. Nanomedical tools could eventually be of help to establish the exact cause of the disease, to better define the disease mechanism and to characterize in vivo the infiltrating cell types. So far, medical interventions have been of limited effect in myocarditis, which might be due to the fact, that all cases have been treated the same way, regardless of the offending agent and the prevalent disease mechanism (direct viral toxicity versus autoimmune reaction). However tailored treatment might be necessary for successful treatment. Nanomedicine could provide the required Trojan horse that brings and delivers potent drugs to places where they are needed, without being harmful to neither nearby unaffected cells nor to other organ systems.

Other cardiomyopathies

Restrictive cardiomyopathies, which are quite rare, occur due to myocardial deposition of amyloid proteins (amyloidosis), iron (hemochromatosis), cerebrosides (Gaucher disease), glycogen (glycogen storage disease); due to the development of intramyocardial fibrous granulomas (sarcoidosis); or due to an intracellular accumulation of glycolipids (Fabry disease). Myocardial restriction leads primarily to an impaired diastolic filling but eventually also to a reduced systolic function of the heart. For most of these cardiomyopathies, medical options are still quite limited. Will Nanomedicine one day provide means to deplete intramyocardial deposits of undesired products?

Hypertrophic cardiomyopathy (HCM), which is often characterized by a marked hypertrophy of the myocardium, as well as arrhythmogenic right ventricular cardiomyopathy (ARVC) is mainly feared because life threatening arrhythmias can occur. Whether nanomedical approaches to stabilize heart rhythm will one day become available also remains speculative.

Nanomedical tools

The development of new biocompatible materials on nanoscale dimensions offers new opportunities for application in diagnostics and treatment of a broad variety of cardiovascular and other diseases. “Intelligent” nanocounters can be specifically targeted to certain cell types (e.g. diseased cells) (16). Special molecules built into the shell of such nanocounters may promote their internalization into targeted cells. Yet other molecules in the shell of nanocounters could make sure that they release their content into the right compartment of a targeted cell (e.g. lysosome, endoplasmic reticulum, cytosol). By such means, high concentrations of a specific marker, dye, or drug can be brought exclusively to those places in the body where they are needed. If for example toxic substances are needed to destroy malignant cells (e.g. cardiac tumors), such substances should only target malignant tumor cells but spare all the other cells in the body.

Substantial research still lies ahead and many questions need to be answered until drugs based on nanomedical approaches can be applied in human medicine; what is the best biocompatible material for a shell? What molecules need to be built into such shells to achieve optimal targeting, optimal internalization and optimal drug release of nanocounters? What are the pharmacodynamics and the pharmacokinetics of such nanocounters? What happens to the degradation product of the nanocounter shells within a living organism?

Despite all these open questions, Nanomedicine has the potential to revolutionize medicine. Therefore cardiologists along with other physicians, look forward to a fascinating future of Nanomedicine.

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Unsolved Medical Problems


Measuring the intrinsic nanomechanics of molecular interactions with micro-cantilever sensors

Thomas Braun*1 and Murali Krishna Ghatkesar2 (DOI 10.3884/0002.4)

Abstract
Microarray techniques are of indisputable importance for today’s research in medicine and biology. Classical microarray sensors depend on the labeling of molecules and do not provide real-time information. In recent years, different micro-array technologies evolved not only measuring in a label-free manner but also providing kinetic information (kinetic microarrays, Braun, Huber et al., 2007). Such techniques involve surface plasmon imaging (Jordan & Corn, 1997), ellipsometry (Wang & Jin, 2003), surface acoustic wave sensors (Gronewold, Baumgartner, Quandt, & Famulok, 2006), nano-wire based sensors (Cui, Wei, Park, & Lieber, 2001) and cantilever based sensors (Hansen & Thundat, 2005; Lang, Hegner, & Gerber, 2006). Note, that all these techniques depend on the activation of a sensor surface with cognitive molecules. This article aims to provide an overview of cantilever based microarray sensors. Cantilevers are extremely thin springboards anchored at one end to a base (Fig 1A). These micro-fabricated structures are traditionally used for imaging (Binnig, Quate, & Gerber, 1986) but also enabled a new type of sensor (Gimzewski, Gerber, Meyer, & Schllitter, 1994; Thundat, Warmack, Chen, & Allison, 1994; Waggoner & Craighead, 2002): the nano-mechanical measurement principles upon which this technique is based work label-free and provide quantitative real-time information in a micro-array format. During recent years a variety of applications for chemical, physical and biological sensing with cantilever technology was presented. In biology, cantilever sensors are proven to robustly measure data for “classical” genomic experiments (“gene fishing”) (Zhang et al., 2006), for proteomic research (e. g. antibody-antigen interaction) (Backmann et al., 2005), macrobiotic growth (Gfeller, Nugaeva, & Hegner, 2005; Nugaeva et al., 2007) and structural changes of membrane proteins (Bálint et al., 2007; Braun et al., 2006).

Here we discuss the principles of cantilever sensors and the unique potential of these devices to characterize molecular interactions.

Keywords: Cantilever, Biosensor, Melittin

Principles of cantilever based sensors

Figure 1. Micromechanical cantilevers and detection modes. (A) Sensor array with 8 cantilevers (white structures). Note the protection bars at the side. Scale bar: 500 µm. (B) Static mode. Surface stress differences between the upper and lower cantilever surface forces the spring bar to bend. (C) Dynamic mode. The adsorption of ligand molecules shifts the resonance frequency of the cantilever to lower values. This frequency shifts are correlated to the mass of adsorbed molecules.

Cantilever sensors are based on a surprisingly simple principle: A mesosscopic bar (cantilever, see Fig 1 panel A) of several hundred micrometers length, about one hundred micrometers width but only 0.3 to 4 micrometer thick represent the transducing platform. These extremely thin structures are able to translate biomolecular recognition processes into a nanomechanical signal (Fritz et al., 2000). This nanomechanical signal is itself converted into an electrical signal, which can be digitized and...
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Figure 2. Schematic of the homemade measurement set-up for combined mode measurements.

For more information see also Braun at al., 2007 (Braun, Ghatkesar et al., 2007). an array of silicon cantilevers was mounted onto a piezo element. A sinusoidal excitation signal generated from a network analyzer swept the requested frequency range vibrating the cantilevers (a). The laser beam deflection detection technique was used to monitor the response of individual cantilevers (2). A frequency analyzer (3) compared input- and output signals and continuously recorded amplitude and phase spectra. A post-processing software (5) was used to extract the mass (dynamic mode). The static mode signal can be directly recorded (6).

recorded (Fig 2). Two different modes for analyte detection are distinguished (Battiston et al., 2001; Braun, Ghatkesar et al., 2007). The static mode (Fig 1 B) relies on the bending of the cantilever bar: nanomechanical interaction changes generate a surface-stress difference between the asymmetrically functionalized cantilever interfaces forcing the beam to bend. The dynamic mode (Fig 1C) detects the mass adsorption to the cantilever by tracking the resonance frequency of the cantilever.

Both modes can be recorded simultaneously as depicted in Fig. 2. Here, the laser deflection technique is used to read-out the cantilever state: The laser beam is deflected at the apex of the cantilever and the actual bending is detected with a position sensitive device (PSD). From this, the static mode bending of the cantilever can be directly recorded. For the dynamic mode, the cantilever is actuated by a piezo-element beneath the cantilever array body. A frequency generator sweeps the frequency spectrum, a frequency analyzer compares the reference signal and the response coming from the PSD. Post-processing software (Braun, Ghatkesar et al., 2007) extracts the desired dynamic mode information such as the mass (Braun et al., 2005).

Measuring the intrinsic nanomechanics of molecular interactions

We propose, that cantilever based sensors offer the possibility to measure the intrinsic nanomechanics of molecular interactions. This differentiates this technology from the other kinetic microarray techniques mentioned at the beginning measuring indirect signals such as a surface plasmon resonance changes.

At least two or three physical properties change, when one molecular partner binds to another (Fig 3): firstly, the masses combine and secondly, the structure of at least one binding partner is altered, transducing the binding into subsequent biological reactions. In the case of ligand activated ion-channels, the membrane potential also changes. We suggest that cantilevers can detect changes in all properties (Hegner & Braun, 2005). Proofs of concept were provided during recent years for the static and dynamic mode: The mass is measured by the resonance frequency of the cantilever (dynamic mode, Fig 3B) as demonstrated with streptavidin-biotin interaction (Braun et al., 2005). Structural changes are quantitatively detected by static bending of asymmetrically functionalized cantilevers (static mode, Fig 3C), as shown with bacteriorhodopsin (Bálint et al., 2007; Braun et al., 2006) and melittin (Pera et al. 2007).

Recently we presented results for combined mode measurements using a Melitin-lipid test-system (Ghatkesar et al., 2008). Melittin is a small peptide (2.84kDa) and is the main constituent of the bee venom responsible for the hemolytic activity of this poison (Raghuraman & Chattopadhyay, 2006). The melittin peptide binds spontaneously to lipid membrane surfaces, where it forms an alpha-helix and inserts into the membrane. Further, the lipid-embedded peptides aggregate and form channels. This process involves nanomechanical changes: the mass of the vesicles increases and the lipid-vesicles expand.
peptides bind specifically to the lipid layer on the cantilever. Fig. $4B$ shows the mass changes as well as the static deflection changes of the cantilever recorded simultaneously. After a buffer (Fig $4B, I$) flush, lipid vesicles were injected (Fig $4B, II$). A clear mass increase was observed and the vesicles pulled the cantilevers upwards by the interaction energy of these spherical structures with the thin spring bar. After a second buffer injection, melittin was flushed through the measurement chamber. Again a mass increase is observed and the cantilevers bends down. Obviously the binding and insertion of the melittin peptide led to an expansion of the vesicles (channel formation) and a relaxation of the cantilever spring. Note that the qualitative results are in excellent agreement with current models of the binding and melittin action on and in lipid bilayers (Mally, Majhenc, Světina, & Žeks, 2007; Raghuraman & Chattopadhyay, 2006; Rex & Schwarz, 1998).

Interestingly, the mass changes follow exactly the injection of the adsorbents (vesicles, melittin) whereas deflection alterations are also observed during the flushing of the chamber with buffer. Such changes we interpret as global structural rearrangements (e.g. melittin aggregation and pore formation in the lipid phase, fusion of neighboring vesicles) taking place on the cantilever surface after vesicle or melittin binding respectively. A proof that we measured independent signals.

In summary, this experiment shows that the simultaneous and direct measurement of nanomechanical (structural) changes and mass adsorption can be performed on the same sensor platform in a liquid environment using cantilever technology. It also demonstrates the high dynamic range of the cantilever sensors, even for complex biological system such as lipid-peptide interaction systems. It is interesting to mention that the dynamic mode is independent of the nature of detected ligands, however the static mode detects specific physical characteristics of the molecular binding system.

Conclusion and Outlook

Cantilever sensors provide a versatile tool for sensing applications (Hansen et al., 2005; Lang et al., 2006; Waggoner & Craighead, 2007). Here we reviewed the capability of these sensors to detect molecular interaction and to provide comprehensive information (mass and global structural changes) of such processes (Ghatkesar et al., 2008). This sensor characteristic is unique and allows intriguing applications in nanomedicine as a new method for drug-screening and future diagnostics.

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References


Preclinical Nanomedicine

Fluorescence in Clinical Nanometrology: its Potential Role in Diabetes Research and Management

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Abstract:
Fluorescence is a key technique in nanometrology and many applications in diabetes are now being researched. The advantages of fluorescence include extreme sensitivity and the ability to measure both intensity and lifetime. The latter is particularly useful for in vivo monitoring. Molecular form and interactions can be assessed by fluorescence resonance energy transfer (FRET) and by environmentally sensitive fluorophores. The main diabetes applications are for improved in vitro diagnostics, continuous glucose sensing, diagnosis and monitoring of tissue complications by molecular targeting and single-molecule detection. Important fluorescent tools for nanometrology are quantum dots, nanosensors composed of fluorescence-based glucose detectors and a variety of imaging techniques such as confocal and fluorescence lifetime imaging microscopy (FLIM).

Keywords: Fluorescence, Nanometrology, Imaging

Introduction
Nanomedicine, defined as the medical application of nanotechnology, involves measurements (nanometrology) and therapies based on nanomaterials, nanosensors, and even the futuristic idea of engineering molecular 'assemblers', machines ('nanorobots') which can reorder matter at a molecular or atomic scale. It is a multi-and interdisciplinary field and is widely anticipated to bring enormous benefits to medical research and clinical practice (1). New measurement methodologies are crucial for the development of Nanomedicine, and fluorescence is emerging as a key element in the toolbox of clinical nanometrology. This review discusses some of the key applications of fluorescence nanometrology, and we use diabetes mellitus as a prime example, because of the world-wide importance of this disease.

The problem of diabetes
Diabetes is an epidemic, with the frequency expected to double in the coming decades. It causes problems not only because of the short-term symptoms and signs of glucose fluctuations (hypoglycaemia and hyperglycaemia) but also because of the serious long-term tissue complications as such as retinopathy, nephropathy and neuropathy, and accelerated atherosclerosis (2). Nanometrology in diabetes is in its infancy but is expected to have an impact in several areas. Mainly, this will likely be in applications of Nanomedicine for in vitro and in vivo glucose sensing, improved in vitro diagnostics of diabetes-related biomarkers, and for the diagnosis and monitoring of diabetes complications, both through targeted molecular imaging and by better understanding of pathological mechanisms (e.g. single-molecule detection) (3).

Why fluorescence?
Fluorescence is caused by the emission of light by a molecule as it relaxes from an electronically excited state. Fluorescence spectroscopy has been a major tool of biochemists and biophysicists for some decades is now used extensively in biotechnology, medical diagnostics, DNA sequencing and forensics. In recent years, it has been particularly researched as a sensing technology, for example for glucose monitoring (4). Fluorescence has many advantages in nanometrology. Extreme sensitivity makes it possible to measure the smallest concentrations of clinical analytes, even down to the single-molecule level (5). Fluorescence also offers the capability of non-invasive detection of molecules in the body when excitation by and emission of near infrared (NIR) light are used, since NIR light passes through several centimetres of tissue (4). Not only fluorescence intensity but also lifetime can be measured (6), with fluorescence decay being especially relevant to in vivo sensing because it is relatively independent of light scattering (which can alter the optical path length in tissues) and altered fluorophore concentration (where implanted fluorophores can diffuse or become encapsulated in the tissues). Fluorescence is also unaffected by electro-active substances in tissues which impair the responses of electrochemical sensors (7). Non-invasive glucose sensing would be a major advance for people with diabetes who currently monitor their blood glucose by obtaining intermittent finger-prick capil-
Fluorescence can also provide information about the structure and microenvironment of molecules, and how these changes in response to analyte variations in health and disease, for example by probing protein conformation with environmentally sensitive fluorophores or by using fluorescence resonance energy transfer (FRET) between a fluorescent donor and an acceptor (6,8).

The use of fluorescence technologies in Nanomedicine has been mainly considered for *in vitro* diagnostics, continuous sensing of analytes in the blood stream, or in some other compartment of the tissues and cells (e.g. glucose in diabetes), or for imaging at sites within the body (e.g. most usually to date, detection of metastases in cancer). But individual molecules can also be both sensed and imaged using fluorescence and this may have important applications in pathology and the understanding of disease.

‘Nanosensor’ is the name give to a nano-sized device (less than about 100 nm) which specifically measures an analyte, and ‘nanobiosensors’ (by analogy with the more commonly known technology of biosensors) consist of tiny devices with a biological recognition element (enzyme, antibody, binding protein etc) in close association with a transducer which converts recognition/binding of the analyte of interest to a signal (electrical current or potential, light etc). Here, we are concerned with fluorophores as transducers which translate differing analyte concentrations into changing fluorescent signals.

**Some components of fluorescence nanometryology**

**Quantum dots**

Quantum dots (QDs) are fluorescent nanocrystals composed of a core of semiconductor material, enclosed within a shell that has a larger spectral band gap. Their cores are usually composed of elements from groups II and VI, e.g., CdSe (most commonly) or groups III and V, e.g., InP, while the shell is typically a material such as ZnS. A typical QD has a diameter of about 2–10 nm which makes it of a size that allows one-on-one interaction with biomolecules such as proteins where the typical size ranges from 1 to 20 nm, and access to tissues within the body for targeting.

QDs have several potential medical applications including sensitive *in vitro* diagnostics (e.g. where the QD is used as an antibody label in an immunoassay), *in vivo* imaging and photodynamic therapy. The diverse applications are attributable to their special optical properties (9-12), like high quantum yield (i.e. they are very fluorescent), the ability to tune fluorescence emission according to QD size (e.g. larger QDs are fluorescent in the NIR range and smaller in the UV range) and broad absorption with narrow emission (e.g. a 7.5 nm CdSe QD can be excited by light of any wavelength from UV to the upper end of the visual spectrum but fluorescence is emitted in a narrow range around 650 nm). QDs are also highly resistant to photo bleaching and chemical degradation, clearly a potential problem in vivo.

There have been several reports of QDs for glucose determination. Tang et al described a novel nanobiosensor QDs-ConA-beta-CDs-AuNPs for the direct determination of glucose in serum with high sensitivity and selectivity. The sensing approach was based on FRET between CdTe QDs as an energy donor and gold nanoparticles (AuNPs) as an energy acceptor. Concanavalin A was conjugated with QDs and thiolated beta-cyclodextrins modified with AuNPs. In the presence of glucose, the AuNP-CD is displaced from the binding sites of ConA, resulting in the fluorescence recovery of the quenched QDs (12).

Several investigators have attempted to monitor the glucose oxidase-catalysed oxidation of glucose using QDs:

\[
\text{Glucose oxidase} \quad \text{Glucose} + \text{O}_2 \rightarrow \text{gluconic acid} + \text{H}_2\text{O}_2
\]

Cavalier-Jaricot et al. (13) reported the quenching of QD fluorescence by the hydrogen peroxide produced by glucose oxidase immobilized with the QDs, and Huang et al. (14) monitored the glucose-related acidity change by QDs associated with glucose oxidase, but both methods are relatively insensitive. In a recent study, Yuan et al. (15) used a biensyme system to ensure better sensitivity, where horseradish peroxidase (HRP) is used to produce benzoquinone (a very efficient quencher of QD fluorescence) from hydroquinone:

\[
\text{HRP} \quad \text{H}_2\text{O}_2 + \text{hydroquinone} \rightarrow \text{benzoquinone}
\]

Very recently an assay based on the energy transfer between a QD and gold nanoparticles has been used to detect protein glycosylation (16), which may have interest in diabetes for monitoring the risk of tissue complications.

**NOCs and PEBBLES**

Nano-optochemical sensors (NOCs) or PEBBLES (Probes Encapsulated By Biologically Localized Embedding) are self-assembled, fluorescent probes with conserved polymer cores that contain multiple elements for the selective localization and measurement of ions or small molecules in living cells. The polymer cores are biocompatible and enable the entrapment or covalent incorporation of combinations of sensing and/or reporter molecules with internal reference reporters for quantitative, radiometric analysis. The small size of the PEBBLE sensors enables them to be inserted into a specific locale within a living cell, and the porous, transparent nature of the matrix allows the analyte to interact with the indicator dye which reports this interaction via a change in emitted fluorescence.

One such sensor for glucose has been reported by Xu et al. (17). The sensors incorporate glucose oxidase (GOx), an oxygen sensitive fluorescent indicator (Ru(dpp(SO$_3$Na)$_2$)$_3$Cl$_2$) and an oxygen insensitive fluorescent dye, Oregon Green 488-dextran or Texas Red-dextran, as a reference for the purpose of ratiometric intensity measurements. The enzymatic oxidation of glucose to gluconic acid results in the local depletion of oxygen, which is measured by the oxygen sensitive ruthenium dye.

**Carbon nanotubes (CNTs)**

There has been interest in biosensors based on CNTs. Their large length-to-diameter aspect ratios provide high surface: volume ratios and CNTs have an outstanding ability to mediate fast electron-transfer kinetics for a wide range of electro-active species, such as hydrogen peroxide or NADH. In addition, chemical functionalization of CNTs can be used to attach a number of desired chemical species, allowing enhanced solubility and biocompatibility of the tubes (18). One example of a fluorescent CNT-based nanosensor for glucose was reported by Liu et al. (19). CdTe QDs with the size of about 3 nm were prepared by first mixing CdTe QDs, CNTs, Nafion, and glucose oxidase in appropriate amounts and then modifying this mixture on the glass carbon electrode.
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Imaging
One of the most widely used applications of fluorescence in Nanomedicine has been for imaging. There are numerous examples in literature of QDs being used for imaging in cancer, reviewed by Zhang et al (20). There are very few examples of use of QDs for imaging for diabetes care. One report uses QD-antibody conjugates directed against cell adhesion molecules in diabetic rats (21) and another uses supramolecular protein nanoparticles that bind a glutamate decarboxylase (GAD65)-specific autoantibody, an early marker of type 1 diabetes (22). Since there is a dearth of information on the use of fluorescent nanoparticles for diabetes, there is tremendous scope for research towards this direction (3).

Nanoparticle capping for improving the biocompatibility of nanosensors in diabetes and other disease

As discussed above, the unique, tunable optical features of nanocrystals like QDs and the possibility of incorporating them into living systems such as tissues and cells has opened the way for the investigation of their potential applications as sensors and imaging tools. However, one concern of the use of nanocrystals or nanoparticles in general in biological systems is the toxicity and potential side effects associated with the core constituents such as cadmium and selenium. Nanoparticles also have unique surface characteristics, such as a large surface area: mass ratio, high reactivity, and a tendency to aggregate and adsorb plasma proteins (23). Another equally challenging problem affecting the in vivo use of nanoparticles is that they could be captured by macrophages before reaching the intended target site.

A common approach for regulating the undesirable interactions of materials with surrounding proteins, tissues and cells and therefore to ensure the safer use of these materials is to modify chemically the surface with less toxic and more biocompatible materials. The capped nanoparticle surfaces can then be further functionalized with, for example, –COOH and –NH2 moieties, allowing post-modification to be later used for bio-conjugation purposes.

Of particular interest for biocompatibility and stability is to cap nanoparticles using molecules containing hydrophilic and biocompatible species such as polyethylene glycol (PEG) (24,25), oligopeptides (26), phosphocholine (27,28), carbohydrates (29) and an inorganic coating of silica (30,31) (Fig. 1). In principle, nanocrystals are best formed and stabilized in nonpolar media, therefore, efficient transferring of nanocrystals into water can be achieved using amphiphilic polymers or molecules which offer possibility of tailoring both the hydrophobic and hydrophilic interactions between the particles and the aqueous solution.

Surface modification can be done by a self-assembly process using thiol-containing capping agents or an aminosilane-based functionization route. Nanoparticles that have the above-mentioned functional exteriors have been reported to be extremely water-soluble and stable, exhibiting minimal non-specific interaction with proteins, and therefore rendering them invisible to microphages (32). Meanwhile, the monolayer at the interface could act as a passivation layer to impede electron, proton and oxygen diffusion to the highly reactive surfaces of the nanoparticles.

Encapsulation of in vivo glucose biosensors
A rapidly advancing area of in vivo biosensor development for medical applications is the use of fluorescently labelled bacterial periplasmic binding proteins for biomolecular detection; this is based on the conformation changes that occur in the protein upon ligand binding, e.g. glucose binding to glucose/galactose binding protein (GBP) (33). Several workers have reported the introduction of a fluorescent label into a mutated galactose/glucose binding protein to form a regentless glucose biosensor, based on both enzymatic and fiber optic transduction or encapsulation strategies. Such immobilization strategies include entrapment of the protein within semi-permeable coatings, biocompatible polymer matrices, or inorganic polymer matrices. The immobilization strategy therefore may ultimately determine the performance of the working biosensor. Efficient surface attachment or immobilization of binding proteins have been achieved by using hydrogels (34) layer-by-layer (LBL) multilayers (35) or microcapsules (36-38) (Fig. 2). One unique feature of these entrapment procedures is that they could render the sensor surface resistant to blood-mediated bio-fouling, thereby overcoming cell adhesion and fibrous tissue encapsulation in response to the body’s self-defence mechanism when implanted in the body.

Fig. 1 Surface modification strategies for capping nanoparticles and improving their biocompatibility. (1) oligosaccharide, (2) PEG (linear or branched), (3) zwitterionic phosphocholine, (4) thiol-containing pentapeptide or hexapeptide, (5) silica. (Size not to scale)

Fig. 2 Entrapping or encapsulating of sensing proteins in nano-structured polymeric assemblies.

An example of a potentially implantable biosensor which is based on protein entrapment is a microcapsule-based fluorescence glucose sensor (39). Here, an electrically requires incorporation of the molecular recognition element into a solid surface for interfacing with a signal detector. This can be achieved by placing proteins within or onto the surface of optical fibres or planar waveguides using immobilization or encapsulation strategies. Such immobilization strategies include entrapment of the protein within semi-permeable coatings, biocompatible polymer matrices, or inorganic polymer matrices. The immobilization strategy therefore may ultimately determine the performance of the working biosensor. Efficient surface attachment or immobilization of binding proteins have been achieved by using hydrogels (34) layer-by-layer (LBL) multilayers (35) or microcapsules (36-38) (Fig. 2). One unique feature of these entrapment procedures is that they could render the sensor surface resistant to blood-mediated bio-fouling, thereby overcoming cell adhesion and fibrous tissue encapsulation in response to the body’s self-defence mechanism when implanted in the body.

Fig. 3 Fluorescence response of LBL-nanoengineered microcapsules composed of a glucose-sensing protein (GBP-badan). The microcapsules were constructed by adsorbing the protein onto spherical templates of calcium carbonate, alternating layers of poly-L-lysine and then poly-L-glutamic acid are applied thereafter, followed by dissolution of the template using EDTA.
trostatic LBL nanoassembly process was used to encapsulate fluorescent-labelled GBP (using the environmentally sensitive dye badan) with nano-engineered multilayers of polypeptides, forming a stable and versatile capsule with tunable permeability. An increase in the fluorescence intensity of the capsules occurs upon glucose binding (Fig. 3).

Other glucose sensing systems have been incorporated in nano-engineered LBL assembled capsules, e.g. a FRET-based assay based on the displacement by glucose of fluorescent-labelled dextran from fluorescent-labelled apo-glucose oxidase (40), and encapsulation in polymer layers.

The instrumentation for fluorescence imaging in manomedicine

Imaging and microscopy allows us to probe (and sometimes affect) the biology of disease with minimal invasion. The nanoscale is well suited to fluorescence microscopy: a 30 nm sensor particle can penetrate a cell membrane and has a billion times its own volume to explore in a 50 μm cell. Although light microscopy is limited by diffraction to a resolution of approximately 200 nm, manipulation of fluorescence both optically and mathematically can allow structures to be detected and localised with sub-diffraction-limit resolution. Fluorescence offers the possibility of whole animal/human, tissue and sub-cellular imaging in conjunction with nanoscale detail, in space and time. A few of the fluorescence imaging techniques employed to image nanostructures in vitro and in vivo are highlighted below.

Epifluorescence and confocal laser scanning microscopy

Epifluorescence describes conventional wide-field fluorescence microscopy, where illumination is through the objective. Emitted fluorescence is collected back through this lens and as a result transmitted light does not reach the detector. Confocal laser scanning microscopy eliminates background fluorescence and enables optical sectioning. This is achieved by scanning a focused laser through the sample plane. Out of focus light is then removed by placing a pinhole at the detector, confocal with the image plane. Use of advanced detectors such as electron-multiplying CCD cameras and photo-switchable fluorophores has improved sensitivity in both these techniques.

Multi-spectral confocal microscopy

Multi-colour images can be generated by using a spectral detector and un-mixing software that picks out emission at narrow wavelength bands (42). A series of images are taken over a spectrum of wavelengths. This can be applied to both wide-field and laser-scanning microscopy and allows multiple dyes to be used. With is technique, Prow et al. envisage a ‘Manomedicine system’, a nanoparticle comprising several functional layers for specific cell targeting, membrane entry, intracellular targeting, biosensing and gene or drug therapy (42). To test the accuracy of intracellular targeting, antibody-labelled polystyrene nanoparticles were introduced to cells. Individual components of live human BJAB cells; the membranes, ER and nuclei, were fluorescently labelled with different coloured dyes. The localization of the particles could then be effectively determined by proximity to the other colour labels.

Fluorescence lifetime imaging (FLIM)

In addition to intensity, fluorescence lifetime can be manipulated to achieve image contrast. Lifetime is a measure of the time a fluorophore remains in an excited state. It can be an adjunct or even more useful quantity than intensity in biological imaging (as noted above) since it is not affected by scatter, probe concentration or bleaching.

Two methods exist to measure time resolved fluorescence. Time domain measurements involve excitation with a short pulse of light and collection of the emission from the long-lifetime state increases with the fluorescence lifetime decay from which a false colour image can be generated, displaying lifetime variation over the sample.

In TCSFC FLIM, complex decays are gathered over time with high spatial resolution (with the possibility of detection at multiple wavelengths). TCSFC FLIM has been used to image FRET-based protein-protein interactions; for a review of progress, see Festy et al. (46). Like confocal microscopy, a laser is scanned in a grid of points across the sample, however in this case the source is a pulsed laser and the emission is captured by a single-photon counting detector. At each pixel there is a lifetime decay from which a false colour FLIM image can be generated, displaying lifetime variation over the sample.

TCSFC FLIM is particularly useful for monitoring the environment of a molecular probe, and we are currently using this technique to sense glucose using the LBL-encapsulated GBP-badan, described above. TCSFC FLIM of glucose nanosensors may be a powerful tool to monitor glucose at many tissue and cell sites in body.

Fig. 4. Two-photon TCSPC FLIM was used to image 5μm peptide capsules containing the glucose sensor, GBP-badan. When attached to GBP, badan emits from two states with different lifetimes. Lifetime is a measure of the time a fluorophore remains in an excited state. It can be an adjunct or even more useful quantity than intensity in biological imaging (as noted above) since it is not affected by scatter, proben concentration or bleaching.

Multi-photon microscopy

This uses short, intense pulses of longer wavelength light to excite the sample, where two or more photons are absorbed by the fluorophore. Infra-red light can then be used to excite a visible spectrum dye, allowing increased penetration depth. A useful review of multi-photon
techniques can be found in Provenzano et al. with reference to tumour metastasis (47). Unlike confocal, multi-photon microscopy does not require de-scanning (passing back through the scanning mirrors and onto a confocal pinhole) to eliminate background fluorescence. The probability of absorption of two photons decreases dramatically with distance from the focal volume and out of focus fluorescence is not generated.

The combination of multi-photon microscopy and FLIM allows deep tissue imaging of fluorescence lifetime. This technique has been applied to FLIM and FRET in cell culture and in animals, and provides the necessary tissue penetration that visible wavelength excitation cannot.

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References


Technologies at Hand

**Quo vadis, affinity?**

Clinical evidences and computer-assisted simulations in the Imatinib saga

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**ABSTRACT**

Imatinib mesylate is a drug currently registered for two major indications: (a) monotherapy in chronic myeloid leukemia (CML) and (b) monotherapy in c-Kit (CD117)-positive unresectable or metastatic gastrointestinal stromal tumors (GISTs). Imatinib mechanism of action is to bind to specific tyrosine kinases (Bcl-Abl in CML and c-Kit in GISTs), thereby blocking the corresponding signaling for cell growth and proliferation of malignant cells in CML and GIST. Imatinib is an effective drug; nevertheless, resistance develops over time in many patients. Although kinase overexpression and gene amplification have been observed, the most common event in resistance is the occurrence of mutation(s) in the corresponding genes. In this work, we report the clinical, biochemical and molecular modeling analyses of some important successful/unsuccessful cases observed during imatinib therapy of our GIST patients. Advanced molecular simulation techniques were applied to study the interactions of the wild-type and mutated receptors with Imatinib at molecular level. KIT expression and phosphorylation was detected in cells transfected with vectors carrying the specific mutant genes. Imatinib treatment demonstrated that some mutations were inhibited by imatinib, while others were insensitive to the drug, but responded to other inhibitors. Modeling of the mutated receptors revealed some mutations substantially modify the protein binding pocket, thus hampering inhibitor binding, whilst others induce only relatively confined structural changes, still compatible with drug binding or are compatible with a different binding mechanism. The results obtained from the clinical/biochemical analysis on mutated receptors testing the actual imatinib inhibitory efficiency coupled with molecular modeling highlighted the strength and weakness of this inhibitor towards c-kit mutated isoforms. Therefore these investigation ensemble gives important information to medical oncologist indicating the most suitable dose for escaping secondary resistance.

Keywords: Target cancer therapy, tyrosine kinase, drug resistance, computational chemistry and simulation

**Introduction**

Since its inception, the study of the molecular basis of cancer has carried with it the promise of more refined, more effective cancer therapies. It has generally been assumed that, because cancers are derived from numerous tissues with multiple etiologies, and as tumor progression carries with it a bewildering and seemingly endless combination of genetic and epigenetic alterations giving rise to a hugely disparate series of diseases, cures for cancer must be as diverse as the diseases themselves. The mantra from the cancer research community has been that cancer is not a single disease for which there will be a single cure, and the task of developing therapies suitable for treatment of the full gamut of cancers is depicted as Herculean and almost impossible. Although cancers are indisputably extremely diverse and heterogeneous, underlyng this variability lies a relatively small number of ‘mission critical’ events whose convergence is required for the development of any and all cancers. Two of them emerge neatly from this list: the lesions that power the relentless proliferation of tumor cells, and the compensatory mutations that arise to ensure their survival. Although neoplasia involves many other processes that also present targets for cancer therapy, in almost all instances, deregulated cell proliferation and suppressed cell death together provide the underlying platform for cancer progression. The challenge before the research community is to identify and understand the molecular anatomy of such pivotal steps in tumor progression and to develop therapies that directly attack these points of convergence.

The ‘conventional approach’ to cancer therapy has been to provide treatment according to the organ or tissue in which the cancer originates. This approach was appropriate when there was only a rudimentary understanding of the molecular origins of cancer and the different intracellular signaling pathways that are perturbed in the various types of cancer (e.g,
breast cancer or lung cancer). Recently, however, the genetic events that lead to cancer have been dissected, and it has become clear that cancer develops as a result of multiple genetic defects, and that individuals with the same type of cancer often have dissimilar genetic defects in their tumors. This finding explains why patients who seem to have similar cancers respond in a heterogeneous manner to anticancer agents and shows clearly the huge obstacle to providing effective treatments for cancer.

Accordingly, in the past decade, cancer therapy has slowly but steadily begun to shift from a ‘one size fits all’ approach to a more personalized approach, in which each patient is treated according to the specific genetic defects in the tumor. Such an individualized approach requires the discovery and development of biomarkers (biological indicators) that help oncologists to decide which patients to treat (prognostic indicators).

Dozens of drugs are used to treat cancer and even more are currently in preclinical or early clinical development. Unfortunately, many factors conspire to limit their effectiveness, including problems of delivery and penetration (1), and a modest degree of selectivity for the very cells they were designed to eradicate. But probably the most important — and certainly the most frustrating — of these limiting factors is drug resistance. Some cancers, such as prostate tumors and melanomas, are intrinsically resistant to most anti-cancer drugs. Others, ovarian carcinoma or small cell lung cancer for example, respond to chemotherapy and often disappear altogether, only to return later as drug-resistant tumors. This is acquired drug resistance, and it is encountered in about 30 per cent of all cancer patients undergoing chemotherapy (2).

Tumor cells are a rapidly moving target because of the instability and consequent plasticity of their genomes. Events such as partial or complete deletion of chromosomes, amplification of genes, translocations or rearrangements of chromosomes, and simple mutations ensure efficient selection and overgrowth of drug-resistant tumor cells during and after chemotherapy.

The importance of being a kinase

Certain cancers are caused by oncogenic primary or ‘driver’ mutations in protein tyrosine kinases (TKs), enzymes which catalyze the phosphorylation of tyrosine residues, are involved in cellular signaling pathways, and regulate key cell functions such as proliferation, differentiation, apoptotic signaling and neureit outgrowth (3). Unregulated activation of these enzymes, through mechanisms such as point mutations or overexpression, can lead to various forms of cancer as well as benign proliferative conditions. Indeed, more than 70% of the known oncogenes and proto-oncogenes involved in cancer code for TKs. TKs represent a diverse and rapidly expanding superfamily of proteins, including both transmembrane receptor tyrosine kinases (RTK) and soluble cytoplasmic enzymes also known as nonreceptor tyrosine kinases (NRTK). Activation of the TK domain of either class of TK enzymes results in interaction of the protein with other signal transducing molecules and propagation of the signal along a specific signal transduction pathway. Activation of transmembrane TKs is typically initiated by binding of a ligand (e.g., hormone or growth factor) to a specific site within the extracellular domain of the receptor. Upon ligand binding, these receptors commonly undergo dimerization, resulting in autophosphorylation of tyrosine residues within the cytoplasmic domain. This autophosphorylation event can occur in trans (between receptor molecules within the dimer) or in cis (within a single receptor molecule in the dimer). These phosphorylation events activate the kinase, thereby increasing its intrinsic TK activity, and produce new binding sites for intracellular adapter molecules that bring signal transduction molecules into close proximity.

The human genome encodes 90 proteins with tyrosine kinase domains (4), and, as mentioned above, many human tumors display aberrant activation of tyrosine kinases caused by genetic alterations. For tumors whose growth is driven by these activated kinases, targeted drugs can potentially inhibit or reverse malignant progression. Clinical studies conducted over the past decade have established that TK inhibitors are safe and therapeutically active in selected populations of cancer patients, and several of these drugs are now part of the standard treatment regimen for specific tumor types. However, kinase ‘addiction’ persists in advanced cancer, and patients who relapse after initially responding to kinase-inhibitor therapy often develop secondary mutations in the target kinase that confer drug resistance without impairing the kinase oncogenicity.

Imatinib: the golden bullet

The discovery of imatinib, the first, effective small-molecule TK inhibitor, is rooted in cytogenetic research performed more than 30 years ago on leukemic cells from patients with chronic myelogenous leukemia (CML). These cells display a characteristic reciprocal translocation between chromosomes 9 and 22 that generates the so-called “Philadelphia (Ph) chromosome.” At the molecular level, this translocation juxtaposes the coding sequences of the BCR gene and c-ABL genes. The c-ABL gene encodes a nonreceptor tyrosine kinase, and the genetic fusion creates an oncoprotein, BCR-ABL, with constitutively active TK activity. BCR-ABL powers the rapid clonal expansion of pluripotent hematopoietic stem cells that underlies CML. The uncontrolled kinase activity of BCR-ABL is sufficient to cause leukemia, making it an ideal therapeutic target. About 15 years ago, Brian Druker and Nicholas Lydon, together with other coworkers at Ciba-Geigy, began the process of identifying a compound that would inhibit the BCR-ABL protein and that ultimately would lead to the approval of Imatinib mesylate (STI-571, Gleevec, Imatinib; Novartis Pharmaceutical, Basel, CH) for CML. Five years ago, the approval of Imatinib, and the identification of the proteasome inhibitor Bortezomib (Velcade; Millennium Pharmaceuticals Inc., Cambridge, MA, USA) as an agent with activity in multiple myeloma, created an enthusiastic frenzy for new therapies that has yet to abate (5-8).

Imatinib (4-[[4-(methylpiperazin-1-yl)-methyl]N-[4-methyl-3-[(4-pyridin-3-yl)pyrimidin-2-yl]amino]-phenyl]-benzamide) is a “promiscuous” tyrosine kinase inhibitor in that it blocks the activity of additional TKs, including the c-KIT receptor and the platelet-derived growth factor receptor (PDGFR). A subset of gastro-
intestinal stromal tumors (GISTs) display mutations in the c-KIT gene and express permanently activated forms of the c-KIT receptor. Patients with such tumors, who are largely unresponsive to conventional chemotherapy, show an excellent response to imatinib (9).

Crystallographic studies of ABL in complexes with Imatinib show that the inhibitor binds to inactive ABL and stabilizes it, thus preventing it from achieving an active form (10). In the KIT receptor, the juxtamembrane cytoplasmic region (Thr544 to Trp580) has a dual role, functioning both as an unphosphorylated autoinhibitory domain of signal transduction and as a substrate for trans-autophosphorylation of Tyr568 and Tyr570, which are the primary sites of autophosphorylation upon stem cell factor ligand binding and dimerization. Mol et al. have co-crystallized both ATP and imatinib with KIT (11,12), and demonstrated that Imatinib binds to autoinhibited KIT in a similar manner to that observed in ABL (see Figure 1).

Interestingly, almost one-third of GISTs lacking mutations in c-KIT have intragenic mutations in the PDGFRα gene, resulting in constitutively active PDGFRα, a finding that potentially explains the clinical responses to Imatinib in GISTs with wild-type c-KIT. Imatinib also exhibits robust clinical activity in several other cancers associated with PDGFR alterations (see Figure 2). These include chronic myelomonocytic leukemia (CMML), which is characterized by the constitutively active TEL-PDGFRα fusion tyrosine kinase; hypereosinophilic syndrome, which is characterized by the FIP1L1-PDGFRα fusion protein; and dermatofibrosarcoma protuberans, which is characterized by a t(17,22) chromosomal translocation leading to constitutive production of PDGF ligand and subsequent PDGFR activation. Together, these results suggest that activating mutations in genes encoding the molecular targets of Imatinib are reliable biomarkers of “kinase dependence” and thus may predict which patients are most likely to benefit from the drug.

Of kit(ten) and men

KIT is a receptor tyrosine kinase that belongs to the Type III TK family (see Figure 3, left). Accordingly, it is structurally similar to other receptor TKs such as PDGFRα and β, CSF1R, (compare Figures 1 and 2) and FLT3. KIT is expressed in hematopoietic stem cells, mast cells, melanocytic cells, germ cells, and interstitial cells of Cajal (ICC). KIT is thought to play a key role in the differentiation of ICCs, and without it, a functional ICC network fails to develop. KIT is a transmembrane receptor (see Figure 3, right) whose extracellular portion binds stem cell factor, which is also known as steel factor, mast cell growth factor, and the Kit ligand. The binding of stem cell factor to KIT causes homodimerization, conformational changes, and activation of its kinase sites. Each KIT receptor then cross-phosphorylates its opposing dimer tyrosine residues (cytoplasmic interface). These phosphotyrosines residues then serve as binding sites for the signal proteins downstream of KIT that include MAP kinase, PI3 kinase, STAT5, Ras, and Jak2. These effectors lead to multiple signal cascades that regulate cell functions such as adhesion, proliferation, differentiation, and apoptosis.

The association between KIT and GIST was first made by Hirota et al. (15), who noted that 78% of GIST expressed both CD34 and KIT. Polymerase chain reaction (PCR) analysis demonstrated that five of six GIST samples had mutations in the juxtamembrane region of the KIT receptor. All five of these mutations activated the KIT receptor without the addition of its substrate stem cell factor. Transfection of the cDNA of these mutant KIT receptors into Ba/F3 murine lymphoid cell line resulted in malignant transformation. Rubin et al. (16) demonstrated that GIST samples showed histological evidence

Figure 1. Cartoon representation of the three-dimensional structure of c-KIT in complex with Imatinib (left) and ATP (right). The secondary structure of the TK portrayed in ribbon style, with the following color code: purple, α-helices; gold, β-sheets; light green, loops. ATP and Imatinib are depicted as atom color-coded sticks: gray, carbon; blue, nitrogen, red, oxygen, cyan, phosphorus. Hydrogen atoms are omitted for clarity. These Figures are adapted from (13).

Figure 2. Cartoon representation of the three-dimensional structure of PDGFRα in complex with ATP (left) and Imatinib (right). The secondary structure of the TK portrayed in ribbon style, with the following color code: orange, α-helices; green, β-sheets; kaki, loops. ATP and Imatinib are depicted as atom color-coded sticks: gray, carbon; blue, nitrogen, red, oxygen, cyan, phosphorus. Hydrogen atoms are omitted for clarity. These Figures are adapted from (14).
are uncommon (20-23) (see figure 4, left). and in the intracellular regions of exons 13
mutations at this site therefore release KiT
tractor of KiT enzymatic site; activating mu-
normally functions as a negative regula-
tor on the membrane region of the receptor which
this site (15,19). Exon 11 encodes the jux-
tamembrane region of the receptor which
in roughly 50-80% of GiST at
analysis has demonstrated that muta-
′
mutation is in the 5
it appears that the most common site of
of GiST, while exon 13 and 1-13 alterations
ments in either progression-free or over-
all survival in this patient population, like
has been observed for CML (25).

Turning gold into tin
Despite the early successes with the TK
inhibitors discussed above, the major-
ity of responding patients will eventually
develop resistance to the drugs. Resis-
tance can be caused by amplification of
the oncogenic protein kinase gene or
other mechanisms but, in a high fraction
of cases, resistance can be traced to the
selection of cancer cells with secondary
mutations in the gene encoding the tar-
geted kinase. Although imatinib mesylate
have dramatic clinical responses in patients with
Kit, initial resistance to therapy ranges from 9-13% (26-28). In
addition, many patients who are treated
with imatinib mesylate may develop an
acquired resistance to imatinib. The resis-
tance mutations often affect amino acids
within the kinase catalytic domain, and
they prevent or weaken interaction of this
domain with the drug. Resistance muta-
tions have been observed in the kinase
domains of BCR-ABL, KiT, and the PDGFR
in the tumor cells of patients treated with
Imatinib (29).

The long and widening role of KIT juxta-
membrane domain
Binding of the stem-cell factor (SCF) di-
er to the extracellular immunoglobulin-
like (Ig) domain of c-Kit causes two c-KIT
TKs to dimerize, and permits the kinase
domain to act in trans as substrate and
enzyme for one another. Thus, the result
of SCF binding is the phosphorylation of
specific tyrosine residues located in c-
KIT juxtamembrane (JXM) regions (e.g.,
Tyr568 and Tyr570). Recently, in an el-
egant work Chen et al. have shown that
the cytosolic JXM region of KIT acts as an
autoinhibitory regulatory domain (30).
Further, as highlighted above, mutations
in the JXM domain are associated with
cancers, such as GiSTs and mastocytosis,
and result in constitutive activation of
Kit. In particular, a human KIT mutation
with a two-residue deletion at position
558 to 559 (i.e., Val558 and Val559) was
extensively reported (9,30-32): intrigu-
ingly, this Δ558/559 deletion mutation in
GiSTs has been demonstrated to be ac-
tivating; at the same time, however, this
mutant form of c-KIT is strongly inhibited
by Imatinib. Concurrently, the missense
Val559Gly mutation was found to be even
more sensitive to Imatinib then its wild-
type counterpart. From the clinical and
biological standpoint, this experimental
observation constitute a stimulating co-
nundrum, which deserves a substantiate
observation.


Technologies at Hand

Figure 3. (see to the top) Human receptor
protein-tyrosine kinases (the kinome). The
prototypic receptor for each family is indicated
above the receptor, and the known members
are listed below. TK members in bold and italic
type are implicated in human malignancies.
An asterisk indicates that the member is de-
void of intrinsic kinase activity (adapted from
(1)). (see below ) Schematic illustration of the
different domains of a receptor TK.

of constitutively active KIT receptor.
Subsequent retrospective studies have
confirmed that activating KIT mutations are
present in 80-90% of GiSTs (17,18).
There are 21 exons in the KIT gene, and
it appears that the most common site of
mutation is in the 5’ end of exon 11. PCR
analysis has demonstrated that muta-
tions appear in roughly 50-78% of GiST at
this site (15,19). Exon 11 encodes the jux-
tamembrane region of the receptor which
normally functions as a negative regula-
tor of KIT enzymatic site; activating muta-
tions at this site therefore release KIT
from its own autoinhibition. Other sites of
mutation are exon 9 (extracellular region)
and in the intracellular regions of exons 13
and 17. Exon 9 mutations appear in 3-38%
of GiST, while exon 13 and 17 alterations
are uncommon (20-23) (see Figure 4, left).
KIT activation appears to be an early step
in the development of GiST. Thus, KIT is
the ideal TK for target therapy.

Imatinib mesylate (see Figure 4, right)
was first used clinically in the treatment of
GiST by Joensuu et al. (24), who treat-
ed a single 50-year-old patient with meta-
static GiST who recurred despite aggres-
sive surgical resection and systemic che-
motherapy. In the following years, four
separate prospective phase I/II clinical
trials have examined the palliative role of
imatinib in a population of patients with
locally advanced or metastatic GIST. The
studies have shown that this small mole-
cule TK inhibitor is relatively safe at doses
up to 800 mg per day and that over half
of patients with locally advanced or met-
astatic GIST will have clinical and radi-
ographic disease stabilization or regression
at short-term follow-up. Unfortunately,
objective tumor response rates have not
been associated with significant improve-
ments in either progression-free or over-
all survival in this patient population, like
has been observed for CML (25).

Figure 4. (Left) Hot-spot mutations of KIT
(red) and PDGFRα (blue) detected in GiST
patients. (Right) 3D visualization of the
chemical structure of Imatinib (atom color-
code as in Figures 1 and 2).
chanics/Poisson-Boltzmann Surface Area (MM/PBSA) free energy calculations (35), to investigate the behavior of isolated wild-type and (∆558-559 and Val559Gly) mutant KIT fragments formed by the JXM residues that fold into a β-hairpin in the native protein structure. 300-ns simulations were performed with cubic periodic boundary conditions at constant temperature (300K) and constant volume, using the AMBER 7 suite of programs (36). The Amber force field (with parm99 parameter set) was used to describe the system (37), and the particle mesh Ewald method was employed for the electrostatic interaction calculation. The SGMD method was used with a local sampling time of 0.2 and a guiding factor of 0.1. This complex computational methodology can be briefly described as follows. Molecular dynamics (MD) is a technique that was developed to enhance systematic motion (Sm) in molecular systems. Sm, which can be described as an average motion over a certain period of time, of a macromolecule is normally very slow as compared to its thermal motion, especially when the molecule is trapped in a local minimum state. By enhancing the slow Sm, a protein spends less time in random walk and in local energy minimum states, so that the folding time scale is reduced. As the theoretical framework underlying this work is described in details in our previous papers, we will only briefly summarized it below (38).

The so-called Molecular Mechanics/Poisson-Boltzmann Surface Area (MM/PBSA) method was used for free energy calculations. To evaluate the conformational energy, we applied the following relationship:

\[ F(\Omega) = E_r(\Omega) + E_p(\Omega) + \sigma S(\Omega) \]

where \( \Omega \) represents a peptide conformation, \( F, E_r, E_p, \) and \( S_A \) are the conformational free energy, intrapeptide interaction, Poisson-Boltzmann solvation term, and solvent accessible surface area, respectively. The procedure for estimating the binding free energy, \( \Delta G_{\text{bind}} \), of the wild-type and the mutated c-KiT proteins in complex with Imatinib was as follows:

\[ \Delta G_{\text{bind}} = G_{\text{complex}} - (G_{\text{c-KiT}} + G_{\text{Imatinib}}) \]

The individual terms of the MM/PBSA method that contribute to the free energy of a molecular binary complex are:

\[ F(\Omega) = E_r(\Omega) + E_p(\Omega) + \sigma S(\Omega) \]

Figure 5. (top, left) Cartoon ribbon of KIT highlighting the kinase domain involved in maintaining an autoinhibited structure: red, JXM β-hairpin domain; blue, control C-helix; yellow, activation domain. (top, right) Highlight of the WT JXM KIT β-hairpin (ball-and-stick atom color-code as in previous Figures). (middle, left) Equilibrated SGMD snapshot of the refolded WT JXM KIT β-hairpin (atom color-code as in previous Figures). Hydrogen bonds detected during SGMD are highlighted in the red panel, and as green lines in the model. (middle right) Superposition of the crystal structure (yellow sticks) and the refolded SGMD snapshot (violet) of the WT JXM KIT β-hairpin. (bottom, left), Equilibrated SGMD snapshot of the refolded ∆ 558-559 JXM KIT β-hairpin (atom color-code as in previous Figures). Hydrogen bonds detected during SGMD are highlighted in the red panel, and as green lines in the model. (bottom, right) Equilibrated SGMD snapshot of the refolded Val559Gly JXM KIT β-hairpin (atom color-code as in previous Figures). Hydrogen bonds detected during SGMD are highlighted as green lines in the model.
\[ \Delta G_{\text{bind}} = \Delta E_M + \Delta G_{\text{solv}} - T\Delta S \] (3)

\[ \Delta F_M, \Delta G_{\text{solv}} \] and \(-T\Delta S\) denote the total of molecular mechanical (MM) energies of the complex in the gas phase, its solvation free energy, and an estimate of the solute entropy, respectively. \(\Delta F_M\) includes van der Waals and electrostatic (Coulombic) energies:

\[ \Delta F_M = \Delta E_{\text{vdW}} + \Delta E_{\text{ Coul}} \] (4)

In the same fashion, the solvation contribution to binding features two terms:

\[ \Delta G_{\text{solv}} = \Delta G_B + \Delta G_R \] (5)

The polar solvation process (i.e., \(\Delta G_B\)) is equivalent to the transfer of a protein from one medium with dielectric constant equal to that of the interior of the protein to another medium with dielectric constant equal to that of the exterior of the protein. This term yields a free energy because it corresponds to the work carried out to reversibly charge the solute, and it is a polarization free energy because the work goes to the polarization of the solvent. The nonpolar solvation contribution to solvation (i.e., \(\Delta G_R\)) includes cavity creation in water and van der Waals interactions between the modeled nonpolar protein and water molecules. This term can be imagined as transferring a nonpolar molecule with the shape of the protein from vacuum to water. Finally, the last parameter yields the change in solute entropy upon association, \(-T\Delta S\).

The good guy (WT) behavior

Now, generally speaking, hydrogen bonds (HBs) are an important property for protein secondary structure description. In particular, a \(\beta\)-hairpin structure has interstrand HBs that contribute to maintain this secondary structure. Indeed, each \(\beta\)-hairpin structure can be uniquely defined by its interstrand HBs; therefore, in this work, for convenience we define a hydrogen bond pattern as one or more interstrand HBs within a given \(\beta\)-hairpin structure. The wild-type (WT) c-KIT \(\beta\)-hairpin has the following sequence (one aminoacid code): Y(546)LQKpmYEVQW

The remaining 4 structures do not belong to this cluster, and have their own interstrand HB pattern, with a smaller number of HBs. Based on frequency of folding and duration of folded periods, it was clear that cluster I represents the major folded structure; moreover, a plethora of other HBs are present in this ensemble, giving rise to a HB network that stabilizes the structure in the optimal conformation for KIT autoinhibition. Last but not least, from Figure 5, middle right it can be easily seen that the cluster I structure is in excellent agreement with the corresponding crystal structure of the JXm domain in the autoinhibited structure of c-KIT (11-12).

At this point of the investigation, a good question rose in our mind: for the WT KIT JXM peptide, was really the \(\beta\)-hairpin the global minimum state? To satisfy our curiosity, from the application of the MM/PBSA we calculated the average of the conformational energy \(F(\Omega)\) and each of its components for each folded species visited during the SGMD simulation. Again, for cluster I (folds 2, 4, 7 and 10, see Table 1) we obtained the lowest values of \(F(\Omega)\) (see Table 1), and this clearly indicated that this cluster represents the free energy minimum state among the conformation searched during this simulation. All other folded species had lower (i.e., less negative!) conformational free energies than the simulation averages, speaking loud and clear about the fact that they are local free energy minimum state.

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The decrease of \(F(\Omega)\) during the \(\beta\)-hairpin refolding stems from the combined changes in intrapeptide interactions and solvent contributions. Indeed, the global minimum structure (cluster I) is not the one with the strongest intrapeptide interaction. It is then the balance of intrapeptide and solvent interactions that determines the conformational free energy: in fact, the solvation effect prevents the peptide from folding to structures whose gain in intrapeptide interaction cannot compensate for loss of solvent interactions, as well evidenced by the \(E_{\text{MM}}\) data listed in Table 1. Said it in other words, the solvation effect restricts the conformational space for the peptide to fold and prevents the peptide from folding into misfolded structures.

The naughty (?) guys behavior

The application of the same procedure to the \(\beta\)-hairpin structure of Δ558/559 of KIT showed that it presents a HB pattern different from that of the WT TK. Also the HB network is almost entirely disrupted in the presence of this deletion mutation. As a result, the structure of the Δ558/559 mutant yielded by the simulation is quite distorted and far from the ideal shape to lock the control c-helix and the activation loop in the proper, closed conformation (see Figure 5, bottom left).

On the contrary, the conservative, missense substitution of a valine with a glycine at position 559 results again in a different HB pattern, but still characterized by three interstrand HBs as in the wt counterpart, and in an overall less distorted and more “wt-like” folding (see Figure 5, bottom right).

Now, the deletion mutation Δ558/559 in KIT is known to be activating, and is found in GISTs; however, GIST patients carrying this mutation respond well if treated with Imatinib. Is there any molecular reason for this? In the affirmative, all other things being equal, the values of the affinity of the mutant KIT isoforms for the inhibitors should provide the rationale. The calculated free energy of binding \(\Delta G_{\text{bind}}\) between WT KIT and Imatinib, as resulting from the application of the MM/PBSA ansatz is equal to \(-10.2 \pm 0.2\) kcal/mol, whereas that of mutant Δ558/559 and Imatinib is \(\Delta G_{\text{bind}} = -12.3 \pm 0.3\) kcal/mol. Therefore, since the affinity of the mutant for Imatinib is more negative, that means that more energy is released from the system upon drug binding, the mutant isoform is a tighter binder of Imatinib than the corresponding WT counterpart. The main reason for such disparity can be traced to the fact that the molecular dimensions of the inhibitor are somewhat too big to result in a snug fit within the pocket formed at the N- and C-lobe interface of the inactive structure of the wt kinase. Accordingly, when the RTK is in the close, inactive form, the distorted JXM conformation causes a global, conformational rearrangement which involves also the ATP-binding pocket, allowing for a larger space to accommodate the inhibitor.

The missense mutation Val559Gly has also been demonstrated to be more sensitive to Imatinib compared to WT KIT. In this case, the slightly different conformation of the JXM influences the drug binding site, allowing for a better Imatinib binding. On the other hand, this mutation has been found to be characterized by a relative inefficiency of constitutive activation. The SGMD simulation clearly supports this finding, as the JXM HBs pattern and the overall form of the refolded JXM

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Table 1. Components of the conformational free energy for cluster I best

<table>
<thead>
<tr>
<th>FOLD</th>
<th>Ep (kcal/mol)</th>
<th>EPB (kcal/mol)</th>
<th>ESA (kcal/mol)</th>
<th>F(Ω) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-246.16 ± 0.85</td>
<td>-209.56 ± 0.80</td>
<td>7.41 ± 0.01</td>
<td>7.83 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-448.3 ± 0.23</td>
<td>-447.78 ± 0.22</td>
</tr>
<tr>
<td>4</td>
<td>-269.80 ± 0.69</td>
<td>-185.36 ± 0.63</td>
<td>7.42 ± 0.01</td>
<td>-447.19 ± 0.20</td>
</tr>
<tr>
<td>7</td>
<td>-266.76 ± 0.62</td>
<td>-187.85 ± 0.53</td>
<td>7.42 ± 0.01</td>
<td>-447.19 ± 0.20</td>
</tr>
<tr>
<td>10</td>
<td>-260.30 ± 0.15</td>
<td>-193.72 ± 0.67</td>
<td>7.53 ± 0.01</td>
<td>-446.49 ± 0.24</td>
</tr>
</tbody>
</table>

domain is well reminiscent of that of the wild type isoform. In summary, the application of advanced molecular simulation techniques to study the folding of WT and mutant Kit JXM domains, and their relative affinity towards Imatinib, in order to find a molecular rationale for clinical evidences, revealed that the β-hairpin of WT KIT is characterized by a 3 HB pattern, and that this structure represent the a global energy minimum, resulting from the balance of intrapeptide and solvation interactions. The structure refolded by simulation is in excellent agreement with interactions. The structure re-folded by simulation is in excellent agreement with the one present in the crystal structure of the closed, inactive form of the rpTK. The Δ558/559 mutant induces a substantial modification in the conformation of the JXM domain which, in turn, results in a shifting of the equilibrium towards the open, active form of the kinase. At the same time, when in the closed form, this JXM carrying the double deletion mutation is able to better accommodate Imatinib, thus accounting for lower values of drug necessary to inhibit this mutant isoform. The missense mutation Val559Gly, on the other hand, is less deleterious since the conformation of the JXM domain in only slightly altered. This fact still allows for a better Imatinib binding when the RP TK is in the closed, inactive form; contemporarily, the similarity of the mutated and wild type domains account for the fact that the mutant protein is characterized by a relatively inefficient constitutive activation.

The story of Mr. X

September 2003: a male patient with c-KIT positive, high risk epithelioid GIST received 6 courses of chemotherapy with Ifosfamide in combination with Imatinib (300 mg), and then continued with a pulse schedule of Ifosfamide (3 times/year) in combination with Imatinib. October 2004: he relapsed at liver. November 2004: he was switched on a new combination chemotherapy of Gemcitabine + Taxotere + Avastin. December 2004: a new computerized tomography (CT) scan and a new magnetic resonance imaging (MRI) performed for the first time at the Istituto Italiano per lo Studio e la Cura dei Tumori of Milan, Italy, revealed further disease progression at liver. December 24th - March 14th: he received Imatinib at 800 mg/day. Therapy was stopped due to the evidence at MRI of progression both at liver and at peritoneum. May 2005: he entered the SU11248 phase II trial A6821026 obtaining stable disease (SD) at liver and PD at peritoneum. MRI and CT highlighted the evidence of some initial response of liver lesions, but peritoneal ones were slightly increasing after one cycle (4 weeks). SU11248 was stopped soon after starting second cycle because of fever and mild respiratory symptoms. Pulmonary CT scan shows lung nodules, consistent with several small metastases, even considering the slight peritoneal progression (respiratory symptoms improved with antibiotics and antifungal agents). August 2005: he started PKC412, a staurosporin derivative that acts as an multi-targeted kinase inhibitor inhibiting several TK isoforms (e.g. FLT3, KDR, PDGFR, KIT) and several clinically relevant mutants thereof, within a compassionate use program.

A positron emission tomography (PET) scan performed on August 31st showed a switch-off of lesions; the MRI, repeated 20 days later, confirmed SD with some signs of tissue response. November 2nd - November 24th: PKC412 was stopped and Imatinib 800 mg/day was reintroduced. While on Imatinib there was an over progression of the disease both at liver and at peritoneal cavity and some soft tissue nodules became clinically evident. November 25th: PKC412 therapy was restarted. Intriguingly, a subcutaneous nodule of the chest wall disappeared after 2 doses of PKC. December 17th: Itraconazole was added to overcome the PK problems through the inhibition of cytochrome P450. January 5th: the CT scan showed a necrotic evolution of the hepatic lesions and a substantial stabilization of the peritoneal lesions. Unfortunately due to GI toxicity (mainly nausea), the dose was decreased and then stopped. Clinically the patient worsened dramatically after having stopped PKC and, eventually, died.

While under treatment, immunostaining for KIT and PDGFRα in the patient cancer tissues was performed following standard protocols. RNA and DNA were extracted and amplified. The sequencing of the cDNA and genomic DNA was performed with an automated sequencing, again following standard protocols. The alignment of sequencing data was done with KIT sequence (NCBI_Xo6aB2) and PDGFRα sequence (NCBI_NM006206). Protein extraction, immunoprecipitation experiments and Western Blotting were performed following standard routines. Site-specific mutagenesis and transfection experiments were performed using COS1 cells, that do not express endogenous KIT. A dual color fluorescent in situ hybridization (FISH) analysis was performed using a SG-labelled PDGFRα + SO-labelled CEPE4, and SG-labelled PDGFRβ + SO-labelled 5p11.2 region.

The immunohistochemical analysis demonstrated a weak positivity for KIT (see Figure 6, top left), while a strong positivity for PDGFRα was detected (Figure 6, top right).

Since the patient did not respond to Imatinib, the molecular analysis was then performed sequencing the hot spot of mutations for KIT as well as the regions for secondary resistance (exons 9,11,12, 13,14 and 17, see Figure 4, left). All these regions resulted WT in sequence. PDGFRα was analyzed for exons 10,12, 14 and 18 and the mutation at position 2520 (A→T) was detected. This mutation involves exon 18, and is responsible for the aminoacidic substitution Asp842Val affecting the activation Loop of the receptor. Since also gene amplification is reported to be one
of the mechanism of Imatinib resistance, FISH analysis was performed, revealing a disomic pattern for both KIT (left) and PDGFRα (right). (bottom) Transient transfection experiments from which the modified receptors (KIT/Asp816Val and PDGFRα/Asp842Val) were found to be insensitive to Imatinib at all applied concentrations (left and middle panel), whilst PDGFRα/Asp842Val resulted to be inhibited by PKC412 (right panel).

The activation-loop represents a hotspot region for activating mutations in class III TKs. Point mutations involving the homologous Asp residue have been already reported for Flt-3 (Asp835) and c-KIT (Asp816). Such an Asp codon is highly conserved in TKs, thus implying some kind of regulatory role.

On these premises, wanted to provide a molecular explanation for this clinical findings, resorting to the use of our computational chemistry toolbox. Then, we first built a three-dimensional model of the PDGFRα receptor, whose crystal structure is currently not available to the scientific community, by applying well-validated set of homology techniques. Then, we applied procedures similar to those described in the first part of this work to spot similarities and differences between c-KIT and PDGFRα. Our models reveal that the side chain of ASP816 in c-KIT is making a H-bond between the carboxylic acid group of Asp816 and the NH of the amidic side chain group of Asn819, which clearly is no longer present when Ala is substituted at this position. Due to the repositioning of the side chain, also other important H-bonds (i.e., with Ala590 and Lys828) are no longer dominant interactions. The calculated free energy of binding for Asp816KIT/Imatinib is $\Delta G_{\text{bind}} = -10.02$ kcal/mol, whereas the corresponding value for Val816KIT/Imatinib is $\Delta G_{\text{bind}} = -6.89$ kcal/mol. Keeping in mind the concepts expressed above, these results clearly indicate that mutation of the key Asp residue, Asp816KIT, is instrumental in destabilizing the inactive state of this protein, resulting in a lesser affinity for Imatinib. Due to the high homology, the stabilizing hydrogen bond network in PDGFRα is very similar.

The calculated free energy of binding for Asp842 PDGFRα/Imatinib is $\Delta G_{\text{bind}} = -10.77$ kcal/mol, whereas the corresponding value for Val842 PDGFRα/Imatinib is $\Delta G_{\text{bind}} = -6.43$ kcal/mol. PKC412 is an ATP competitive inhibi-

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In summary, the molecular and biochemical analyses of a GIST patient refractory to Imatinib therapy but responding to treatment with PKC412 demonstrated the presence of the missense mutation Asp842Val in the PDGFRα receptor. This aminoacidic substitution is responsible for the overexpression of an activated PDGFRα receptor. Transient transfection experiment demonstrated the Imatinib insensitivity of Asp842Val/ PDGFRα, while it was inhibited by PKC412. For this reason, the patient was treated in a compassionate use program with this compound. The efficacy of PKC412 was detectable by lesion switch off, observed by PET scan. The molecular modeling experiments clearly revealed that the substitutions Asp842Val in PDGFRα and the homologous in KIT, Asp816Val, are responsible for the induction of the conformational changes of the receptors towards their active/open conformation, which has a less affinity for Imatinib. Since PKC412 targets the active kinase form, the mutation favor equilibrium shifting towards this kinase state. In this state, the residue 842 does not appear to be involved in any particular intramolecular interaction and, hence, the Asp842Val mutation does not interfere with PKC412 binding to PDGFRα.

**Conclusions**

This work reports only two of the vast array of works in which molecular modeling techniques can be usefully coupled to well-consolidated experimental routines to unveil the molecular mechanisms eventually presiding at the successes or failures of target therapy. From the ideal standpoint, it would be extremely beneficial to be able to analyze as many tumor tissues form patients participating in

![Figure 6](image-url) (top) Immunohistochemical analysis of a GIST showing weak positivity for KIT (left), and strong positivity for PDGFRα(right). (middle) Dual color FISH analysis revealing a disomic pattern for both KIT (left) and PDGFRα (right). (bottom) Transient transfection experiments from which the modified receptors (KIT/Asp816Val and PDGFRα/Asp842Val) were found to be insensitive to Imatinib at all applied concentrations (left and middle panel), whilst PDGFRα/Asp842Val resulted to be inhibited by PKC412 (right panel).

![Figure 7](image-url) (top) Molecular model of the inactive form of Asp816 (WT) (left) and the mutant Val816 KIT (right) in complex with Imatinib. The proteins are depicted as green ribbons, the drug is in atom-colored stick-and-ball, and the residue is in atom-colored sticks. Hydrogens are omitted for clarity. (bottom) Molecular model of the active form of Asp816 (WT) (left) and the mutant Val816 KIT (right) in complex PKC412. The proteins are depicted as yellow ribbons, the drug is in atom-colored stick-and-ball, and the residue is in atom-colored sticks. Hydrogens are omitted for clarity.
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clinical trials as possible in order to study, by computer simulations, all the molecular features that correlate with sensitivity or resistance to these molecularly targeted agents. Should these conditions be achieved, future research on molecular target therapies, a fundamental branch of Nanomedicine, could focus on the identification of new therapies and targets, improved selections of tumors sensitive to these active principles, and the rational design and optimization of combined therapies. The resulting new wave of discoveries will undoubtedly concur to transform oncology from its current, widespread status to a true, personalized medicine.

Acknowledgments
Such a longstanding and fruitful activity in this stimulating field would have never happened if many, superb collaborators had not put all their efforts in each single project. It is a formidable task to thank all of them. However, let me take the offer of course, science cannot survive without financial support. The continuing generous contributions from the Italian Association for Cancer Research, the Italian Ministry of Health, and the Swiss Cantonal Ticino are gratefully acknowledged.

References
The Spiritualization of Science, Technology, and Education in a One-World Society

Martin Erdmann*

Abstract

Western Christianity has always provided a rational and moral basis for the development of science and technology, including clinical nanomedicine. Yet this sensible basis has been strongly disputed for about half a century. This paper will outline some of the pivotal reasons why influential intellectuals in England and America, mostly in the later part of the 20th century, concluded that irrationality would be a better foundation for the scientific enterprise.

Aldous Huxley envisioned a future world society totally controlled by an elite group of scientists. His best-known fictional work explicating this dire prospect bore the title *Brave New World*. Years later he would “revisit” his prognostications only to conclude that he had underestimated the rate of change realizing his darkest fears. Turning to mysticism, both in its meditative and drug-induced varieties, he prepared the way for the burgeoning Human Potential Movement which was initially formed at the Esalen Institute, Big Sur, CA in the early 1960s. The electrical engineering professor at Stanford University Willis W. Harman, who had gotten involved in researching the cognitive and societal effects of LSD consumption, conducted seminars at Esalen on “Human Potentialities”. Under his directorial supervision at the Stanford Research Institute a scientific study entitled “Changing Images of Man” was carried out from 1972-74 with the purpose of changing the “conceptual premises underlying Western society”, including a radical modification of the rational worldview of western scientists. As the president of the Institute of Noetic Sciences from 1977 to 1996, Harman advocated openly a mystical outlook on life claiming that a spiritual approach to scientific research and technological development would greatly enhance our understanding of the monistic unity of the universe.

Keywords: Aldous Huxley, Brave New World, The perennial philosophy, mysticism, LSD, Captain Albert M. Hubbard, totalitarianism, scientific research, Stanford Research Institute, Changing Images of Man, Willis W. Harman, Gorbachev, cosmic conscience

Introduction

In bygone centuries, science, technology, and education in Western society have benefited greatly from the foundation of rational thinking which the Christian faith affords. It seems logical, therefore, that the development of a highly sophisticated and complex field such as clinical Nanomedicine will be best facilitated by a continued affirmation of the Christian view of a rational and moral universe. However, a strong thrust to discard this foundation is observable since the early 1960s.

Inspired in part by Aldous Huxley’s publications and his advocacy of psychedelic drugs, intellectuals such as Willis W. Harman have begun to emphasize irrational “intuitive knowledge” (gained usually by meditative — mind-emptying — exercises or the use of hallucinogens) as a more congenial basis for scientific and technological progress. Calling for a new metaphysic of science/technology, the proponents of the Human Potential Movement perceive the religious heritage of the West, based on Christian premises, as the greatest impediment of an evolving “cosmic conscience”. In gaining a more comprehensive understanding of the spiritual and material processes of the universe, a mystically inclined elite of technically enhanced human mutants would be able to usher in a homogeneous world socialist society, perhaps not altogether different from the one envisioned in *Brave New World*.

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Brave New World

The famous novelist Aldous Huxley (1894-1963) expressed his growing dissatisfaction with Western civilization in the dystopian satire *Brave New World* (1932).* Despite its frightful tone and horrific outlook, it was destined to become his most acclaimed literary work. Masterfully depicting the inhumanity prevalent in a technocratic society — which had outlawed art and religion while intimately controlling the private affairs of its citizens — the
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The author's disdain for the spiritual emptiness of modernity broke frequently to the surface in his narrative. The novel depicted the dark side of futuristic totalitarianism. To distract the people's attention from the grim reality of being serfs, the governing regime entertained them in the movie theatre by "feeles". Men were allowed to enjoy the questionable pleasures of "pneumatic girls". In order to produce an artificial sense of both tranquility and ecstacy, an ample supply of the potent psychedelic "soma" was handed out regularly to the suppressed population. It was an "[e]uphoric, narcotic, pleasantly hallucinant ... all the advantages of Christianity and alcohol; none of their defects." While perverted sexual behaviour was encouraged, normal human reproduction was considered immoral and embryos were produced by artificial fertilisation in factories; a life-support mechanism referred to as a "bottle" took the place of a mother's womb.

In Brave New World Revisited (1958), Huxley expanded on his earlier prognostications of a dictatorial future society in which the ruling elite would employ highly effective brain-washing techniques — "orthodoxies drummed in by nightly courses of sleep teaching" — to subdue their subjects' rebellious inclinations. To submit willingly to the imposed confines of a completely organized society, the hapless victims of a "scientific caste system" would undergo "methodical conditioning". These mind-bending procedures would be supplemented by regular doses of chemically induced happiness. The need to use unmitigated violence would then be a disagreeable aspect of past tyrannies:

Under the relentless thrust of accelerating over-population and increasing over-organization, and by means of ever more effective methods of mind-manipulation, the democracies will change their nature; and quaint old forms — elections, parliaments, Supreme Courts and all the rest — will remain. The underlying substance will be a new kind of non-violent totalitarianism. All the traditional names, all the hallowed slogans will remain exactly what they were in the good old days. Democracy and freedom will be the theme of every broadcast and editorial — but democracy and freedom in a strictly Pickwickian sense. Meanwhile the ruling oligarchy and its highly trained elite of soldiers, policemen, thought-manufacturers and mind-manipulators will quietly run the show as they see fit!

In 1961 the University of California, San Francisco Medical Center sponsored a conference to discuss the societal effects of technology. Never wont to pass up an opportunity to point to his anticipated vision of a "scientific dictatorship of the future", Aldous Huxley, one of the featured conference speakers, said the following:

There will be in the next generation or so a pharmacological method of making people love their servitude and producing dictatorship without tears, so to speak. Producing a kind of painless concentration camp for entire societies, so that people will in fact have their liberties taken away from them but will rather enjoy it, because they will be distracted from any desire to rebel — by propaganda, or brainwashing, or brain-washing enhanced by pharmacological methods. And this seems to be the final revolution.

Chemical Ecstasy

In The Doors of Perception (1954), Huxley published the detailed elucidations of his mystical experiences after the British psychiatrist Humphrey Osmond had introduced him to the hallucinatory drug mescaline (derived from the cactus peyote) a year earlier:

The man who comes back through the Door in the Wall will never be quite the same as the man who went out. He will be wiser but less cocksure, happier but less self-satisfied, humbler in acknowledging his ignorance yet better equipped to understand the relationship of words to things, of systematic reasoning to the unfathomable Mystery which it tries, forever vainly, to comprehend.

Huxley explicitly referred to H. G. Wells' phrase "Doors in a Wall" which had originally described the taking of drugs in death cult rituals. Both authors knew that the use of hallucinatory substances had always been an essential part of the initiatory rites of ancient mystery cults. The priesthood of the Isis Cult was especially keen on using drugs to induce in its initiates euphoric and transcendental experiences. Nearly a decade earlier, in 1946, Huxley's annotated anthology of mystical writings had appeared under the title The Perennial Philosophy indicating his intimate knowledge of the mystical tradition of ancient, medieval and eastern spirituality.

Captain Albert M. Hubbard and Gerald Heard were present when Huxley took another dose of mescaline in 1955. At the time he was writing the sequel to The Doors of Perception, which he named later Heaven and Hell. In a letter to Dr. Osmond he wrote, "Your nice Captain tried a new experiment — group mescalination ... Since I was in a group, the experience had a human content, which earlier, solitary experience, with its Other Worldly quality and its intensification of aesthetic experience, did not possess ... It was a transcendental experience within this world and with human references." Shortly thereafter Hubbard convinced Huxley to try LSD. In Acid Dreams Lee and Shain state, "Huxley and Hubbard shared a unique appreciation of the revelatory aspect of hallucinogenic drugs. It was Hubbard who originally suggested that an LSD-induce mystical experience might harbour unexplored therapeutic potential."

Huxley harboured a deep-seated aversion to Christianity. In a conversation with Timothy Leary, a former lecturer in psychology at Harvard University and front man of the Hippie counter-culture, Huxley seemed confident that his advocacy of psychedelics would overcome any resistance to his ideas of large scale social engineering: "These brain drugs, mass produced in the laboratories, will bring about vast changes in society. This will happen with or without you or me. All we can do is spread the word. The obstacle to this evolution, Timothy, is the Bible." Fully agreeing with the opinion of his mentor, Leary, in his autobiographical account of the Harvard University Psychedelic Drug Project Flashbacks, added the following remarks:

We had run up against the Judeo-Christian commitment to one God, one religion, one reality, that has cursed Europe for centuries and America since our founding days. Drugs that open the mind to multiple realities inevitably lead to a polytheistic view of the universe. We sensed that the time for a new humanist religion based on intelligence, good-natured pluralism and scientific paganism had arrived.

Human Potential

In 1960 Huxley was diagnosed with cancer; his delicate constitution, ravaged...
already by his drug-habit, began to deteriorate visibly in the years following. Undeterred the British littérature accepted in the same year he fell sick an invitation by the University of California, San Francisco Medical Center to deliver a lecture on “Human Potentialities”. Although “we are pretty much the same as we were twenty thousand years ago,” said Huxley, “we have “in the course of these twenty thousand years actualized an immeasurable number of things which at that time for many, many centuries thereafter were wholly potential and latent in man.” Following these remarks, he put forth the theory that humans possess within themselves still other unrealized abilities which need to be discovered and actualized by utilizing appropriate techniques and chemicals. “The neurologists have shown us that no human being has ever made use of as much as ten percent of all the neurons in his brain. And perhaps, if we set about it in the right way, we might be able to produce extraordinary things out of this strange piece of work that a man is.” Soon thereafter Aldous began to write his final work, the utopian novel Island, and lecture regularly on “Human Potentialities” at the Esalen Institute.

Esalen Institute

Attending the “Human Potentialities” lecture at the San Francisco Medical Center, Richard Price was fascinated by Huxley’s appeal to explore the hidden powers of the human psyche. In a letter to Huxley, Price’s friend Michael Murphy wanted to know how to tap into the hidden powers of the mind. In response Huxley highlighted the complementarity of science and mysticism and pointed to the writings of ancient mystics and eastern swamis as the source of his own inspiration.

In 1962, Price and Murphy established a retreat centre, the Esalen Institute; at Big Sur, California and asked Willis W. Harman, known for his LSD research, to lead the first conference on human potentiality called “The Expanding Vision”. In the late 1950s Harman had volunteered to be one of Captain Albert M. Hubbard’s early test cases in psychedelic drug research. Hubbard, a high-level OSS officer in the Second World War and an undercover agent for several agencies including the FDA and FBI in the 1950s, was put in charge of studying the therapeutic potential of LSD. Of particular interest to him were the drug’s mind-altering potencies, supposedly producing a harmonious state of being. The societal implications of an extensive use of LSD in reducing civil strife were considered advantageous if scientifically proven. To be able to conduct his research more efficiently, Hubbard asked Myron J. Stolaroff and Paul Kurtz to set up the International Foundation for Advanced Studies (IFAS) in Menlo Park, CA as an institutional base. Also involved with the IFAS were Charles and Ethel Savage, Robert Mogan, and James Fadiman. Stolaroff served as its president from 1960 to 1970 while being the executive administrator for a research group conducting clinical studies on the cognitive effects of psychedelics. The research findings of the experiences of about 350 volunteers, who had taken LSD and mescaline under strict supervision, were published in six scientific papers.

Quickly moving from the experimental stage to an administrative post, Harman accepted the vice-presidency of the IFAS and guided the organisation through mounting public criticism in the early 1960s. The exorbitant fee of $500 for a single session of high-dose psychedelic therapy had stirred up bad publicity at a time when the FDA began thinking about outlawing the usage of LSD. In late 1962, the Foundation released its first academic paper “The Psychedelic Experience: A New Concept in Psychotherapy”. Its abstract read in part as follows: The authors, by the simultaneous administration of massive doses of lysergic acid diethylamide (LSD) and mescaline, tried to produce a unique experience for the patient which is to be so profound and impressive that it changes the patient’s own evaluation of his past life experiences and consequently may lead him to establish new values and a more realistic frame of reference than had been established before. The experience, in a broad sense, is not unlike a religious conversion.

Hubbard’s connections to the political establishment in Washington, D.C., aided by his secret service credentials, strengthened Harman’s resolve to continue the research program at IFAS until its final demise in 1965. Keenly interested in continuing his sociological studies of the psychedelic subculture and its relationship to the political upheavals of the New Left, Harman accepted the directorial position at the Stanford Research Institute’s Educational Policy Research Center.

Stanford Research Institute

The Stanford Research Institute (SRI; later renamed SRI International) was incorporated by the trustees of Stanford University in 1946 as a non-profit research institute similar to the more established Mellon and Battelle Institutes. Originally a centre of innovation to support economic development in northern California, it became one of the world’s largest contract research organisations. Some of Stanford’s major clients included at first different branches of the US military — such as the Department of Defence Directorate of Research and Engineering and the US. Department of Defence Office of Aerospace Research.

SRI quickly assumed a vital role in the development of new military technology being used by the Defence Advanced Research Projects Agency (until 1972 and from 1993-1996 known as ARPA: Advanced Research Projects Agency). Its computer network, a precursor of the Internet, linked thousands of processing consoles, including those of the CIA, U.S. Army Intelligence, The Office of Naval Intelligence, Bell Telephone Laboratories, Rand, MIT, Harvard and UCLA, with one another. The Institute’s database functioned as the “library” of the entire system, cataloguing all DARPA documentation.

Outside of the defence sector, the Institute offered a diverse palette of research projects such as the SRI Business Intelligence Program to a growing number of prestigious clients. Among corporations contracting its advanced research services were Bank of America, Bechtel Corporation, Blyth, Eastman Dillon, Hewlett Packard, McDonnell Douglas Corporation, TRW Company, and Wells Fargo Bank.

Bereft of the invaluable services of Captain Al M. Hubbard, who had semi-retired and moved to British Columbia, Willis W. Harman succeeded in reactivating his former mentor in October 1968. Outlining the reasons for his invitation to Hubbard, Harman wrote: Our investigations of some of the current social movements affecting education indicate that the drug usage prevalent among student members of the New Left is not entirely undesigned. Some of it appears to be present as a deliberate weapon aimed at political change. We are concerned with assessing the significance of this as it impacts on matters of long-range educational policy. In this con-
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nection it would be advantageous to have you considered in the capacity of a special investigative agent who might have access to relevant data which is not ordinarily available.\textsuperscript{44} Hubbard agreed to work as a well-paid “security officer” at SRI. “Although,” Harman admits, ‘Al never did anything resembling security work.’ Hubbard was specifically assigned to the Alternative Futures Project, which performed future-oriented strategic planning for corporations and government agencies. Harman and Hubbard shared a goal ‘to provide the [LSD] experience to political and intellectual leaders around the world.’ Harman acknowledges that ‘Al’s job was to run the special [LSD] sessions for us.’ According to Dr. Abram Hoffer, ‘Al had a grandiose idea that if he could give the psychodelic experience to the major executives of the Fortune 500 companies, he would change the whole of society.’\textsuperscript{45} Six years later Harman explained why he had appreciated Hubbard’s expertise as a “special investigative agent” so highly: “His services to us consisted in gathering various sorts of data regarding student unrest, drug abuse, drug use at schools and universities, causes and nature of radical activities, and similar matters, some of a classified nature.”\textsuperscript{46} In meting out high compliments to Hubbard, Harman was well aware of the invaluable service the Captain had rendered to the research team at SRI — which was commissioned in 1972 to write an academic study identifying, “insofar as possible, what changes in the conceptual premises underlying Western society would lead to a desirable future”.\textsuperscript{47}

Changing Images of Man

The Charles F. Kettering Foundation\textsuperscript{48} provided the funding for the May 1974 report “Changing Images of Man”. SRI’s Urban and Social Systems Division prepared the 319-page mimeographed report\textsuperscript{49} under the general guidance of executive director Harvey L. Dixon and Willis W. Harman, by then director of the Center for the Study of Social Policy. Twelve years later Pergamon Press published the academic study in its Systems Science and World Order Library series.\textsuperscript{50} The original report was prepared by a team of thirteen researchers\textsuperscript{51} and supervised by a panel of six advisors, including anthropologist Margaret Mead, Yale University physician Henry Margenau, and British Intelligence operative Geoffrey Vickers.\textsuperscript{52} Although the final editorial responsibility lay with the SRI staff, the report was thoroughly reviewed by eighteen additional academicians such as Ervin Laszlo, Carl R. Rogers, and B. F. Skinner, before it was released.\textsuperscript{53}

Spiritual Dimension

In pointing to Elise Boulding’s essay in Appendix A\textsuperscript{54} (“An Alternative View of History, The Spiritual Dimension of the Human Person, and a Third Alternative Image of Humanness”), the report prognosticated a seismic change in the thinking of Western intellectuals. The obsolete pursuit of industrial progress, said the authors, needed to be abandoned in favour of a renewed dedication to religious mysticism. They concluded that despite its abundant material benefits, the incessant quest for industrial and technological development in modern society was harmful to the anticipated future of humankind’s spiritual evolution:

Many of our present images appear to have become dangerously obsolete, however … Science, technology, and economics have made possible really significant strides toward achieving such basic human goals as physical safety and security, material comfort and better health. But … many of these successes have brought with them problems of being too successful – problems that themselves seem insoluble within the set of societal value-premises that led to their emergence. Improved health, for example, has caused population increases which exacerbate problems of social organization, food distribution, and resource depletion. Our highly developed system of technology leads to higher vulnerability and breakdowns. Indeed the range and interconnected impact of societal problems that are now emerging pose a serious threat to our civilization … if such projections of the future prove correct, we can expect the problems associated with multifold trend will become more serious, more universal, and to occur more rapidly than will growth of the trend itself.\textsuperscript{55}

Therefore, said the SRI report, a fundamental and radical alteration of the industrial-technological self-understanding of humans would be needed to create a more harmonious world society: “Some characteristics of an adequate image of humankind for the post-industrial future were derived: (1) by noting the direction in which premises underlying the industrial present would have to change in order to bring about a more ‘workable’ society; (2) from examination of the ways in which images of humankind have shaped societies in the past; (3) from observation of some significant new directions in scientific research.”\textsuperscript{56}

Willis W. Harman

Willis W. Harman (1919-1997) was the leading social scientist and futurist at SRI in the late 1960s and early 1970s, and previously a professor of electrical engineering and systems analysis at Stanford University. He led the Institute of Noetic Sciences from 1977 until late 1996. Apollo astronaut Edgar Mitchell and oil billionaire Paul N. Temple established the Institute of Noetic Sciences in 1973 to promote the vision of “expanding knowledge of the nature and potentials of the mind, and applying that knowledge to the advancement of health and well-being for humankind and the planet.”\textsuperscript{57} As the author of several books — including Creative Work: The Constructive Role of Business in a Transforming Society (with John Hornmann), An Incomplete Guide to the Future, and Global Mind Change and co-editor of The New Business of Business: Sharing Responsibility for a Positive Global Future (with Maya Porter) — Harman’s influence as a prime change agent of his time in science, technology, education\textsuperscript{58}, and business\textsuperscript{59} was felt around the globe.

The central ideas of Harman’s urgent plea to adopt a holistic outlook on life can be gleaned from his essay “Bringing About the Transition to Sustainable Peace” (Part One: “A Changing Worldview”):

This emerging trans-modern worldview, involves a shift in the locus of authority from external to ‘inner knowing.’ It has basically turned away from the older scientific view that ultimate reality is “fundamental particles,” and trusts perceptions of the wholeness and spiritual aspect of organisms, ecosystems, Gaia and Cosmos. This implies a spiritual reality, and ultimate trust in the authority of the whole. It amounts to a reconciliation of scientific inquiry with the “perennial wisdom” at the core of the world’s spiritual traditions. It continues to involve a confidence in scientific inquiry, but an inquiry whose metaphysical base has shifted from the reductionist, objectivist, positivist base of 19th- and 20th-century science to a more holistic and transcendent metaphysical foundation.

... The core of the current challenge to
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In the last 35 years there has been increased interest in chemical substances that change the quality and characteristics of normal everyday consciousness, particularly through such drugs as lysergic acid, mescaline, psilocybin, and others. These drugs, referred to as psychedelics, hallucinogens, or psychoactive chemicals, expand or contract the field of consciousness; they seem capable of enhancing perceptions and sensations, giving access to memories and past experiences, facilitating mental activity, and producing changes in the level of consciousness, including what are reported as transcendent experiences of a religious or cosmic nature.

In Alternative Educational Futures, Harman stated further that what Aldous Huxley had called “The Perennial Philosophy” is “present in the Rosicrucian and Freemasonry traditions and meant that “man can under certain conditions attain to a higher awareness, a ‘cosmic consciousness,’ in which state he has immediate knowledge of a reality underlying the phenomenal world.” Harman further highlighted Lawrence Frank’s remark that “a social order which tolerates such wide-ranging pluralism of norms must seek unity through diversity.” To achieve this goal:

“Nothing less than a new guiding philosophy will do. Ferkiss [1969] outlines three basic and essential elements for such a new philosophy: a ‘new naturalism,’ ‘the new holism,’ ‘and the new immanence.’ Educational experiences must be contemplated which are akin to psychotherapy … that result in a felt realization of the inevitability of one inseparable world, and a felt shift in the most basic values and premises on which one builds one life. In a sense, this means bringing something like ‘person-changing technology’ into the educational system (e.g., meditation, hypnosis, sensitivity training, psychodrama, yoga, etc.).”

The “modus operandi” of how Harman envisioned this “new guiding philosophy” to mould the thinking of the world’s population was outlined by him in a World Goodwill (Lucis Trust) Occasional Paper, “For a New Society, a New Economics,” published in the April, May, and June 1987 issues of Development Forum, by the United Nations Division for Economic and Social Information and the United Nations University. In this essay, Harman referred to Abraham Maslow’s “self-ac-

THE SCIENTIFIC WORLDVIEW CAN BE TAKEN TO BE ‘CONSCIOUSNESS,’ WHICH HAS COME TO BE A CODE WORD FOR A WIDE RANGE OF HUMAN EXPERIENCE, INCLUDING CONSCIOUS AWARENESS OR SUBJECTIVITY, SELECTIVE ATTENTION, INTUITION, CREATIVITY, RELATIONSHIP OF MIND TO HEALING, SPIRITUAL SENSIBILITY, AND A RANGE OF ANOMALOUS EXPERIENCE AND PHENOMENA … THE EPISCOPOLOGY WE SEEK WILL RECOGNIZE THE PARTIAL NATURE OF ALL SCIENTIFIC CONCEPTS OF CAUSALITY … IN SOME ULTIMATE SENSE, THERE IS NO CAUSALITY — ONLY A WHOLE EVOLVING.

“SPACESHIP EARTH”

In 1993, the Centre for Educational Research and Innovation (CERI) [14], an intergovernmental organization connected with the UN, published Alternative Educational Futures in the United States and in Europe. [14] The Ford Foundation and the Royal Dutch Shell Group of companies financed the study. In this book, Willis W. Harman stated that educators have a responsibility to preside over “a shift from a parochial to a ‘one world’ view of ‘Spaceship Earth’ … Emergent change, not homeostasis, is the order of the day … it is apparent that ‘new’ values are currently challenging the traditional ones.” [15] Harman was remarkably successful in bringing about this dramatic “shift.” In his capacity as consultant to the White House National Goals Research Staff, he formed and led a team to assist the U.S. Office of Education in efforts to apply the newly emerging discipline of futures research to guiding the nation’s policies in education and educational research. [16] Harman has been a prominent participant at Mikhail Gorbachev’s “State of the World” forums. In a PBS interview in April 2001 the former chairman of the Soviet politburo revealed that his vision of “perestroika” was inspired by the “spaceship earth” concept. [17]

In 1992, the Esalen Institute sponsored a lecture tour of Mikhail Gorbachev in the United States. On 6 May 1992, Gorbachev delivered a speech in Fulton, Missouri where Winston Churchill had identified the ideological and political barrier separating the capitalist from the communist nations as the “Iron Curtain”. Speaking to the more than 20,000 people gathered on the campus of Westminster College and thousands more who listened to the speech live in 132 countries around the world, former Soviet President closed the curtain on the Cold War with his speech, “The River of Time and the Imperative of Action.” [18] At this historical site the Gorbachev noted that “[a]n awareness of the need for some kind of global government is gaining ground, one in which all members of the world community would take part.” [19] After pointing out the grave problem of “exaggerated nationalism” which had “already led to much bloodshed” he presented the solution of a “global international security system.” [20] Yet the worst of dangers faced by every human being would be the accelerated destruction of the environment. Gorbachev referred specifically to the following ecological crises: “Global climatic shifts, the greenhouse effect, the ozone hole, acid rain, contamination of the atmosphere, soil and water by industrial and household waste, the destruction of forests.” [21] Gorbachev concluded his speech with an appeal to imbue the United Nations with indisputable authority: “However, I believe that the new world order will not be fully realized unless the United Nations and its Security Council create structures, taking into consideration existing United Nations and regional structures, which are authorized to impose sanctions and make use of other measures of compulsion, especially when the rights of minority groups are being particularly violated.” [22] At the September 1995 “State of the World Forum” in San Francisco, which was organised by the Gorbachev Foundation, the former Soviet leader called for a “global brain trust” consisting of great thinkers “who are widely respected as well as global citizens”. They should be put in charge of monitoring “social change” and “focus on the present and the future of our civilization”. These proposals were identical with those of the Esalen Institute which had suggested for some time the creation of a Council of Wise Persons. To accomplish this immensely important work, the future world rulers would need to tap into the cosmic reservoir of a higher intelligence.

“COSMIC CONSCIOUSNESS”

Turning to the subject of psychedelic drugs, the SRI study Changing Images of Man referred to Masters and Houston’s work Varieties of Psychedelic Experience (1966). [23] In producing changed levels of consciousness certain effects of hallucinogens on the brain are perceived “as transcendent experiences of a religious or cosmic nature”. [24]
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ualization” theories and postulate that “a respiritualization of society is taking place, but one more experiential and non-institutionalized, less fundamentalist and sacerdotal, than most of the historically familiar forms of religion. With this change comes a long-term shift in value emphasis.” In conclusion, he averred that “there may indeed be a conflict between dogmatic esoteric religion and positivist science. However, there is not an inevitable conflict between the esoteric ‘perennial wisdom’ of the world’s spiritual traditions and a science based on certain metaphysical assumptions.”

Conclusion

Since the publication of the academic study Changing Images of Man in 1974 (1982) the spiritualization of science, technology, and education has unquestionably made great strides. Its proposed change from a traditional value system based on analytical and rational thinking to a holistic view which imagines all aspects of intellectual pursuit to be in harmony with the mystical underpinnings of monism has led to the emergence of a global community having a heightened sense of cosmic spirituality that supposedly permeates all existence. We believe, however, that a scientist or technician who is dedicated to the advancement of the theoretical and practical knowledge of humankind, especially in the area of clinical Nanomedicine, should avoid adopting an irrational methodology in this research. Otherwise, Aldous Huxley’s dystopian vision of a “scientific dictatorship of the future” may come true after all.

References

b Ibid., 37, passim; see also online edition: http://www.huxley.net/bnw/index.html
cl Ibid., 47.
tion: “But then there are the various other methods one can think of which, thank heaven, as yet have not be used, but which obviously could be used. There is for example, the pharmacological method, this is one of the things I talked about in BNW. I invented a hypo-
thetical drug called SOMA, which of course could not exist as it stood there because it was simultaneously a stimulant, a narcotic, and a hallucinogen, which seems unlikely in one sub-
stance. But the point is, if you applied several different substances you could get almost all these results even now, and the really interesting things about the new chemical substances, the new mind-changing drugs is this, if you looking back into history it’s clear that man has always had a hankering after mind changing chemicals, he has always desired to take holidays from himself, but the, and, this is the most extraordinary effect of all that every nat-
urally occurring narcotic stimulant, sedative, or hallucinogen, was discovered before the dawn of history, I don’t think there is one single one of these naturally occurring ones which modern science has discovered.”
e Aldous Huxley, Brave New World, 55. f Ibid., 4, passim.
h Ibid., I. Over-Population.
ibid.
j Ibid., XII. What Can Be Done? See also Aldous Huxley, “The Ultimate Revolution”: “Tradition-
ally it has been possible to suppress individual freedom through the application of physical coercion through the appeal of ideologies through the manipulation of man’s physical and social environment and more recently through the techniques, the cruder techniques of psychological conditioning. The Ultimate Revolution, about which Mr. Huxley will speak today, concerns itself with the development of new behavioral controls, which operate directly on the psycho-physiological organ-
isms of man. That is the capacity to replace external constraint by internal compulsions. ...
[Huxley] Well now in regard to this problem of the ultimate revolution, this has been very well summed up by the moderator.”
k See Aldous Huxley, “The Ultimate Revolu-
tion”: “Whereas my own book which was writ-
ten in 1932 when there was only a mild dicta-
torship in the form of Mussolini in existence, was not overshadowed by the idea of terror-
ism, and I was therefore free in a way in which Orwell was not free, to think about these other methods of control, these non-violent meth-
ods and my, I’m inclined to think that the sci-
entific dictatorships of the future, and I think there are going to be scientific dictatorships in many parts of the world, will be probably a good deal nearer to the brave new world pattern than to the 1984 pattern, they will a good deal nearer not because of any humanitarian qualms of the scientific dictators but simply

because the BNW pattern is probably a good deal more efficient than the other.”


n Aldous Huxley first offered the term “psychedelic” at a meeting of the New York Academy of Sci-
ences in 1957. He said the word meant “mind manifesting” (from “mind”, νους, and “manifest-
q Aldous Huxley, The Perennial Philosophy (London: Chatto & Windus, 1946) dustcover flap: “The Perennial Philosophy is an attempt to present this Highest Common Factor of all theologies by assembling passages from the writings of those saints and prophets who have approached a direct spiritual knowledge of the Divine and who have recorded not only the method of that approach but also the clarity and tranquility of sol they derived from it. Mr. Huxley quotes from the Chinese Taoist philosophers, from followers of Buddha and Mohammed, from the Brahmim scriptures and from Christian mystics ranging from St. John of the Cross to William Law...”.
r Aldous Huxley, Heaven and Hell (London: Chatto & Windus, 1956).
s See Martin A. Lee & Bruce Shlain, Acid Dreams, 43-46, 52.
t Ibid., 46.
u Timothy F. Leary, Flashback: An Autobiogra-
v Ibid.
w Cited in Jeffrey J. Kripal, Esalen. America and the Religion of No Religion (Chicago: The Uni-

press.uchicago.edu/Misc/Chicago/459399.

html
x http://www.esalen.org/
z Willis W. Harman dedicated his book Global Mind Change (1998) to Hubbard among oth-
ers: “I wish to dedicate this book to four per-

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sions who have profoundly affected the latter part of my life: Alfred M. Hubbard ..."

aa See Walter Truett Anderson, The Upstart Spring, passim. The IFAS is also referred to as the International Federation of Advanced Study; see e.g., Martin A. Lee & Bruce Shlain, Acid Dreams, 156.

ab Stolaroff stated the following in The Secret Chief (Ben Lomond CA: Multidisciplinary Association for Psychedelic Studies, 1990) 50: “You see, again, a spiritual trip is what’s involved here. This I have to say -- it’s the only way I know how to talk about it -- what I do and even how do I do it is not up to me. I’m guided. I can’t define that, I can’t explain it. If God didn’t want me to do it, He would have stopped me a long time ago. I have a lot of faith that that’s true. At the same time I keep a close eye on my integrity and my security... We’re all in it together.” http://www.maps.org/secretchief/scchpt2.html


eh Willis W. Harman, Director, Educational Policy Research Center, SRI, to Dr. A. M. Hubbard, October 2, 1968, as cited in Martin A. Lee & Bruce Shlain, Acid Dreams, 156.

a1 See Todd Brendan Fahey, “The Original Captain Trips”: "His services were eventually recruited with Willis Harman, then-Director of the Educational Policy Research Center within the Stanford Research Institute (SRI) of Stanford University. Harman employed Hubbard as a security guard for SRI. http://www.fargone-books.com/high.html

aj Willis Harman, Director, Center for the Study of Social Policy, SRI, “To Whom It May Concern,” January 14, 1974, as cited in Lee & Shlain, op. cit., 156.

ak O. W. Markley & Willis W. Harman, eds., Changing Images of Man. Prepared by The Center for the Study of Social Policy/SRI International (Oxford, New York, Toronto, Sydney, Paris, Frankfurt: Pergamon Press, 1982) xvii. al http://www.kettering.org/about/history.aspx - "The Charles F. Kettering Foundation was founded in 1932 to sponsor and carry out scientific research for the benefit of humanity. Inspired by the open-mindedness and creative philosophy of its founder, the American inventor Charles F. Kettering, the foundation’s work has expanded to include research on education, international affairs, and democracy."

am Contract Number UHR 489-215O, Policy Research Report No. 441,74 an O. W. Markley & Willis W. Harman, eds., Changing Images of Man. The General Editor of the Systems Science and World Order Library was Ervin Laszlo, who also published, besides two of his own books, E. Jantsch, The Self-organizing Universe: Scientific an Human Impli-


aq Ibid., xv. ar Ibid., 152 fn.: „Note: See also Elise Boulding’s compelling statement of “The Spiritual Dimension of the Human Person” in Appendix A.” Prof. Boulding taught at the Institute of Behavioral Science at the University of Colorado.

as Ibid., 219ff.

at Ibid., 4, 5-6, 9.

at Ibid., 220.

av “Noetic” comes from the Greek word for intuitive knowing. In its article „The Higher Self Gets Down to Business“ Christianity Today reported the following on Harman’s understand-

ings of „noetic“ science: „Intuition was ... for Harman ... the very means of connect-

ing to the one Universal Mind. Nor was vis-

ualization merely a means of clarifying goals, but of altering material reality. Both intuition and visualization were central in the evolution of consciousness that Harman envisioned.” http://www.christianitytoday.com/workplace/articles/ct-2002-002-1.34.html

av Paul N. Temple has been a member of the institute board of directors since 1973 and served as its chairman from 1983 to 1999. He is a businessman, investor and a graduate of Princeton University and Harvard Law School. He and Diane Temple are founders of the Temple Awards for Creative Altruism, administered by the institute. ax See http://www.ions.org

ay Cit. in W. W. Harman, Global Mind Change. The Promise of the 21st Century (San Francisco, CA: Berrett-Koehler Publishers, ‘1998) ix. See also http://www.bkconnection.com/BB/Prod-Details.aspx?D=1767502999 - “Global Mind Change, first published in 1988 and revised and expanded by the author just before his death in 1997, connects every major field of human endeavor in its exploration of the possibilities for social transformation through internal change. Harman, whose career spanned both the technical (electrical engineering) and psychological sciences, examines the role of consciousness in five areas: o Mature science, which validates subjective, religious, and spiritual insights along with objective data as a way of describing reality; o Spirituality and consciousness research, which shows the compatibility between the world’s religions and the insights of thousands of years of exploration of consciousness; o Health and healing, where the mind’s role is increasingly recognized as a crucial influence on human wellness; o Psychology and psychotherapy, where research into unexplained phenomena and exceptional mental and physical abilities proves the only human limits are those we believe in o Economics and management, where managers are utilizing brain-mind research to release employees’ creativity, and corporations are addressing global issues of poverty, security, and the environment.” az Harman was a founder of Futures Research Group at Stanford University and a board member of Planetary Citizens, two goals of which are to redesign education for global

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awareness and to give the U.N. the authority to act on behalf of the common will of humanity.
ba In 1988 Harman co-founded the World Business Academy.
bb http://www.newciv.org/ISSS_Primer/semi
bc CERI was established by the Organization for Economic Cooperation and Development (OECD) in 1968.
bd Centre for Educational Research and Innovation (CERI), Alternative Educational Futures in the United States and in Europe: Methods, Issues and Policy Relevance (Paris: Organisation for Economic Co-operation and Development, 1972). This report was prepared by the CERI as Volume 8, background report No. 12, of Proceedings from the Conference on Policies for Educational Growth, organised by the OECD in Paris, France, June 3-5, 1970. Abstract: This book contains four papers by noted educational planning experts that, together, cover practically all the implications of undertaking ‘futurological’ studies in education. Louis Emmery, in his „Alternative Educational Futures and Educational Policy-Planning,” summarizes the three papers that comprise the remainder of the document and stresses the importance of viewing alternative educational futures in the context of policy planning or „second generation” educational planning. Torsten Husen then describes three major purposes for exploring alternative educational futures. In the third paper, Warren Ziegler develops a taxonomy consisting of five models, which purports to synthesize the current practice of American educational planning as it views the future. Finally, Willis Harman focuses on alternative future states of American society that represent, in some sense, alternative dominant belief and value systems. See http://eric.ed.gov/ERICWeb-Portal/custom/portlets/recordDetails/detailsmini.jsp?_nfpb=true&_ERICExtSearch_ SearchValue_o=ED072508&ERICExtSearch_ SearchType_o=no&accno=ED072508
be ibid., 167.
bf ibid., 171.
bg “The Impetus for Change in the Soviet Union” – PBS interview conducted 04/23/2001; http://www.pbs.org/wgbh/commanding-heights/shared/minitextlo/int_mikhailgorbachev.html: „And therefore a reformist leadership was necessary, and that leadership came in 1985 when we started to lay down our plans for our country, perestroika and new thinking for the International Community. The new thinking postulated [that] we are one planet, one human civilization. There are others living in the world, so why should we act in a way that could blow up our planet, our spaceship Earth?”
bi ibid.
bj ibid.
bk ibid.
bl ibid.
bo O. W. Markley & Willis W. Harman, eds., Changing Images of Man, 92.
bp ibid.
 bq CERI, Alternative Educational Futures, 173.
Blood-brain barrier in neurodegenerative diseases: perspectives for Nanomedicine


Abstract
The blood-brain barrier (BBB) is the most restrictive barrier in the body, presenting a formidable obstacle to drug delivery into the brain. In neurodegenerative diseases such as Alzheimer’s disease (AD), Parkinson’s disease (PD), and amyotrophic lateral sclerosis (ALS) therapeutic trials have frequently failed. One of the reasons involved may be that the therapeutic agents are not able to reach their target in effective concentrations due to the restricted anatomical access to the brain. Selective delivery of diagnostic and therapeutic agents across the BBB using nanotechnological carrier devices such as liposomes or nanoparticles is a potentially safe and minimal invasive strategy to overcome the BBB. In addition, there is evidence for a dysfunction of the BBB itself in various neurodegenerative disorders that also offers perspectives for a restorative therapy with nanomedicinal devices. We review the literature on the potential role of the BBB in neurodegenerative diseases and discuss recent developments in nanomedicine and their potential for targeted drug delivery across the BBB in neurodegenerative diseases.

Keywords: Blood-brain barrier, Neurogenerative disorders, Nanogels, Nanomedicinal devices

Introduction
Neurodegenerative diseases such as different subtypes of dementia, Parkinsonian syndromes, or motor neuron diseases form an important challenge to diagnostic and therapeutic progress in neurology. Though clinically heterogeneous, these diseases share a chronic progressive course that frequently leads to severe, debilitating symptoms. Furthermore, while proceeding from different pathophysiological origins, they all share the common pathology of neurodegeneration, characterized by neuroaxonal damage, apoptosis and glial cell reaction (1-3).

In the past, a multitude of therapeutic trials in neurodegenerative diseases have failed. For example, potentially promising agents like ciliary neurotrophic factor (CNTF) that demonstrated therapeutic effects in several mouse mutants with motor neuron degeneration failed to live up to expectations in subsequent clinical trials in humans (4). One of the reasons might be that neuroprotective agents are not able to reach the target cells in effective concentrations due to the restricted anatomical access to the central nervous system (CNS). The CNS is separated from the blood space by the blood-brain barrier (BBB) which constitutes a highly effective barrier system restricting the entry of serum proteins (5). Because the BBB also forms a restrictive obstacle to systemically administered therapeutic agents, an important step towards an effective treatment of neurodegenerative diseases would be a strategy to overcome this barrier system. Biocompatible nanomaterials such as liposomes, nanoparticles, dendrimers, or polymeric micelles are candidate agents to act as pharmaceutical drug delivery devices that could facilitate the penetration of the BBB (6-8).

In addition, several studies indicate that the function of the BBB and the architecture of the BBB itself is altered in neurodegenerative diseases, but the pathophysiological relevance remains largely unclear so far (9-14). (9-14). We review the literature on the potential role of the BBB in neurodegenerative diseases. We furthermore discuss recent developments in Nanomedicine and their potential role for targeted delivery of drugs across the barrier systems in the treatment of neurodegenerative diseases.

Methods
We searched MEDLINE and EMBASE up to January 2008, for studies on neurodegenerative diseases and nanomedicine, using the following keywords: “neurode-
unsolved medical problems

Anatomy of the blood-brain barrier

The BBB is the most restrictive barrier in the body, preventing the access of most small molecules and nearly all macromolecules into the CNS (5). The concept of an anatomical barrier separating blood and CNS first emerged from the studies of Ehrlich (1882) and Goldman (1923) who introduced trypan blue into the blood and observed that only brain and CSF remained unstained (15). The role of this barrier system is to protect the brain from the outside environment and to maintain homeostasis of the brain. The BBB consists of a monolayer of capillary endothelial cells, a basement membrane, and of three types of cells surrounding the blood vessels with their processes: astrocytes, oligodendrocytes, and neurons (Fig. 1). The first element of the barrier is the capillary wall consisting of a monolayer of endothelial cells with a luminal membrane on the inside of the vessels and the abluminal membrane on the brain site, separated by an interval of 300 Å (30 nm) from the basement membrane (15). The non-fenestrated capillary endothelium forms the functionally most important part of the BBB. The cell membranes of the endothelial cells are connected to each other by tight junction protein complexes known as zonulae occludentes. The tight junctions of the BBB consist of different integral membrane proteins including occludins, claudins, junctional adhesion molecules, and associated cytoplasmatic proteins (16). The presence of tight junctions and the lack of fenestrae severely restrict paracellular transport. Accordingly, any transport of molecules to the brain must occur via the transcellular route by passive diffusion or various active transport mechanisms (17-21) as shown in figure 1. The bi-directional transport across the BBB can be classified into five main categories (17-21) that are given in table 1.

The basement membrane (300-500 Å) which is composed of extracellular matrix and includes gap junctions allowing the passage of smaller solutes (15). Another part of the satellite cells closely located to the neurons are oligodendrocytes. Besides forming the myelin sheaths in the CNS, oligodendrocytes also take part in the formation of the glial sheath covering neuronal and vascular cells.

A further element of the BBB is the extracellular space with a width of about 200 Å that is labyrinthically ramifying between neurons, glial cells, and capillaries. It allows the unrestricted passage of ions and substances of colloidal size (i.e. ≥ 1 nm) (23). The final element of the BBB is the plasma membrane of the neuronal cell, which is built from protein and lipoprotein molecules (15).

Nanomedicinal devices for BBB passage

Strategies to overcome the BBB include direct intraventricular administration of drugs into the cerebrospinal fluid (CSF) as well as temporary pharmacological BBB disruption. Drugs administered directly into the ventricular system show a very fast elimination due to a high turnover rate (24) which contrasts with a very slow diffusion of most drugs across the brain parenchyma. Consequently, this approach was mainly effective for drugs whose site of action is located in close proximity to the surface of the brain such as opioids (25). Disruption of the BBB using substances which induce an osmotic pressure (26) is the plasma membrane of the neuronal cell, which is built from protein and lipoprotein molecules (15).

Table 1: BBB Transport Systems (according to (21))

<table>
<thead>
<tr>
<th>Transport mechanism</th>
<th>Transporters and their substrates</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrier-mediated transport</td>
<td>• Glucose transporter 1 (GLUT1): hexoses • Monocarboxylate transporter 1 (MCTs): lactate, pyruvate, ketone bodies • L-facilitative transporter: neutral amino acids • y-facilitative transporter: cationic amino acids • Transports for nucleosides, purines, amines, and vitamins • Peptide transport systems (PTS): e.g., encephalin and arginine-vasopressin</td>
<td>Provides the brain with nutrients and energy sources</td>
</tr>
<tr>
<td>Active efflux transport</td>
<td>• ATP-binding cassette (ABC) transporters, such as P-glycoprotein (ABCs, P-gp)</td>
<td>Clearance of abundant and toxic substrates in the brain interstitial fluid</td>
</tr>
<tr>
<td>Ion transport</td>
<td>• Sodium pump (Na+, K+ -ATPase) • Sodium-potassium-two chloride cotransporter • Chloride-bicarbonate exchanger • Sodium-hydrogen exchanger • Sodium-calcium exchanger</td>
<td>Regulates pH and ion concentrations for sodium dependent carriers and homeostasis in endothelial cells and the brain interstitial fluid</td>
</tr>
<tr>
<td>Receptor-mediated transport</td>
<td>• Specific protein BBB receptors: transferrin, leptin, immunoglobulins, insulin, and others</td>
<td>Mediates influx or efflux of proteins across the BBB</td>
</tr>
<tr>
<td>Caveolae-mediated transport</td>
<td>• Endocytosis and transcytosis of ligands and receptors from the plasma membrane (enriched in caveolins)</td>
<td>Remains to be explored</td>
</tr>
</tbody>
</table>

proteins, e.g. laminin and collagen, separate endothelial cells from its neighboring cells. In general, it offers no barrier to the passage of molecules and proteins as big as ferritin (200 Å) (22). Therefore, the basement membrane can be considered as part of the extracellular space of the brain parenchyma.

The vascular system is separated from the neuronal system by a sheath made up from processes of neuroglial cells including astrocytes and oligodendrocytes. The astrocytes dominate the transport route from capillaries to the neuron as seen in electron microscopy (15). Their processes variously called pedicles, end-plates, or foot-plates form a sheath covering the neurons, dendrites, axons, and capillaries. This glial sheath consists of multiple processes of the same or different astrocytes

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Fig. 1. The blood-brain barrier (BBB) with bi-directional transport mechanisms:
1 - carrier-mediated transport; 2 – efflux transport; 3 – ion transport; 4 – receptor-mediated transport; 5 – transcytosis. Tj = Tight junction; ZO = Zonula occludens.
pressure like mannitol or vasoactive agents like bradykinin, vascular endothelial growth factor, or serotonin leads to a shrinkage of capillaries of the endothelial cells and opens a passage from the bloodstream to the brain so that the influx of drugs to the brain increases but may also facilitate access of other unwanted and potentially toxic molecules (16). After several hours the normal concentration of urea or mannitol in the blood is reestablished, the cells regain their initial size and the BBB is sealed again (26,27). Furthermore, transient BBB opening may cause fluid imbalances leading to vasogenic brain edema (28).

In comparison, selective delivery of diagnostic and therapeutic agents across the BBB using nanotechnological devices is a potentially safe and minimal invasive strategy (8). Recent advances in the field of nanotechnological drug delivery devices include liposomes, nanoparticles, nanogels, polymeric micelles, and dendrimers.

Liposomes are spherical vesicles formed by phospholipid bilayers in aqueous solutions. They can be either small unilamellar vesicles having sizes below 100 nm or large multilamellar vesicles with a size of 100 nm to several microns (16). Water-soluble compounds of drug molecules can be incorporated into the aqueous compartment, while lipophilic compounds can be embedded into the lipid bilayers. Liposomes may be composed of very different lipids depending on the required characteristics, but phospholipids and cholesterol are the most frequent components. A general problem regarding liposomes is that they are physiologically cleared from the blood circulation by the reticuloendothelial system (RES). Extended circulation can be achieved by reduction of the particle size (< 100 nm) and by liposome surface modification using substances like polyethylene glycol (PEG) (8). PEG-coated liposomes were also observed to have a better penetration through the brain tissue when compared to uncoated formulations (26). To target liposomes into the CNS, they can be further modified using monoclonal antibodies to glial fibrillary acidic protein or human insulin receptors (29,30). Immunomodification of liposomes was successfully applied to deliver drugs such as daunomycin, doxorubicin, and digoxin into the brain in animal models as well as humans (31-33). So far, studies using liposome-mediated drug delivery in neurodegenerative diseases are lacking. Furthermore, expectations may be reduced by studies reporting brain accumulation of liposomes after previous BBB opening by osmotic or vasoactive agents (34,35). Drugs can be also linked to specific polymeric ligand such as saccharides or proteins. Such so called “prodrugs” can also result in enhanced BBB permeation (36-38). The conjugation of specific ligands allows the drug to exploit receptor mediated pathways through this cellular barrier, mimicking endogenous systems. The increase of the hydrophobicity increases the ability to diffuse passively across the barrier. Despite the success of prodrugs, this approach is very specific and has to be adjusted to every compound.

Nanoparticles (NPs) are solid colloidal particles made of insoluble biocompatible and/or biodegradable polymers (8). Drug molecules can be entrapped within the NP core (nanocapsule), embedded in the matrix, absorbed or covalently attached to the surface of the carriers (16). NPs applied as drug delivery devices to the brain need to be smaller than 100 nm and stable in the blood, as well as to avoid the RES (6,16). Similar to liposomes, NPs can evade the RES by modification of the carrier surface with surfactants or PEG which increase their ability to resist opsonization (39). NPs conjugated with the metal chelator defereroxamine were observed to cross the BBB and reduce the metal load in neuronal tissue (40). This could be of interest with regard to alleviate the effects of oxidative damage in neurodegenerative diseases like ALS (8). So far, the mechanisms of entry of NPs into the brain remain unclear, with in vitro studies indicating an involvement of phagocytotic processes (42). The feasibility of using NPs as drug carriers across the BBB was demonstrated in animal models for drugs such as tubocurarine (42), doxorubicin (43), and loperamide (42), which are not able to cross the BBB under physiological conditions. In a rat model for Parkinson's disease (PD), nanoparticles were shown to prolong the half-life of bromocriptine in the brain (44). In Alzheimer's disease (AD), a significant increase in rivastigmine uptake was achieved using poly(n-butylcyanoacrylate) (PBCA) nanoparticles (46). Caution may derive from studies reporting a NP-associated disruption of the tight junctions of brain capillary endothelial cells (8,47).

For polymeric nanoparticles, the surface characteristics, presumably in combination with the polymeric matrix, are the key factors for the passage to the brain. Particularly PBCA-based particles coated with amphiphilic (and therefore stabilizing) molecules like polysorbates like Tween® 80 or poloxamers like Pluronic® F68 have been reported to successfully pass the BBB (48,49). PBCA is known to be a biodegradable polymer and is therefore perfectly suitable for sustained release applications. The polysorbates on the particle’s surface leads to an increased adsorption of specific plasma proteins of the apolipoprotein family which is discussed as one factor for facilitating the BBB passage by disguising the particles as LDL particles which are endocytosed by the endothelial cells without immediate effluxing (50-53). The PBCA nanoparticles could be loaded with drugs, e.g. dalgargin as an antinociceptive drug that is acting on neurons in the CNS but does not cross the intact BBB. It has been clearly shown that the dalgargin-loaded PBCA particles significantly enhance the antinociceptive effect, however the fate of the particle and the drug after intravenous application is not yet clarified. Fact is, that the adsorbed drug reaches the brain while elsewhere no therapeutic effect could be observed. Also the use of other drugs is described, e.g. the dipeptide kytorphin, loperamide, tubocurarine, the NMDA receptor antagonist MRZ 2/576, and doxorubicin (see review (54)).

Other polymeric matrices like poly(methylmethacrylate) (PMMA) coated with polysorbates can be found in the brain, but also in other non-RES tissues as heart, kidneys or muscles (55). Usually a successful passage through the BBB is indicated in vivo experiments by the effect of a particle associated drug (42,56,57) or by radioactive labeling (58-60) but very rarely with fluorescence markers (61,62).

Most of the nanoparticles are produced in an anionic emulsion polymerization process. The drawback of this method is that dextran is used as stabilized in large quantities (100 wt% compared to the polymer) leading to a high loading of the surface with dextran, that only limited solid contents of ≤3% can be achieved and that the drug has to always be adsorbed after the completion of the polymerization.

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Using the miniemulsion polymerization process allows one to produce PBCA nanoparticles with well-defined sizes, molecular weights, low surfactant loads, and at high solid contents (10%) with the possibility to directly introduce fluorescent or other markers and a wide variety of drugs before the polymerization process (63) and introduce functionalities on the particles’ surface (64). For such fluorescent polysorbate 80-coated poly(n-butylcyanoacrylate) (PBCA) nanoparticles it was found in vivo studies, that nanoparticles enter the endothelial cells of the brain and the retina of rats (65).

Another candidate carrier device are polymeric micelles (PMs) (66-70). PMs form spontaneously in aqueous solutions of amphiphilic block polymers and have core-shell architecture (8). With a size of 10 to 100 nm in diameter, PMs include a core composed of hydrophobic polymer blocks (like polypropylene glycol, poly-caprolactone etc.) and a shell composed of hydrophilic polymer blocks (like polyethylene glycol). The core can incorporate considerable amounts of water-insoluble drugs preventing premature drug release and degradation. The shell stabilizes the PMs and prevents interactions with serum proteins and untargeted cells (8). After reaching target cells, the drug is released from the PMs via diffusion. Several clinical trials are under way to evaluate PMs for delivery of anti-cancer drugs (71,72).

Nanogels are another type of nanomaterials that could function as drug-delivery devices across the BBB (8,73). They consist of cross-linked polymer networks that frequently combine ionic and non-ionic chains. These networks can incorporate charged molecules of drugs that bind to oppositely charged ionic chains. Nanogels were shown to enhance the transport of oligonucleotides into the brain in vitro and in vivo (8,73). The mechanism of nanogel-mediated delivery of oligonucleotides apparently involves transcytosis across brain capillary endothelial cells (8). Nanogels are currently investigated for CNS delivery of several low-molecular mass compounds and biomacromolecules (74).

Dendrimers are repeatedly branched polymer molecules containing a cascade of branches grown from one or several cores with a diameter of about 1.5-15 nm (75). They are made up of the core, to which the branches are attached, the shell of branches surrounding the core, and the multivalent surface formed by the endings of the branches (76). During the dendrimer synthesis, various solutes can be entrapped within the dendrimer core. Several studies evaluating intratumoral delivery of dendrimer conjugates with anti-cancer agents to glioma showed dendrimers as promising drug-delivery devices across the BBB (77,78). In addition, dendrimers used as scaffolds for the amino acid residues 16-20 of amyloid-beta (Aβ) were shown to potentiate inhibition of Aβ aggregation and may therefore have a perspective as future therapeutic agents (79). However, there is evidence that dendrimers in some cases may have toxic effects including a permanent disruption of the BBB (8).

The role of the BBB in neurodegenerative diseases

The most common chronic neurodegenerative diseases include AD, PD, and amyotrophic lateral sclerosis (ALS). To date, there is no drug available to cure any of these disorders in human diseases or in animal models, and many promising agents failed in clinical trials possibly due to the effectivity of the BBB. On the other hand, recent morphological studies revealed abnormalities in the brain microvasculature involving the integrity of the BBB in AD and in ALS, as described in the following. Therefore, on closer examination of the BBB function in neurodegenerative diseases it seems important to note that therapeutic intervention not only has to deal with an impermeable BBB for drug delivery to the brain but also faces the problem of a dysfunction of the BBB at least in a sub-group of the patients. Reports on an altered BBB based on pathological elevations of the CSF/serum albumin ratio, however, have to be considered with caution (24), because the BBB and the CSF-blood barrier are different barrier systems that show significant differences in their morphology with different transport and filtration abilities (80).

Alzheimer’s disease (AD)

AD is characterized by a progressive decline of cognitive functions associated with accumulation of neurotoxic Aβ in the brain parenchyma and in blood vessels (81). Although current therapy with cholinomimetic agents that increase synaptic acetylcholine (cholinesterase inhibitors) or drugs that modulate potentially neurotoxic calcium influx into the cells (blockers of the N-methyl-D-aspartate receptor) improve cognitive performance in dementia they are lacking proven neuroprotective effects.

Nanomedicinal approaches in AD

Some studies indicate that the BBB itself may be a target for therapeutic intervention in AD aiming to lessen the burden of Aβ in the brain. Aβ produced in the brain may be cleared through the BBB by efflux receptors to be metabolized in the liver instead of being deposited in senile plaques (82). On the other hand the transcytosis of plasma-derived Aβ across the BBB might be suppressed through inhibition of influx receptors (83). The perspective of immunotherapy is another argument of interest in BBB function in AD, as the intact BBB is a potential barrier to an effective therapy with anti-amyloid immunoglobulins (84).

First approaches to lessen the burden of Aβ plaques in AD brain by nanomedicinal devices include in vivo studies on metal chelators, which are able to solubilize Aβ deposits, conjugated with nanoparticles to pass the BBB (40,85), and an in vivo study in mice with the model drugs thioflavin S and thioflavin T that where released from nanoparticles in the brain and selectively targeted fibrillar Aβ (86).

BBB dysfunction in AD

Most studies agree that the frequency of BBB dysfunction in AD is about 20% (87-89), with one study observing it to be more frequent in male patients (90). The mechanisms underlying BBB dysfunction in AD remain a matter of speculation, although an effect of amyloid angiopathy on the BBB may be the most obvious candidate (89). Deposition of Aβ occurs in arterioles in the brain causing cerebral amyloid angiopathy. Recent studies have found that Aβ deposition also occurs in capillaries in lesion-prone regions in AD brains. Thus, the BBB may be injured secondarily (91,92). Another contributor to BBB impairment in AD may be high plasma homocysteine. As homocysteine is known to be toxic to vascular endothelial cells, elevated homocysteine levels could contribute to BBB impairment in AD and other subtypes of dementia (93). It furthermore has been suggested that BBB dysfunction in AD may be related to vascular risk factors such as hypertension and heart disease (94), though this observation has not been confirmed by all studies (89). The consequences of BBB dysfunction include an increased influx of plasma-derived molecules, proteins, and blood cells into the brain. The BBB dysfunction is of great importance for the treatment of AD, as it may require the use of directly BBB penetrant drugs.
dysfunction in AD are also not entirely clear. A beneficial effect seems unlikely as BBB dysfunction in AD was observed to correlate with rates of disease progression, including annual change on MMSE and annual ventricular volume change (89). Interventions that aim to lessen the burden of $\beta\beta$ in the CNS therefore might be beneficial for recreation of proper BBB function.

Parkinson’s disease (PD)

Idiopathic PD is a chronic progressive movement disorder resulting from degeneration of dopamine-producing nerve cells in the mid brain (99). The reduction of normally high concentrations of dopamine in the basal ganglia leads to muscular rigidity, tremor, and bradykininess, i.e. slow movements and difficulties in walking. Pharmacologic attempts to restore dopaminergic activity have been successful in alleviating many of the symptoms of this disorder. Dopamine, however, does not cross the BBB and has no therapeutic effect if given into the peripheral circulation, but its immediate metabolic precursor levodopa (3,4-dihydroxy-L-phenylalanine) does penetrate the brain via the L1 facilitative transporter (18). The effective treatment of PD with levodopa is therefore an excellent example of how BBB transport systems can be used to deliver therapeutics across the BBB. Although levodopa positively influences mobility of PD patients and lowers the mortality rate due to PD, it does not stop the progression of the disease and has long-term complications. Several dopamine agonists have also been developed and many lead to clinical benefit. Unlike levodopa, they do not require enzymatic conversion to an active metabolite and do not compete with other substances for active transport into the blood and across the BBB. However, as other symptomatic therapies, these agents still do not address the continuous loss of affected neurons. Future therapeutic strategies in PD therefore include neuroprotection and neurorestoration of degenerating dopaminergic neurons in the mid brain and of downstream neurons in the basal ganglia.

Nanomedicinal approaches in PD

Because increased oxidative stress is associated with neurodegenerative disorders such as PD and ALS, the placing of antioxidants at specific sites of the neurodegenerative process might be promising. NPs with antioxidative properties such as cerium oxide have been found to provide a nanopharmacological approach (96), but interference in the basal free radical signaling by NPs may be deleterious (97). Recently, a bone-marrow derived macrophage system carrying NPs was developed to deliver antioxidative catalase across the BBB to PD-affected brain regions (98): to overcome the problem of intracellular enzyme degradation within the macrophages, catalase was packaged into a block ionomer complex with a cationic block copolymer. These “nanozymes” were shown to be transported effectively to the brain in an animal model of PD (98).

Another interesting aspect is the degradation of aggregated alpha-synuclein (99), the main component of PD-typical Lewy bodies in affected brain regions (100), but this will be a matter of further research.

BBB dysfunction in PD

There are few reports that BBB might be altered in individual PD patients on the basis of microvascular changes in post-mortem PD brains, such as thickening of capillary basement membrane (13) and an altered shape of capillaries in affected areas of the brain (101). The underlying mechanisms are unknown, but it might be a secondary phenomenon in the context of neuroinflammation and release of highly reactive oxygen species (102). There are also reports on an altered reverse transport from the brain site to the blood site in PD patients. An in vivo study with positron emission tomography using a substrate for the P-glycoprotein transporter that transports substances from the brain site across the BBB (103) suggested an impaired BBB function in the parkinsonian midbrain (14). However, there is no satisfactory explanation why this reverse transport mechanism should be altered in a regionally specific fashion (104), but it points out that clearance mechanisms across the BBB have to be taken into account in the development of new treatment strategies.

Amyotrophic lateral sclerosis (ALS)

ALS is characterized by progressive selective degeneration of motor neurons in the brain, brainstem and spinal cord. Progressive paralysis across nearly all motor functions finally results in respiratory failure and death within 2-5 years after diagnosis. The only drug approved by the Food and Drug Administration for the treatment of ALS is a neuroprotective blocker of glutamate release, i.e. riluzole (2-aminog-2-trifluoromethoxy)benzothiazole). The neuroprotective effects of riluzole are mediated by at least four independent mechanisms, G-protein dependent, sodium-channel dependent, blockade of post-synaptic excitation amino acid receptor and pre-synaptic inhibition of glutamate release in nerve endings (105). In two therapeutic trials, riluzole prolonged survival by three to six months (106, 107), but treatment only slightly slowed the decline of strength of limb muscles. Despite promising treatment effects in mice that show an ALS phenotype based on a familial defect of superoxide dismutase 1 (SOD1), several other glutamate antagonists, antioxidants, neurotrophic factors, immunomodulatory approaches, and antiviral agents have failed for the treatment of ALS in humans (www.alsa.org) (108,109).

Nanomedicinal approaches in ALS

So far, no definite nanomedicinal approaches have been developed for the treatment of ALS, but the application of antioxidative NPs as mentioned in the PD section may also be applicable in future studies in ALS.

BBB dysfunction in ALS

Alterations of the BBB in the course of human ALS have not been reported so far, but recent studies gave evidence for a compromised BBB in brain and spinal cord of G93A SOD1 mice on the basis of alterations of the capillary ultrastructure (10,110). The authors found degenerated endothelial cells with swollen mitochondria, multiple layers of endothelial cells, and thickened basement membranes (110). Although tight junctions appeared intact in early as well as in late disease stages, leakage of intravenously applied Evans blue dye indicated dysfunction in endothelia and basement membranes in this mouse model (20). Other groups reported reduced levels of tight junction proteins (11,12) and reductions of the total length of capillaries in the spinal cord of SOD1 mice as one of the earliest pathologic events in the cascade of ongoing neurodegeneration (11). These studies indicate that, at least in familial ALS forms, alterations of the BBB most likely precede neuronal degeneration. Therefore, the dysfunction of the BBB itself also offers prospects for early intervention with nanomedicinal strategies in ALS.
Unsolved Medical Problems

Conclusion

The BBB protects the brain from the outside environment while at the same time creating a formidable obstacle for brain drug delivery. Management of the BBB remains a challenge for the development of more effective treatment strategies in neurodegenerative diseases. A promising approach to overcome the BBB is the use of nanotechnological carrier devices such as liposomes or NPs. However, the use of nanotechnology for drug delivery across the BBB is still in its infancy and studies investigating their efficiency and safety in patients with neurodegenerative diseases are lacking. Even though, several promising studies in patients with malignant brain tumors highlight the huge potential of the nanotechnological approach for future treatment strategies in neurodegenerative diseases.

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Nanomedical approaches in Parkinson’s disease

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Abstract

In the aging population of many countries, neurodegenerative diseases like Parkinson’s disease (PD) are becoming an increasing burden. Therefore, early diagnosis, therapy and ultimately disease prevention is essential. However, until now, treatment in PD is only symptomatic and curative therapy is still missing. Possible diagnostic approaches are detecting biomarkers and molecular mechanisms leading to PD. Therapeutic goals for PD are neuroprotection such as preventing free radical injury and lowering brain metal ions through the administration of chelators as well as neuroregeneration (e.g., activation of neuronal progenitor cells) and more effective drug delivery systems through the blood brain barrier. A key role in developing such new diagnostic and therapeutic tools in PD will play nanotechnology. This review gives an overview of new nanomedical approaches in diagnostic and therapeutic tools in PD.

Key words: Neurodegenerative disease, Parkinson’s disease, nanotechnology, nanodiagnostics, atomic force microscopy, drug delivery system

Search strategy and selection criteria

We searched Biosis, Embase and PubMed, for English articles on Parkinson’s disease using the keyword “Parkinson’s disease”, “Nanomedicine”, “nanotechnology”, “nanoparticle” together with other keywords including “alpha synuclein”, “nanodiagnostic”, “atomic force microscopy”, “imaging” and several other keywords relevant to every section. We largely selected publications in the past 5 years but did not exclude important older publications. Selection criteria also included a judgement on the novelty of studies and their relevance for physicians.

Introduction

Parkinson’s disease (PD) is one of the most common neurodegenerative disorders (2). The prevalence of PD in industrialised countries is estimated at 0.3% of the general population and about 1% of the population older than 60 years (1-3). Clinical signs of PD include paucity of dopamine in the brain areas that receive dopaminergic inputs from those neurons. Dopamine replacement therapy with levodopa has been the mainstay of symptomatic treatment of PD. Although levodopa offers effective symptom relief at all stages, its risk of inducing motor complications has led many to advocate alternative drugs for initiation in suitable patients.

PD lacks definite early diagnostic approaches and effective cure at the present. Possible diagnostic approaches are detecting biomarkers and molecular mechanisms leading to these neurodegenerative disorders. A promising role in developing such new diagnostic tools plays nanotechnology, especially if diagnosis is not achievable by other approaches: nanosensors could be used for precise detection of biomarkers and atomic force microscopy, a tool for single molecule imaging, offers more opportunities for biomolecular observation and characterisation. Nanotechnology is also expected to have a impact in development of new types of therapeutic tools i.e. nanodevices or nanomaterials for preventing free radical injury, lowering brain metal ions through the administration of chelators as well as neuroregeneration and more effective drug delivery systems through the blood brain barrier. Furthermore nanodevices and nanomaterial can stimulate and respond to target cells with a high degree of specificity to induce desired physiological responses with minimal undesirable effects.

This review encompasses nanotechnologies used for molecular research and for diagnostic as well as therapeutic approaches in PD.

Parkinson’s Disease

Parkinson’s disease (PD) is a neurodegenerative disorder clinically characterized by tremor, bradykinesia, rigidity, and postural instability (4;5). The clinical diagnosis of PD relies on history, physical examination, and improvement of symptoms and signs with dopaminergic treatment (6).

PD is considered to result primarily from abnormalities of the basal ganglia. The basal ganglia include the striatum (caudate nucleus and putamen), the external and internal pallidal segments, the subthalamic nucleus, and the substantia nigra with its pars reticulata (SNr) and pars compacta (SNC). They participate in anatomically and functionally segregated loops that involve specific thalamic and cortical areas (7,8). The neuropathologic correlate of PD is mainly a progressive dopaminergic Cella loss in the SNr (9) and a degeneration of the nigrostriatal dopaminergic pathway, although more widespread abnormalities have been observed (7). Furthermore, the neuropathology of PD is characterized by accumulation of...
Lewy bodies (7). The main component of Lewy bodies are alpha-synuclein fibrils. Alphasynuclein is an unstructured protein of 140 amino acids that is present in presynaptic terminals. This protein plays an important role in synaptic plasticity and neurotransmitter release (7). Misfolding and self-assembly of alpha-synuclein has been implicated in the pathogenesis of PD (10). The main cause of nonfamilial PD is probably the direct or indirect interaction of alpha-synuclein with environmental factors in a way that promotes aggregation of this protein (11-13). Alpha-synuclein can adopt different aggregated morphologies, including oligomers, protofibrils and fibrils (14-16). The small oligomeric aggregates have been shown to be particularly toxic (17;18). Current therapy for PD is focused primarily on ameliorating the dopamine deficiency in the rain (19). Most patients with PD require levodopa therapy for adequate symptomatic control of the symptoms 3 to 5 years after the diagnosis of PD. However, disease progression and long-term oral treatment with levodopa, with or without concomitant dopamine agonist therapy, may lead to the development of motor fluctuations and dyskinesias. These longterm side effects are related to the short action of the drug which results in a pulsatile stimulation of dopamine receptors (20). Thus continuous stimulation of dopamine receptors is highly desirable. Some studies demonstrate that maintenance of continuous levels of levodopa via pumped intravenous infusion ameliorates “on/off” motor fluctuations (21). An ideal in vivo drug delivery system would be able to determine when and if a dose is needed and then deliver it automatically. Dopamine agonists and monoamine oxidase (MAO) B inhibitors offer effective relief of the motor features of PD in early and more advanced disease and are associated with a low risk for motor complications. However, they are not as potent as levodopa (29). Another therapeutic approach may be antibodies that neutralize the neurotoxic aggregates of alpha-synuclein without interfering with beneficial functions of onomeric alpha-synuclein (22;23).

Nanotechnical research approaches on molecular mechanisms of PD
Atomic force microscopy (AFM) (24) has become a widespread methodology in research on molecular mechanisms of PD (10;25-31). AFM is a very important tool for the observation of high-resolution three-dimensional structures and has the advantage of easy sample preparations compared with electron microscopy or X-ray fiber diffraction. In situ AFM under liquid offers the possibility of examining alpha-synuclein fibrils under physiological conditions in a time-dependent manner (26).

Using AFM, diverse morphologies of the alpha-synuclein fibrils depending on different experimental conditions have been shown (25). AFM has also been performed to investigate the self-assembly (27) and the structural properties (26) of the alpha-synuclein fibrils: In order to investigate the structural properties of alpha-synuclein fibrils in solution, Zhang and coworkers (26) used two different AFM imaging modes: tapping mode and contact mode. In the in situ contact mode, AFM experiments alpha-synuclein fibrils quickly broke into fragments and a similar phenomenon was found using tapping mode AFM in which alphasynuclein fibrils were incubated with guanidine hydrochloride. The alpha-synuclein fibrils kept their original filamentous topography for over 1 hour in the in situ tapping mode AFM experiments. These results provided indirect evidence on how beta-sheets assemble into alpha-synuclein fibrils on a nanometer scale (26). Zhang and coworkers (27) were also able to show that digestion of the flexible peptide chains by protease could significantly slim the alpha-synuclein fibrils with remaining of the filamentous cores resistant to proteolysis, whereas disruption of the interchain interactions by denaturant broke down the fibrils into articles which may be a possible therapeutic approach (27).

In order to get more insight into the in vitro process of alpha-synuclein fibril assembly Segers-Nolten and coworkers (30) explored different contrast modes of high resolution AFM on fibrils formed by the wild-type alpha-synuclein protein, and by the familial disease-related A30P, E46K and A53T disease mutant alpha-synuclein protein. From quantitative analysis of height images measured in tapping mode AFM, they obtained data that are compatible with a twisted hierarchical assembly model for all protein variants (31). The E46K mutant displayed the most distinctly irregular and smallest periodicity. The modulation depth for all mutants was very similar (30). AFM was also used to study the mechanical properties of alpha synuclein fibrils: contact force of 15-20 nN was applied while scanning in contact mode, resulting in characteristic deformation of protein fibrils with a periodicity corresponding to the modulation observed in tapping mode. They concluded that hierarchical assembly model may not be the exclusive mechanism of alpha-synuclein fibril assembly, but that multiple modes of fibril assembly play a role in alpha-synuclein fibril formation (30).

In order to characterize misfolding and self-assembly of alpha-synuclein dimers, Yu and coworkers applied in a recent study (10;28) single molecule probing technique. Using AFM force spectroscopy approach, they were able to detect protein misfolding via enhanced interprotein interaction. Moreover, lifetime of dimers formed by misfolded alpha-synuclein was possible to measure. Their data suggest that alpha-synuclein dimers are practically static and thus can play a role of aggregation nuclei for the formation of aggregates. Importantly, two different dissociation channels were detected suggesting that aggregation process can follow different pathways (10;28).

Segers-Nolten and coworkers (29) studied the interaction of the enzyme tissue transglutaminase (tTG) with alpha-synuclein protein variants at physiologically relevant concentrations and investigated the properties of the tTG-alpha-synuclein in oligomers using AFM. tTG catalyzing cross-link formation between protein-bound glutamine residues and primary amines, has been found colocalized with Lewy bodies, suggesting a role for tTG in the pathophysiology of PD (32;33). Cross-linked alpha-synuclein appeared to correlate with PD progression, indicating that tTG cross-linking may change the structure of monomeric alpha-synuclein resulting in altered functionality. In vitro investigations on the effect of tTG demonstrated preferential formation of monomeric intramolecular cross-links at relatively low alpha-synuclein concentration (34). Segers-Nolten and coworkers reported that AFM revealed morphologically similar structures for oligomers from all alpha-synuclein variants; the extent of oligomer formation was found to correlate with tTG concentration. These data suggested that tTG bound equally effective to wild-type and disease mutant alpha-synuclein variants. Furthermore they proposed that tTG cross-linking imposes structural constraints on alpha-sy-
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nuclein, preventing the assembly of structured oligomers required for disruption of membranes and for progression into fibrils. In general, cross-linking of amyloid forming proteins by tTG may prevent the progression into pathogenic species (29).

Nanodiagnostics for PD

Biosensors

As PD is characterized by a severe depletion of the in vivo dopamine pool (35), the ability to sensitively and selectively measure the concentration of dopamine (DA) could potentially be used for molecular diagnosis of PD. The ability to physiologically determine the concentration of DA could also be useful to design therapeutics and to evaluate their therapeutic efficacy toward PD (36). DA can be easily oxidized electrochemically at conventional electrodes, which have been used to detect the neurotransmitter both in vitro and in vivo (37-41). However, there are a number of problems with electrochemical methods due to the nature of the oxidative electrode reaction of DA. One of the primary problems is that the concentration of DA in the extracellular fluid of the caudate nucleus is extremely low (0.01-1 mM) for a healthy individual and in the nanomolar range for patients with PD (38,42) while the concentrations of the major detection interferents, e.g., ascorbic acid, are several orders of magnitude higher and the interferents undergo oxidation within the same potential window as DA. Ali and coworkers (43) recently demonstrated that DA can be electrochemically detected with high sensitivity and selectivity by modifying the electrode surface with a thin layer of in situ polymerized single-stranded DNA/poly(anilineboronic acid)/carbon nanotube composite and a thin layer of the highly permselective Nafion film, a so-called layer by layer DA sensor. Since direct oxidation of DA is avoided in this approach, the associated problems with direct oxidation, such as DA regeneration, were prevented. The interference from ascorbic acid is diminished by coating a thin layer of Nafion on top of the composite. Furthermore, the DNA-wrapped, single-walled carbon nanotubes in the composite not only greatly improved the electrochemical activity of the composite in physiologically relevant solutions but they also increased the effective electrode surface area and, therefore, the density of boronic acid groups available for DA binding. These features significantly enhanced the sensitivity for DA detection (43).

Other biosensors consist of ultralong aligned multi-walled carbon nanotube (MWNT) bundles synthesized using water-assisted chemical vapor deposition on aluminum (Al) and iron (Fe) coated silicon wafer with ethylene and argon/hydrogen gas as carbon source and buffer gas respectively (44). These electrodes were able to sense very low concentration of ascorbic acid (approximately 0.7 mM) and DA (approximately 1.87 mM) (44).

Concerning DA sensors, most methods have focused on nanotube coatings of large electrodes and slower electrochemical techniques (45,46) that are not conducive to measurements in vivo. Swamy and coworkers (47) investigated carbon-fiber microelectrodes modified with single-walled carbon nanotubes for the co-detection of DA and serotonin in vivo. Nanotube-modified microelectrodes showed significantly less fouling after exposure to serotonin than bare electrodes. Furthermore, the nanotube-modified electrodes were used to monitor stimulated DA and serotonin changes simultaneously in the striatum of anesthetized rats after administration of a serotonin synthetic precursor. This study showed that nanotube-coated microelectrodes can be used with fast scanning techniques and are advantageous for in vivo measurements of neurotransmitters because of their greater sensitivity and resistance to fouling (47).

In order to detect nanomolar levels of DA and uric acid (UA) in the presence of excess of ascorbic acid, Mathiyarasu and coworkers (48) developed a sensor consisting of gold nanoparticles and a conducting polymer, poly-3, 4- ethylenedi-oxythiophene (PEDOT). The PEDOT matrix has been shown to detect DA and UA (selectivity) and the nanometer-sized gold particles allowed nanomolar sensing of DA and UA (sensitivity). Thus, it was possible to detect nanomolar levels of DA and UA in presence of excess of ascorbic acid (48).

Another approach to determine DA has been shown by Lin and coworkers (49). They used high performance liquid chromatography coupled with microdialysis sampling and electrochemical detection (HPLC-EC). In the HPLC-EC, a multi-wall carbon nanotube electrode chemically modified with carboxyl groups (MWNT-COOH CME) was used as the working electrode for determination of DA. The results indicated that the MWNT-COOH CME enabled efficient electrocata-

lytic oxidation of DA with relatively high sensitivity and stability and long life. Peak currents for DA were linearly dependent on concentration in the range 5.0×10-9 to 5.0×10-5 mol L−1 and the calculated detection limit was 2.5×10-9 mol L−1. This method had been successfully used to measure DA in rat striatal microdialysate. In order to study the physiological effect of nitric oxide (NO) on striatal release of DA, 0.5 mmol L−1 sodium nitroprusside (SNP) was continuously perfused into rat striatum. This resulted in a 46% increase in DA basal level (49).

Nanoimaging in PD

Anomalous iron homeostasis has been proposed to be involved in the selective loss of dopaminergic neurons from the SNc in PD (50,51). Indeed, iron specific accumulation in the NC is associated with PD (52-54). However, the lack of analytical technique with sufficient spatial resolution prevents the investigation of iron distribution in neurons. To investigate the subcellular distribution of iron in DA producing neurons, Ortega and coworkers (55) used a chemical nanoimaging system with a 90 nm spatial resolution using synchrotron-based X-ray fluorescence (88 nm X-ray beam). This unique spatial resolution, combined to a high brightness, enabled chemical element imaging in subcellular compartments. Using this nanoimaging technique, they could show that iron accumulates into DA neurovesicles. In addition, inhibition of DA synthesis resulted in a decreased vesicular storage of iron (55).

Furthermore, imaging of iron distribution was performed in a recent study (56) in subcellular compartments of dopaminergic cells at high spatial resolution using the X-ray fluorescence anaprobe recently developed at the European Synchrotron Radiation Facility. High spatial resolution was obtained using the concept of a secondary source focused to a 90 nm probe by multilayer mirrors bent in Kirkpatrick - Baez geometry. This original setup was applied for trace metal mapping of single dopaminergic cells. This cellular model was able to differentiate upon exposure to nerve growth factor and to extend neurite-like processes. Two important results were obtained. First, iron was distributed in a granular form into dopamine vesicles, found mainly in primary neurite outgrowths and distal ends. Second, thin neurite-like processes produced by differentiated cells accumulated copper, zinc, and to a minor
L-dopa (LD) is converted by neuronal aro-
nanotechnical approaches in therapy of PD of trace metals in neurochemistry (56).

**Neuroprotection**

Another therapeutic approach for PD is neuroprotection: Selective delivery of antioxidants to the Snc can potentially attenuate oxidative stress and as such increase survival of dopaminergic neurons. Batrakova and coworkers (59) developed a bone-marrow-derived macrophage (BMM) system to deliver catalase to PD-affected brain regions in a PD animal model. To preclude BMM-mediated enzyme degradation, catalase was packaged into a block ionomer complex with a cationic block copolymer, polyethyleneimine-poly(ethylene glycol) (PEI-PEG). The self-assembled catalase/PEI-PEG complexes, “nanozymes”, were to 100 nm in size, stable in pH and ionic strength, and retained antioxidant activities. Cytotoxicity was negligible over a range of physiologic nanozyme concentrations. Nanozyme particles were rapidly, 40-60 min, taken up by BMM, retained catalytic activity, and released in active form for greater than 24 h. In contrast, “naked” catalase was rapidly degraded. The released enzyme decomposed microglial hydrogen peroxide following nitrated alphasynuclein or tumor necrosis factor alpha activation. Following adoptive transfer of nanozyme-loaded MM to 1-methyl 4-phenyl 1,2,3,6-
tetrahydropyridine-intoxicated mice, ca.
6.0% of the injected dose were found in brain. Thus, cell-mediated delivery of nanozymes can reduce oxidative stress in animal models of PD (59).

Another neuroprotective approach in PD is the use of fullerenols. Fullerenols have been shown to possess antioxidant properties but they also inhibit the activity of glutamate receptors and exert antiapoptotic effects. Studies on one class of these compounds, the malonic acid C60 derivatives (carboxyfullerenes), indicated that they were capable of rescuing mesencephalic dopamine neurons from toxin-induced degeneration (60). Despite these promising results in vitro, in vivo data are scarce.

**Neuroregeneration**

A further therapeutic approach for PD offers non-viral gene delivery (61-65). Promising candidates are amino-termina-
ted organically modified silica (ORMOSIL) nanoparticles that are able to condense, protect, and deliver plasmid DNA within cells (66-69). The anionic plasmid DNA is induced electrostatically to form a complex with the cationic amino groups on the ORMOSIL nanoparticles, (average di-
ameter of the complexes: 30 nm). Once inside the cells, the plasmid is thought to be released following the destabilization of the plasmid nanoparticle complex in the acidic environment of intracellular compartments such as endosomes and lysosomes. In a recent study the possibility of in vivo gene transfer in the CNS has been explored (67) using direct, stereotaxic injection of the ORMOSIL-DNA nanoparticle complexes (nanoplexes) into the mouse brain. Initially, a simple reporter gene expression using the plasmid encod-
ing the enhanced green fluorescent protein (pEgfp) was tested in the Snc region of the brain. Immunofluorescent detection using specific monoclonal antibodies to enhanced green fluorescent protein (EGFP) resulted in observation of robust EGFP expression of neuron-shaped cells in the Snc (67).

**Immunological approach in PD therapy**

Since misfolding of alpha-synuclein into specific toxic morphologies is essential in the progression of PD and other related diseases, identification of the toxic forms of alphasynuclein and prevention of their accumulation are important for under-
standing the progression of this disease and for developing a therapeutic strate-
gy. Emadi and coworkers (70) utilized in their study a biopanning technology com-
bining phage display technology and AFM to isolate individual single chain antibody fragments which bound to a specific tar-
get morphology of alpha-synuclein (71). AFM was used to visualize the target mor-
phology and to monitor the panning process. Using only a minimal amount of the target antigen, they were able to isolate a single chain antibody fragment (scFv) that specifically bound to the oligomeric form of alpha-synuclein. The scFv was able to inhibit alpha-synuclein cytotoxicity when co-incubated with alpha-synuclein and also when added to performed oligo-
meric aggregates (71).

**Nanosurgery in PD**

Surgical interventions for PD have been shown to be beneficial for refractory symp-
toms. Thalamotomy and thalamic stimu-
lation are considered safe and effective procedures to treat tremor. Pallidotomy

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and pallidal stimulation primarily reduce dyskinesia, and have minimal effects on bradykinesia and rigidity. Studies indicate that subthalamic nucleus stimulation improves levodopa-induced ‘off’ period function, decreases ‘off’ time, and reduces dyskinesia (72). However their role is limited to being the ‘means of last resort’ due to the high risk of potential complications and limited long-term efficacy. It must be noted that these procedures are not without their potential for complications. Possible adverse side effects of surgery include brain haemorrhage, infarction, seizures, and even death (73). Equipment malfunctions can include lead breakage or other hardware failure and pulsergenerator malfunction (74).

A recent study reported on neural interface using vertically aligned multiwalled carbon anotube (CNT) pillars as micro-electrodes. Functionalized hydrophilic CNT microelectrodes offered a high charge injection limit (1-1.6 mC/cm²) without faradic reactions. The first repeated in vitro stimulation of hippocampal neurons with CNT electrodes has been demonstrated by Wang and coworkers (75). These results suggest that CNTs are capable of providing far safer and more efficacious solutions for neural prostheses than previous metal electrode approaches (75).

Conclusion
Applications of nanotechnology to PD have already significant effects, which will continue in the future. Short-term progress has benefited in vitro and ex vivo studies of neural cells, often supporting or augmenting standard technologies. These advances contribute to both our basic understanding of cellular neurobiology and neuropathology, and to our understanding and interpretation of neuropathology. Although the development of nanotechnologies designed to interact with the nervous system in vivo is slow and challenging, they will have significant, direct clinical implications. Nanotechnologies targeted at supporting cellular or pharmacological therapies or facilitating direct physiological effects in vivo will make significant contributions to clinical care and prevention.

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