Nanoscience and Technology

Stergios Logothetidis *Editor*

Nanomedicine and Nanobiotechnology

NanoScience and Technology

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Nanomedicine and Nanobiotechnology

With 75 Figures

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Preface

This book presents the state-of-the-art laboratory, scientific and clinical aspects of nanotechnologies, nanomaterials, and tools for medical applications. It gives a broad overview of nanomedical utilities in order to achieve breakthroughs in health care, ranging from nanoparticle drug delivery, diagnostics, regenerative medicine, nanomaterials for advanced medical implants, nanodentistry, and pharmaceutics to toxicity issues. The different pillars of the nanomedicine field are highlighted, in respect to clinical needs for the accurate diagnosis and effective treatment of human diseases. It also presents a spectra of nanoscale imaging modalities for hemocompatibility and cytotoxicity assessment of nanostructured materials implemented in the medical field. The authors, having a distinguished expertise in the academic and industrial world, take an interdisciplinary approach of medicine, biology, pharmacy, physics, chemistry, engineering, nanotechnology, and materials science, and as an outcome, this book will provide the cutting-edge data on nanomedicine, in a comprehensive and simple way. Thus, this book will be of great value to researchers, graduate students, and medical doctors who want to enhance their knowledge and expertise in the field of nanomedicine.

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Contents

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Chapter 1
Nanomedicine: The Medicine of Tomorrow

S. Logothetidis

Abstract Nowadays nanotechnology has become a technological field with great potential since it can be applied in almost every aspect of modern life. One of the sectors where nanotechnology is expected to play a vital role is the field of medical science. The interaction of nanotechnology with medicine gave birth to a completely new scientific field called nanomedicine. Nanomedicine is a field that aims to use the nanotechnology tools and principles in order to improve human health in every possible way. Nanotechnology provides monitoring tools and technology platforms that can be used in terms of detection, diagnostic, bioanalysis and imaging. New nanoscale drug-delivery systems are constantly designed with different morphological and chemical characteristics and unique specificity against tumours, offering a less harmful approach alternative to chemo- and radiotherapies. Furthermore, nanotechnology has led to great breakthroughs in the field of tissue engineering, making the replacement of damaged tissues and organs a much feasible procedure. The thorough analysis of bio and non-bio interactions achieved by versatile nanotools is essential for the design and development of highly performed medical implants. The continuous revolution in nanotechnology will result in the fabrication of nanostructures with properties and functionalities that can benefit patient's physiology faster and more effectively than conventional medical procedures and protocols. The number of nanoscale therapeutical products is rapidly growing since more and more nanomedical designs are reaching the global market. However the nanotoxic impact that these designs can have on human health is an era that requires still more investigation. The development of specific guidance documents at a European level for the safety evaluation of nanotechnology products in medicine is strongly recommended and the need for further research in nanotoxicology is identified. Ethical and moral concerns also need to be addressed in parallel with the new developments.

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1.1 Introduction

Nanoscale is generally considered to be at a size below 0.1 μm or 100 nm (a nanometre is one billionth of a metre, 10^{-9} m). Nanoscale science (or nanoscience) studies the phenomena, properties and responses of materials at atomic, molecular and macromolecular scales, and in general at sizes between 1 and 100 nm. In this scale, and especially below 5 nm, the properties of matter differ significantly (i.e. quantum scale effects play an important role) from that at a larger particulate scale. Nanotechnology is then the design, the manipulation, the building, the production and the application, by controlling the shape and size, of the properties (responses) and functionality of structures, devices and systems of the order of less than 100 nm.

Nanotechnology is considered an emerging technology due to the possibility to advance well-established products and to create new products with totally new characteristics and functions with enormous potential in a wide range of applications. In addition to various industrial uses, great innovations are foreseen in information and communication technology, biology and biotechnology, medicine and medical technology, metrology, etc. It is anticipated that nanotechnology can have an enormous positive impact on human health. Relevant processes of living organisms occur basically at nanometre scale; elementary biological units such as DNA, proteins or cell membranes are of this dimension. By the means of nanotechnology, these biological units are going to be better comprehended so that they can be specifically guided or directed. Miniaturization down to nanometre scale provides to become an essential feature of biomedical products and procedures in postgenomic era. Nanoscale devices could be 100–10,000 times smaller than human cells but are similar in size to large biomolecules such as enzymes and receptors. Nanoscale devices smaller than 50 nm can easily enter most cells, and those smaller than 20 nm can move out of blood vessels as they circulate through the body.

Huge aspirations are coupled to nanotechnological developments in modern medicine (Nanotechnology, Biotechnology, Information Technology & Cognitive Science – *NBIC* developments). The potential medical applications are predominantly in diagnostics (disease diagnosis and imaging), monitoring, the availability of more durable and better prosthetics, and new drug-delivery systems for potentially harmful drugs [1, 2], as shown in Fig. 1.1. For example, nanoscale diagnostics are expected to identify in the becoming, giving the opportunity to intervene specifically prior to a symptomatically detected onset disease.

Biomedical nanotechnology presents revolutionary opportunities in the fight against many diseases. An area with near-term potential is detecting molecules associated with diseases such as cancer and diabetes mellitus, and neurodegenerative diseases, as well as detecting microorganisms and viruses associated with infections, such as pathogenic bacteria, fungi and HIV viruses. For example, in the field of cancer therapy, promising novel nanoparticles will respond to externally applied physical stimuli in ways that make them suitable therapeutics or therapeutic delivery systems. Another important field of application for nanotechnology

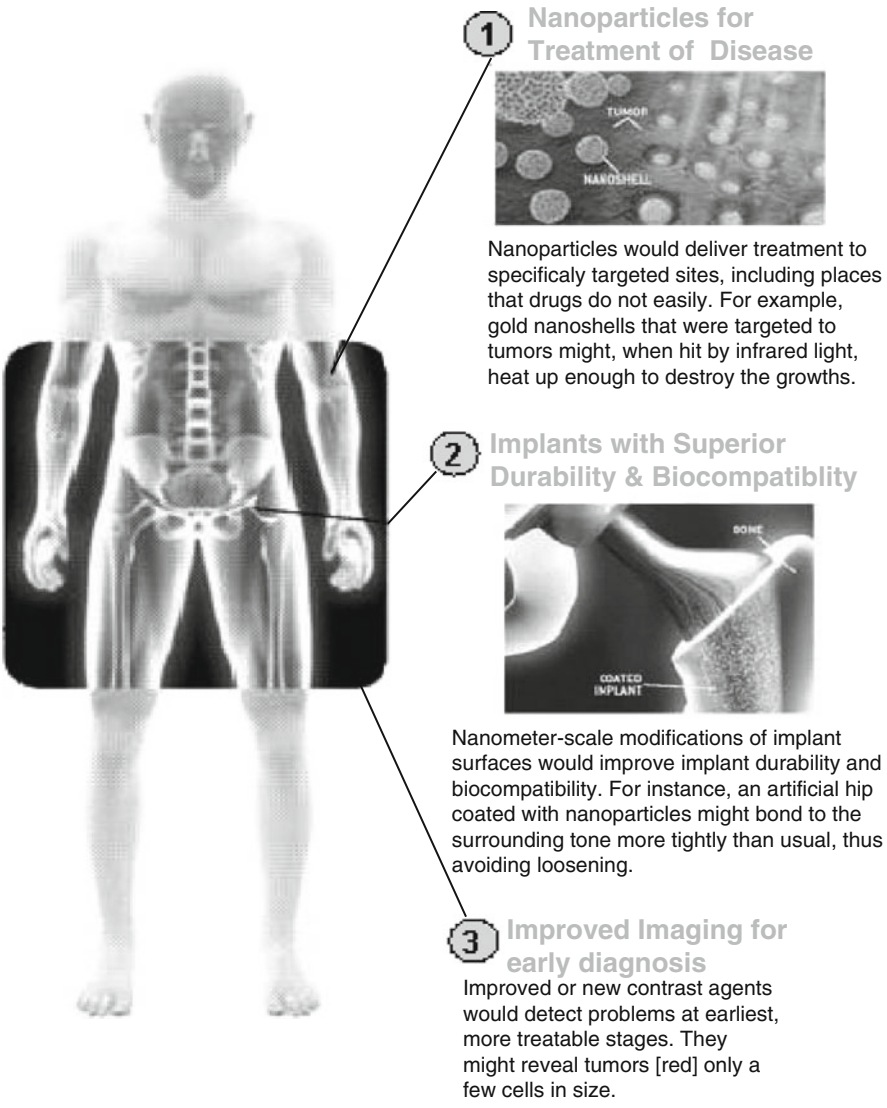

Fig. 1.1 Great developments are expected in medicine with the use of nanotechnology, such as (1) use of nanoparticles for the treatment of diseases, (2) implants with superior durability and biocompatibility and (3) improved imaging for early diagnosis

is biomaterials used, for example, in orthopaedic implants or as scaffolds for tissue-engineered products. Nanotechnology might yield nanostructured surfaces preventing non-specific protein adsorption. Control of surface properties at nanolevel was shown to increase the biocompatibility of the materials [3].

While products based on nanotechnology are actually reaching the market, sufficient knowledge on the associated toxicological risks is still lacking. Reducing the size of structures to nanolevel results in distinctly different properties. In addition to the chemical composition, which largely dictates the intrinsic toxic properties, very small size appears to be a dominant indicator for drastic or toxic effects of particles. From a regulatory point of view, a risk management strategy is already a requirement for all medical technology applications [2].

In order to discuss the advances of nanotechnology in modern medicine, we presented in Sect. 1.1 the terms and concepts of nanoscale and nanotechnology, and the relevant process and relation to living units. The impact of nanomaterials and nanoparticles in medicine is presented in Sect. 1.2, followed by a description of nanotechnology tools in medicine in Sect. 1.3. The impact of nanotechnology in medicine and medical technology is presented in Sect. 1.4, first with the introduction of nanomedicine and the "nanorobots", and then through some of myriad applications in diagnosis and treatment (such as biocompatibility and implants, cardiology, cancer, theranostics, etc.). In Sect. 1.5, a reference to the possible risks for human health is given.

1.2 Nanomaterials and Nanoparticles in Biomedical Applications

Novel nanomaterials and nanoparticles are envisaged to have a major impact on a number of different relevant areas. Materials with high performance and unique properties can be produced, which traditional synthesis and manufacturing methods could not create. Future nanoparticles should act as drug-delivery and drug-targeting systems. Due to their smallness, they are not recognized by the human body, migrate through cell membranes beneath a critical size and are able to pass the blood–brain barrier. These characteristics are used to develop nanoscale ferries, which transport high potential pharmaceutics precisely to their destination. There are different kinds of nanoparticles which are suitable to be applicable in drug and gene delivery, probing DNA structures, etc., and are categorized as liposomes, polymer nanoparticles (nanospheres and nanocapsules), solid lipid nanoparticles, nanocrystals, polymer therapeutics such as dendrimers and fullerenes (most common as C60 or buckyball, similar in size of hormones and peptide a-helices) and inorganic nanoparticles (e.g. gold and magnetic nanoparticles).

Carbon nanotubes (diameter of 1–20 nm, as shown in Fig. 1.2a) and *inorganic nanowires* exhibit extraordinary mechanical, electric, electronic, thermal and optical properties, offering the electronic industry properties that few materials platforms could ever hope to match. Carbon nanotubes, magnetic iron oxide nanoparticles

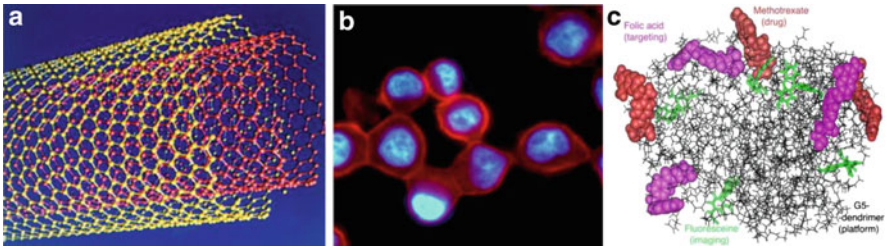

Fig. 1.2 Representative types of nanomaterials and nanoparticles: (**a**) Carbon nanotube, (**b**) human breast cancer cells tagged with quantum dots and (**c**) dendrimer [4]

and gold-coated silica nanoshells can transform electromagnetic energy into heat, causing a temperature increase lethal to cancer cells merely by increasing the magnetic field or by irradiation with an external laser source of near-infrared (NIR) light at the very location where these nanoparticles are bound to or internalized within tumour cells [3]. CNTs can be used as potential drug carriers as well. Pharmaceutical cargos are bound to nanotubes where specific biomolecules that target specific cell types are also attached [5].

Quantum dots (nanometre-sized semiconductor nanocrystals with superior fluorescent properties, as shown in Fig. 1.2b) possess remarkable optical and electronic properties that can be precisely tuned by changing their size and composition, due to their very small size (2–10 nm). Due to their relatively inexpensive and simple synthesis, quantum dots have already entered the market for experimental biomedical imaging applications. Quantum dots can be made to emit light at any wavelength in the visible and infrared ranges and can be inserted almost anywhere, including liquid solution, dyes, etc. These novel nanostructures can play an important role in future biomedical imaging and diagnostics. A hypothetical approach proposes the simultaneous usage of many quantum dots with different physicochemical properties for imaging applications. In particular, this would require a complicated system where a variety of surface ligands with unique specificity for different targets in patient's body are attached to each different quantum dot. So the resulting quantum dot–ligand conjugates would be used as imaging agents for a multiple-target in vivo detection application like the one shown in Fig. 1.3 [3, 4, 6, 7].

Dendrimers (complex almost spherical macromolecules with diameter 1–10 nm, shown in Fig. 1.2c) have improved physical, chemical and biological properties compared to traditional polymers. Some unique properties are related to their globular shape and the presence of internal cavities, offering the possibility as medical nanovehicles. Dendrimers have a tree-like structure where a central nucleus is surrounded by a large number of branches where a variety of molecules, including drugs, can be attached. Less than 5 nm in diameter, dendrimers are small enough to slip through tiny openings in cell membranes and to pass vascular pores and tissues in a more efficient way than bigger polymer particles. The architectural structure of dendrimers can be easily controlled during the synthesis process making them ideal candidates for drug-delivery applications. In experiments reported in Cancer

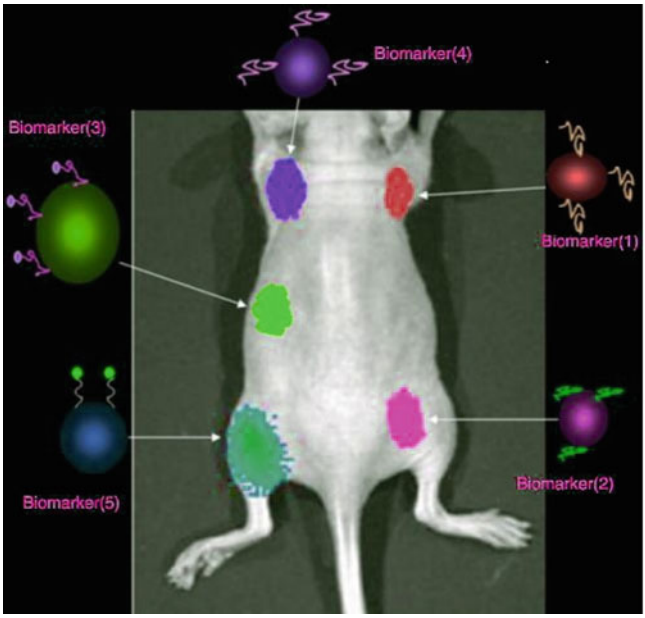

Fig. 1.3 A hypothetical application of quantum dot systems with bounded ligands that can be used for in vivo detection of different target sites simultaneously [6]

Research, University of Michigan, scientists attached methotrexate, a powerful anticancer drug, to branches of the dendrimer (Fig. 1.2c). On other branches, they attached fluorescent imaging agents and a vitamin called folic acid [3,4]. Apart from activity against tumours, dendrimer-mediated pharmaceutical approaches against bacteria and viruses have been demonstrated as well [8].

Nanoparticles, as shown in Fig. 1.4, being the fundamental elements of nanotechnology, can be applied in various ways, for example as fluorescent biological markers or as markers for detection of proteins, probing of DNA structures and separation and purification of biological molecules and cells, and they can also be used for magnetic resonance imaging (MRI) enhancement, tumour destruction via heating, tissue engineering and drug or gene delivery. As an example, two kinds of nanoparticles that are suitable to be applicable at least in drug delivery will be described: First, gold nanoparticles (3–20 nm), which are gold composites with dielectrical cores and golden shells. By choosing the right ratio of core to shell diameters, the particle can be tuned to absorb highly in the NIR, and by irradiation with such wavelength, the particle can be heated, even in deeper skin areas. If the particles are embedded in a temperature-sensible hydrogen matrix, the matrix will collapse and the included agents will be released at a critical temperature. Second, magnetic nanoparticles with controllable sizes between 2 and 30 nm can be coated with biological molecules to make them interact with or bind to a biological entity. Due to their magnetism, they can be manipulated by an external magnetic field

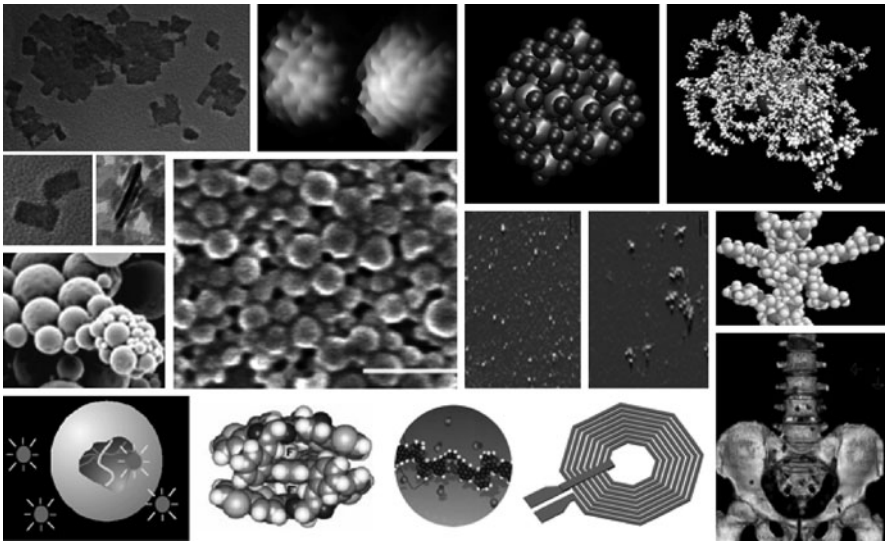

Fig. 1.4 Various nanoparticles and dendrites and their medical applications

gradient, thereby providing a controllable means of "tagging" or addressing the biological entity. They can be made to deliver a package (an anticancer drug, or a cohort of radionuclide atoms) to a targeted region of the body. The magnetic particles can be provided with energy from the exciting external field and can be heated up, making them good hyperthermia agents, delivering toxic amounts of thermal energy to targeted bodies, such as tumours. Finally, experiments where nanoparticles are used as carriers for anticancer drugs and antibiotics have shown promising results [9, 10]. Further analysis of the different nanostructured systems that are used in medicine is presented in Chap. 2.

For applications to medicine and physiology, these nanomaterials, nanoparticles and devices can be designed to interact with cells and tissues at a molecular (i.e. sub-cellular) level with a high degree of functional specificity, thus allowing a degree of integration between technology and biological systems not previously attainable. It should be appreciated that nanotechnology is not in itself a single emerging scientific discipline but rather a meeting of traditional sciences such as chemistry, physics, materials science and biology to bring together the required collective expertise needed to develop these novel technologies [11]. On the other hand, due to advances in biochemical research and molecular biology, diseases can be put down to molecular abnormalities. Molecular imaging should detect the corresponding molecular signatures of diseases and use it for medical diagnosis. This should ideally lead to diagnosis and therapy before occurrence of symptoms. In molecular imaging, an imaging molecule is coupled to a transport molecule or particle, which possesses a targeting unit (e.g. special receptors, ligands or peptides). The target finding system should be a specific molecular marker of a certain disease;

thus, the contrast medium accumulates within the sick tissue. Molecular imaging is developed for several diagnostic procedures such as magnetic resonance, ultrasonic imaging and nuclear and optical imaging technologies.

In addition to these examples of individual nanoparticles, novel biomaterials can be constructed using structural surface modifications of macro-, micro- and nanomaterials. Control of surface properties at nanolevel was shown to increase the biocompatibility of the materials by favouring the interaction of living cells with the biomaterial, especially by their beneficial effect on cell adhesion and proliferation [3]. Apart from the structural modifications, sometimes the surface of the biomaterials needs to be coated with specific molecules as well. For instance, in many cases, implants can have a harmful effect on patient's health due to the occurrence of undesirable interactions between the synthetic construct and the cells and molecules located at the area of implantation. This major problem can be eliminated by modifying the surface of the implant with a wide variety of functional molecules including proteins, peptides, extracellular matrix (ECM) and enzymes that enhance the biocompatible properties of the surface itself. Furthermore, such modification techniques have been used in order to immobilize biomolecules on surfaces that are used as biosensors for diagnostics like DNA and protein microarrays [12]. Extended presentation of surface biofunctionalization approaches is included in Chap. 3.

1.3 Nanotechnology Principles and Tools in Medicine

Different methods for the synthesis of nanoengineered materials and devices can accommodate precursors from solid, liquid or gas phases and encompass a tremendously varied set of experimental techniques. A detailed presentation of these is beyond the scope of this review. In general, however, most synthetic methods can be classified into two main approaches: "*top-down*" and "*bottom-up*" approaches and combinations of them. "*Top-down*" (photolithography, microcontact printing) techniques begin with a macroscopic material or a group of materials and incorporate smaller scale details into them, whereas "*bottom-up*" (organic-synthesis, self-assembly) approaches begin by designing and synthesizing custom-made molecules that have the ability to self-assemble or self-organize into higher order mesoscale and macroscale structures [11].

There are several nanotechnology-based synthesis techniques of these materials. For example, carbon nanotubes are developed by electric arc discharge, laser ablation and chemical vapour deposition techniques. Various inorganic nanotubes are developed by arc discharge and laser ablation, as well as through appropriate chemical reactions. Nanowire properties can differ distinctly from those of their corresponding crystalline bulk materials, though some properties are similar. Nanowires can be synthesized using a large variety of materials such as metals, e.g. Ag; semimetals, e.g. Bi; semiconductors, e.g. CdS; and superconductors. The most common synthesis methods are template-assisted synthesis, including

vapour and electrochemical deposition, and vapour–liquid–solid growth, especially successful for semiconductor nanowires. Dendrimers were first synthesized by an iterative synthetic methodology. The iterative sequence of reaction steps leads to a higher generation dendrimer after each iteration. The creation of dendrimers, using specifically designed chemical reactions, is one the best examples of controlled hierarchical synthesis, an approach that allows the "bottom-up" creation of complex systems. The functional end groups can be modified for various purposes, including sensing, catalysis or biochemical activity.

Other advanced applications of micro- and nanotechnology in medicine are the microchip-based drug-delivery systems, which are devices incorporating micrometre-scale pumps, valves and flow channels. They allow controlled release of single or multiple drugs on demand. Micro- and nanotechnology-based methods (e.g. UV-photolithography, reactive ion etching, chemical vapour deposition and electron beam evaporation) can be used for the fabrication of these silicon-based chips.

A myriad of studies are available for applications of micro- and nanotechnologies in chips for medical molecular diagnostics. Keywords are, for example, DNA microarrays (gene chips), protein microarrays (protein chips), lab-on-a-chip devices (Fig. 1.5a) and cell chips. Basically, these devices or systems are constructed using techniques inspired from micro/nanoscale fabrication methods that are used for processing, manipulation, delivery, analysis or construction of biological and chemical entities. Inkjet printing methods are used in DNA microarrays for human genomics and in protein microarrays (or protein chips), which are useful for molecular diagnostics. For the subsequent readout detection, either fluorescence- or radionuclide-based markers, or surface plasmon resonance spectroscopy can be applied [3].

In order to study and explore these rich and complex systems, highly sophisticated experimental, theoretical and modelling tools are required. Especially, the visualization, characterization and manipulation of materials and devices require sophisticated imaging and quantitative techniques with spatial and temporal

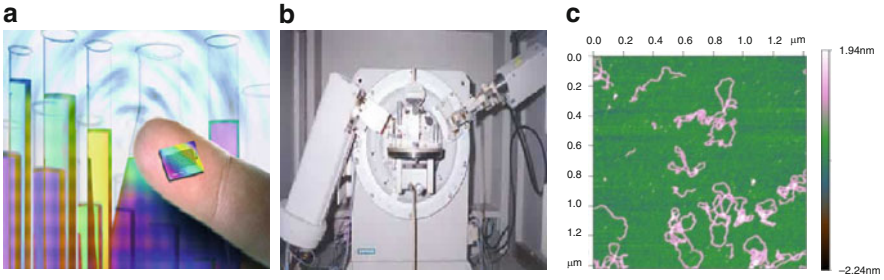

Fig. 1.5 (**a**) Lab-on-a-chip, for quick test results using very small samples. A lab-on-a-chip miniaturizes all the steps needed to process a medical sample and detect disease, (**b**) X-Ray Diffractometer of the Laboratory for Thin Films – Nanosystems & Nanometrology of AUTh, (**c**) plasmid DNA in buffer solution imaged with AFM semi-contact technique [13]

resolutions on the order of 10^{-6} (a micron – a red cell is 7 microns) and below to the molecular level. In addition, these techniques are critical for understanding the relationship and interface between nanoscopic and mesoscopic/macroscopic scales, a particularly important objective for biological and medical applications. As such, further nanotechnological advances will necessitate parallel progress of these physical characterization techniques. Examples of important tools available at the moment include highly focused (i.e. 1–2 μm) synchrotron X-ray sources and related techniques that provide detailed molecular structural information by directly probing the atomic arrangement of atoms (Fig. 1.5b); scanning probe microscopy (STM, AFM, etc.) that allow three-dimensional-type topographical atomic and molecular views or optical responses (SNOM) of nanoscale structures (Fig. 1.5c); in situ monitoring techniques that allow the monitoring and evaluation of building block assembly and growth [4]; ellipsometry, an optical method, with the capability of measuring in liquid environment (e.g. protein solution) to study protein and cell adsorption on solid surfaces [14] – it has been employed to discriminate and identify bacteria at the species level and is very promising for analytical purposes in biochemistry and medicine [14–18]. More specific information regarding the synchrotron X-ray sources and related techniques can be found in Chap. 4

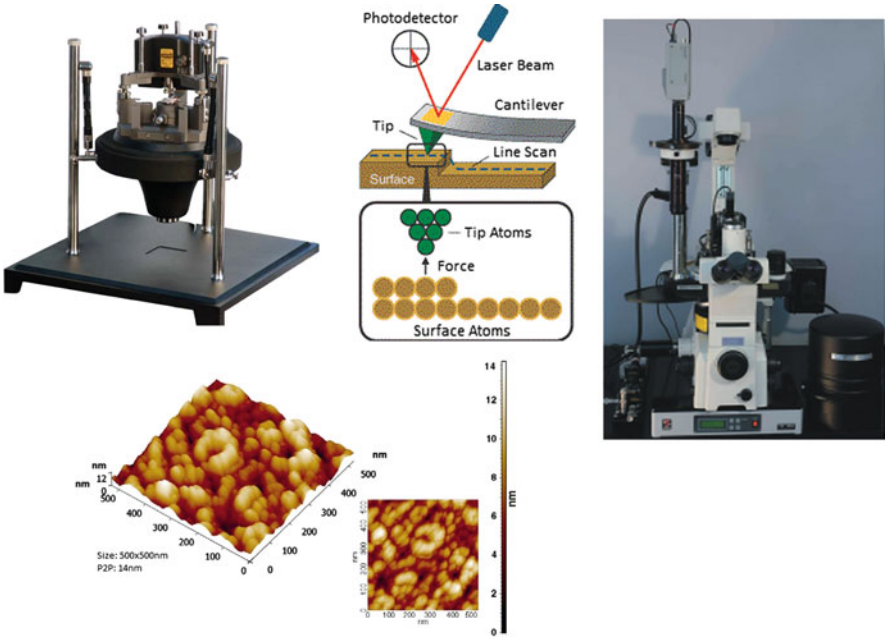

Fig. 1.6 (**a**) Atomic force microscope and how AFM works [13], (**b**) AFM topography image of fibrinogen on amorphous carbon thin film after 5-min incubation time, with a 500 × 500-nm size focus on one of the fibrinogen molecular cluster – shape features (Inset: the 2D equivalent image) and (**c**) scanning near-field optical microscope for reflection and fluorescence from scales below 50 nm [13, 20, 21]

Imaging is becoming an even more important tool in the diagnosis of human diseases. Imaging at cellular, and even sub-cellular and molecular, level is still largely a domain of basic research. However, it is anticipated that these techniques will find their way into routine clinical use. Atomic force microscopy (AFM) and AFM-related techniques (e.g. scanning near-field optical microscopy – SNOM) have become sophisticated tools, not only to image surfaces of molecules or sub-cellular compartments, but also to measure molecular forces between molecules. These techniques substantially increase our knowledge of molecular interactions [19]. Figure 1.6a, b shows the SNOM and AFM microscopes respectively. Figure 1.6c is an AFM topography image of a cluster of fibrinogen molecules adsorbed on amorphous carbon thin film, after incubation for 5 min. The adsorbed cluster has preserved the morphological characteristics of the protein molecule [20]. Through these techniques, protein adsorption is studied, in order to shed some light on and to understand its mechanism, and improve the properties of the potential materials and coatings that are going to be used for biomedical applications.

Scientists have developed analytical tools to examine the biological cells in great detail. We now understand better how biological structures function in general at intracellular level. However, we still do not know how to build nanostructures or "nano-"biomachines that are compatible (i.e. biocompatible) with living organ, tissues, cells and biochemical systems so that they safely operate inside the body. Once these questions are answered, we will be able to design better diagnostic tools and engineer structures for better treatment of diseases.

1.4 Nanotechnology and Nanomedicine: New Medical Approaches

1.4.1 "Nanomedicine"

The convergence of recent advances in nanotechnology with modern biology and medicine has created the new research domain of nanobiotechnology. The use of nanobiotechnology in medicine is termed nanomedicine. Thus, nanomedicine is an offshoot of nanotechnology, referring to highly specific medical intervention at the molecular scale for *therapeutic purposes* (involving curing diseases or repairing damaged tissues), and for the development of *diagnostics* for rapid monitoring, targeted cancer therapies, localized drug delivery, improved cell material interactions, scaffolds for tissue engineering and gene delivery systems. Successful research and development in nanomedicine where ultimately patients can benefit from these new technologies require the interaction of a multitude of disciplines including material science and engineering, cellular biology and clinical translational research. Many scientific as well as economic activities are expected to accelerate medical research

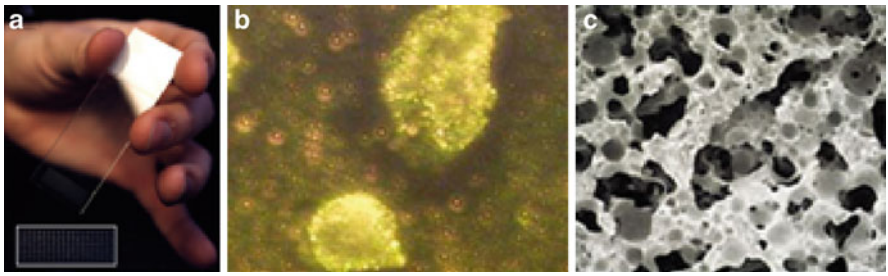

Fig. 1.7 (**a**) DNA chip: glass plate containing thousands of genes to be studied. Comparison of its size with a human hand. *Inset*: glass plate detail, (**b**) image of gold nanoparticles sticking to cancer cells and (**c**) electron microscope image of highly porous OSferion, an artificial bone replacement material with excellent biocompatibility [22]

and development. Several medical devices [3] have already benefited from recent developments in micro-nanotechnology and are in use or are currently being commercialized (Figs. 1.5 and 1.7). Nanomaterials and biological structures are approximately of the same size, which allows for unique interactions between biological systems and synthetic materials for analytical, diagnostic and therapeutic applications.

Nanomedicine can focus on several topics, such as *Engineering Topics* including, for example, peptide nanoparticles for medical applications, the transition from semiconductors to biochemistry in the lithography industry; *Clinical Applications* (like nanomedicine and protein misfolding diseases); *Topics in Genetics* (e.g. nanostructured probes for gene detection in living cells, detecting UV damage to individual DNA molecules with AFM, etc.); *Topics in Diagnostics*, with its main focus on early diagnosis in vitro and in vivo; *Policy and Commercialization Topics*, including initiative in nanomedicine to focus efforts in research, development and applied nanotechnology for improving the diagnostics, therapeutics and treatment of cancer; *Experimental Research Topic*s, which are an important basis for preclinical study, like nanodiagnostic imaging; *Topics on Basic Nanomedicine, Pharmacology Topics* and *Topics on Oncology and on Toxicology* [23].

The long-term goal of nanomedicine research is to characterize quantitative molecular-scale components known as nanomachinery, as well as to precisely control and manipulate nanomachinery in cells to improve human health, understand the cellular mechanisms in living cells and develop advanced technologies for early diagnosis and treatment of various diseases. Thus far, the assessment of single-molecule properties in living cells has been restricted by either the size of the probe or the photobleaching of the small fluorescent labels. The significance of this research lies in the development of a platform technology that will influence nanoscale imaging approaches designed to probe molecular mechanisms in living cells.

1.4.2 "Regenerative Medicine"

It refers to the medical field that by means of tissue-engineering approaches leads to the restoration of cell populations, tissues and organs that have lost their functionality due to a variety of reasons either genetic or physiologic. The main goal here is the construction of scaffolds that are able to stimulate molecular cascades that activate procedures and factors responsible for tissue regeneration [24]. The synthetic constructs that will be used as scaffolds should meet the below requirements. First, it should be biodegradable and biocompatible in order to prevent possible long-term negative side effects on host organism. Second, it should be structurally designed in a way that promotes the migration, attachment and development of the cells from which the tissue will be reformed [25]. Apart from ex vivo fabricated scaffolds, scientists can use living material from the host organism such as cells and proteins to create a scaffold that shows much higher biocompatibility compared to synthetic structures that may trigger the immune system of the patient [26]. Nowadays, it is also possible to replace differentiated cells with natural or genetically modified stem cells that are still undifferentiated and can be converted into almost any desired cell type with proper cell culture protocols [27, 28]. However, further research is required in order to understand better the range of interactions that occur when a scaffold is placed into the host's micro-environment. For this reason, a third alternative approach that includes the construction of complete functional ex vivo engineered tissue that is then implanted into the patient has gradually developed. This approach requires the usage of specific experimental devices that are called bioreactors and provide a restricted environment in which the tissue engineering takes place. By applying different parameters and by using different substrates and cells, it is possible to form tissues with unique structure and functionality each time. The field of bioreactor-mediated tissue engineering has a promising potential because of its low cost since the experimental conditions can be easily optimized [29–32]. Detailed presentation of the different approaches and techniques of tissue-engineered regenerative medicine is included in Chap. 2.

1.4.3 "Nanorobots" and Nanodevices

Such future devices are, for example, the artificial mechanical red blood cell or "respirocyte" (spherical shape of 1-μm diameter) and an artificial mechanical white blood cell of microscopic size, called a "microbivore" (3.4-μm major axis diameter and 2.0-μm minor axis diameter). The "respirocyte" is expected to be able to deliver more oxygen to the tissues than natural red blood cells and to manage carbonic acidity. Primary medical applications of respirocytes would include transfusable blood substitution, partial treatment for anaemia, lung disorders, enhancement of cardiovascular/neurovascular procedures, tumour therapies and diagnostics, prevention

of asphyxia, artificial breathing, etc. The primary function of "microbivore" is to destroy microbiologic pathogens found in the human bloodstream using a digest and discharge protocol. Microbivores are expected to be up to ~1,000 times faster acting than either unaided natural or antibiotic-assisted biologic phagocytic defences and able to extend the therapeutic competence of the physician to the entire range of potential bacterial threats, including locally dense infections. The "microbivores" would be removed from the body once their mission was completed.

Medical "nanorobots" may also be able to intervene at the cellular level, performing in vivo cytosurgery. The most likely site of pathologic function in the cell is the nucleus – more specifically, the chromosomes. In one simple cytosurgical procedure called "chromosome replacement therapy", a "nanorobot" controlled by a physician would extract existing chromosomes from a particular diseased cell and insert new ones in their place, in that same cell. If the patient chooses, inherited defective genes could be replaced with non-defective base-pair sequences, permanently curing a genetic disease. Engineered bacterial "*biobots*" (synthetic microbes) may be designed to produce useful vitamins, hormones, enzymes or cytokines in which a patient's body was deficient or to selectively absorb and metabolize harmful substances such as poisons and toxins into harmless end products [33].

1.4.4 Biocompatibility and Orthopaedic Implants

An important field of application for nanotechnology in medicine is the biomaterials, used, for example, in orthopaedic implants or as scaffolds for tissue-engineered products. If the design of a hip implant, for instance (Fig. 1.1), is carried out at nanolevel, it might become possible to construct an implant which closely mimics the mechanical properties of human bone, preventing stress shielding and the subsequent loss of surrounding bone tissue [3]. ECM provides an excellent three-dimensional web of intricate nanofibres to support cells and present an instructive background to guide their behaviour. It takes a variety of forms in different tissues and at different stages of development in the same tissue. This diversity arises through combinations of specific molecular interactions and geometrical arrangements of collagens, elastins, proteoglycans and adhesion proteins, such as fibronectins and laminins. Unwinding the fibres of ECM reveals a level of detail unmatched outside the biological world. Each fibre hides clues that pave the way for cells to form tissues, as complex as bone, liver, heart and kidney. A key challenge is to capture the degree of complexity that is needed to replicate functionally the ECM of natural tissue. Nevertheless, we are still a long way from recreating the molecular architecture of the ECM and the dynamic mechanisms by which information is revealed in response to challenges within the local environment. Nanostructuring of materials provides a powerful mechanism to encourage and direct cell behaviour, ranging from cell adhesion to gene expression, thus enhancing their biocompatibility, by dictating the desirable interactions between cells and

materials. The question of how cells detect and respond to nanofeatures remains unresolved yet. However, there are early findings where the promotion of one cell type over another, such as osteoblasts (bone-forming cells) over osteoclasts (bone-resorbing cells), to stimulate bone growth, will be important in reducing aseptic loosening and failure of implants. It has been found that not only the scale of topography (5 nm to micrometre scale) modulates cell behaviour, but also the type of ordered topography (e.g. ridges, steps, grooves, pillars and pits) and even their symmetry (e.g. orthogonal or hexagonal packing of nanopits) [34,35]. Furthermore, surface modifications at nanolevel of biomaterials or their coatings might greatly enhance the biocompatibility by favouring the interaction of living cells with the biomaterial, especially by their beneficial effect on cell adhesion and proliferation. Together with the control of nanoporosity allowing vascularization and the growth of cells inside the biomaterial, the nanostructured surfaces of biomaterials also allow the creation of novel types of scaffolds for tissue-engineered products [3].

1.4.5 Nanotechnology in Cardiology

Nanotechnology has various applications in the field of cardiology research not only for diagnostic but also for therapeutical purposes [36]. On the therapeutical scope, minimally invasive treatments for heart disease, diabetes and other diseases are a desirable goal for scientists, and there is hope for it, because of the use of nanotechnology. More precisely, a team led by Paul Grayburn of Baylor University Medical Center, and Ralph Shohet of the University of Texas Southwestern Medical School, in Dallas, TX, has demonstrated that ultrasound-targeted microbubble destruction (Fig. 1.8) can deliver genes that stimulate the growth of new blood vessels in rat heart [37].

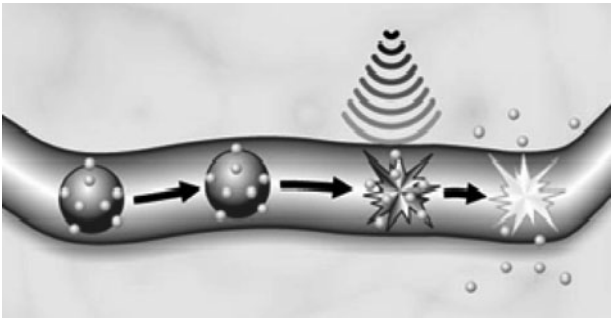

Fig. 1.8 Gas-filled microbubbles (*purple*) covered with DNA (*green*) pass harmlessly and uneventfully through blood vessels until they are exposed to ultrasound. Then, the bubbles burst, causing not only the release of the DNA but also the opening of holes in the cells that line the vessel [37]

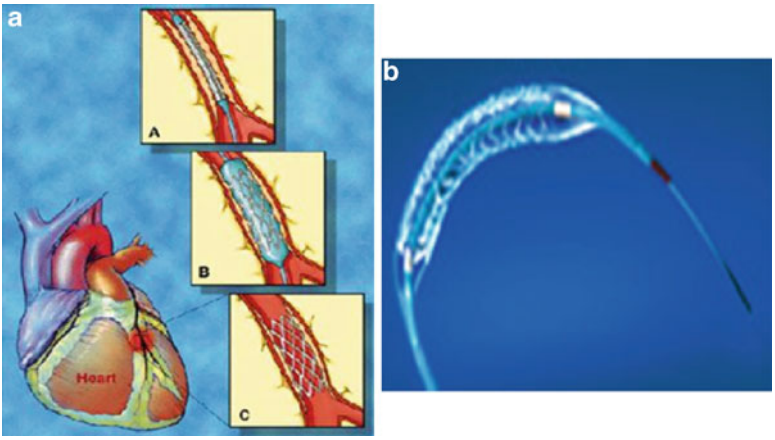

Fig. 1.9 (a) Percutaneous transluminal coronary angioplasty with stent implantation, (**b**) drug-eluting stent

Cardiovascular gene therapy could be realized roughly as follows: identification of a protein whose presence causes blood vessels to form, produce and package strands of DNA that contain the gene for making the protein and delivering the DNA to heart muscle. Of those steps, the last is the most challenging. In the late 1990s, physicians and physicists hit on the idea of using ultrasound contrast agents to deliver DNA, which can be seen in Fig. 1.8. If the ultrasound is intense enough, the bubbles can burst with sufficient force to breach the membranes of nearby cells. And if the bubbles are coated with DNA, their destruction releases the DNA, enabling it to enter the cells through the holes forced open by the burst [37].

Nanotechnology also plays a key role in the interventional therapeutic approach of *atherosclerosis and coronary Artery disease* (CAD), by improving the biocompatibility of intracoronary stents and by regulating the main limit factors for *percutaneous transluminal coronary angioplasty* (PTCA) at a molecular level via nanoparticles (Fig. 1.9a). The PTCA introduced in the late 1970s has been one of the most important treatment strategies for CAD, and its landmarks were the implantation of a metallic stent introduced in the late 1980s and the production of drug-eluting stents (Fig. 1.9b) in the early 2000s.

The major drawback of PTCA has been the occurrence of restenosis of the treated vessels, due to neointima proliferation and the negative remodelling of the artery, resulting in renewed symptoms and the need for repeated intervention in up to 50% of patients. The introduction of intracoronary bare metal stents reduced the restenosis rate within 6 months; however, a smaller portion of the patients (20–30%) still suffered of the so-called in-stent restenosis. Recently, drug-eluting stents loaded with the anti-proliferative compounds paclitaxel and rapamycin have led to the reduction of restenosis rate to 1–3% at 1 year.

However, "in-stent" restenosis remains the major limiting factor of percutaneous interventions for CAD. This is the reason that makes nanotechology necessary

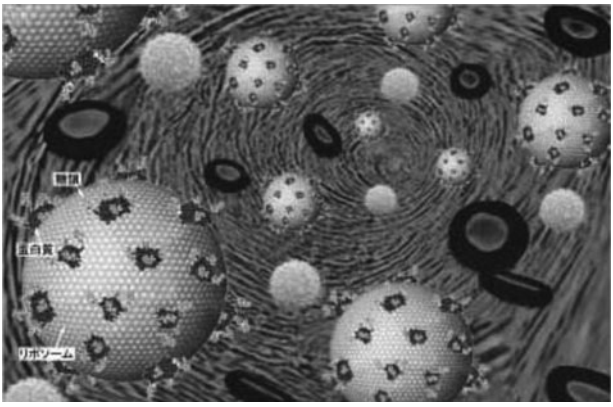

Fig. 1.10 Molecules such as drugs targeting the area of arterial wall undergoing PTCA with stent implantation

for improving biocompatibility of stents. Many nanocoatings have been evaluated in vivo and in vitro, and have been proposed to improve the biocompatibility of metallic stents or to serve as matrix for drug delivery.

Recent research data involving surface modifications of these prostheses at nanoscale as well as the loading of an anti-proliferative and anti-inflammatory drug onto a stent via nanoparticles such as liposomes may lead to the prevention of early thrombus formation and late neointima development, which are the major side effects of PTCA with stent implantation [38].

Going beyond drug-eluting stents, many nanoparticle carrier systems may be developed in order to transfer molecules via blood stream that block both neointimal hyperplasia and negative remodelling. In Fig. 1.10, we can see these molecules that can be used as stand-alone and that are not necessary to be loaded onto stents, and their potential molecular targets could be endothelial cells, vascular smooth muscle cells, adventitial fibroblasts, leucocytes and platelets which are the main cells involved in acute thrombosis and neointimal hyperplasia after PTCA with stent implantation.

In particular, in a mouse model of vascular injury, the injection of endothelial progenitor cells was associated with endothelization of the injured segment of the artery and with reduced neointimal proliferation. Many anti-apoptotic agents such as caspase inhibitor or an anti-apoptotic gene targeting against the activation of vascular smooth muscle cells, transferring via nanoparticles to the arterial wall being injured by stent implantation, might reduce restenosis.

In general, various inhibitors of growth factors secreted by activated platelets such as PDGF, Il-1, TGF-β and inhibitors of proinflammatory agents released by leucocytes upon activation (e.g. monocyte chemoattractant protein-1) could be used as anti-thrombotic and anti-restenotic agents. It can be concluded that a highly effective molecular coronary intervention by means of nanotechnology may eliminate the need for stents themselves [39].

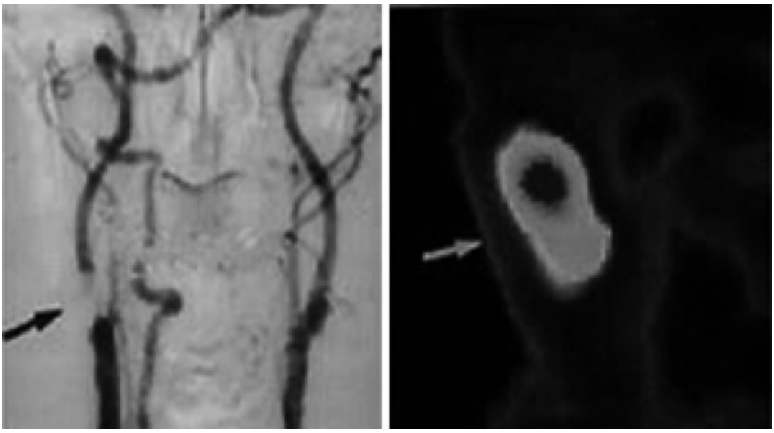

Fig. 1.11 Atherosclerotic lesions labelled by bispecific antibodies targeted with very high specific-radioactivity nanopolymers [36]

Diagnosis of cardiovascular diseases is an application of recent advances in nanotechnology as well. Many monoclonal antibodies, peptides and carbohydrates for non-invasive targeting of atherosclerotic lesions, myocardial necrosis, brain infarction and various tumours can be used for their detection. As an example, an antibody specific for the proliferating smooth muscle cells of the human atherosclerotic plaque was standardized for imaging experimentally induced atherosclerotic lesions in rabbits. The antibody after successful preclinical trials in Northeastern University is already being used for clinical studies in Italy and Spain [40]. Figure 1.11 illustrates very small atherosclerotic lesions in vivo, using bispecific antibodies targeted with very high specific-radioactivity nanopolymers.

1.4.6 Nanotechnology Against Cancer

Nanotechnology may have an impact on the key challenges in cancer diagnosis and therapy. Diagnosing, treating and tracking the progress of therapy for each type of cancer have long been a dream among oncologists, and one that has grown closer thanks to parallel revolutions in genomics, proteomics and cell bio1ogy. Nanotechnology's greatest advantage over conventional therapies may be the ability to combine more than one function.

Recently, there is a lot of research going on to design novel *nanodevices* capable of detecting cancer at its earliest stages, pinpointing its location within the human body and delivering chemotherapeutic drugs against malignant cells. The major areas in which nanomedicine is being developed in cancer involve (a) *early detection of tumour* (developing "smart" collection platforms for simultaneous analysis of cancer-associated markers and designing contrast agents that improve the resolution

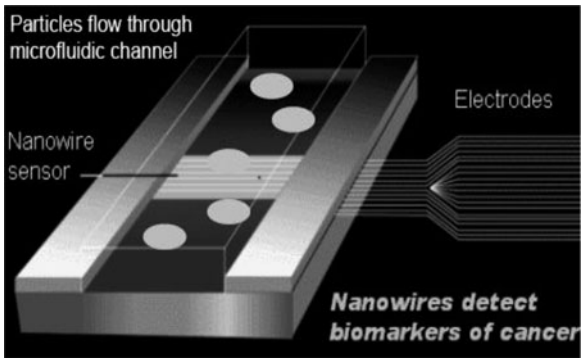

Fig. 1.12 A representation that explains how nanowires can be used for detection of cancer biomarkers

of tumour area compared with that of the nearby normal tissues) and (b) *cancer treatment* (creating nanodevices that can release chemotherapeutic agents).

Tumour diagnostics and prevention are the best cure for cancer, but failing that, early detection will greatly increase survival rates with the reasonable assumption that an in situ tumour will be easier to eradicate than one that has metastasized. Nanodevices and especially nanowires can detect cancer-related molecules, contributing to the early diagnosis of tumour. Nanowires having the unique properties of selectivity and specificity can be designed to sense molecular markers of malignant cells. They are laid down across a microfluidic channel and allow cells or particles to flow through it. Nanowires can be coated with a probe such as an antibody or oligonucleotide, a short stretch of DNA that can be used to recognize specific RNA sequences.

Proteins that bind to the antibody will change the nanowire's electrical conductance and this can be measured by a detector. As a result, proteins produced by cancer cells can be detected and earlier diagnosis of tumour can be achieved [41] (Fig. 1.12).

Nanoparticle contrast agents are being developed for tumour detection purposes. Labelled and non-labelled nanoparticles are already being tested as imaging agents in diagnostic procedures such as nuclear MRI [42–44]. Such nanoparticles are paramagnetic ones, consisting of an inorganic core of iron oxide coated or not with polymers like dextran. There are two main groups of nanoparticles (1) superparamagnetic iron oxides whose diameter size is greater than 50 nm and (2) ultrasmall superparamagnetic iron oxides whose nanoparticles are smaller than 50 nm [45,46]. Moreover, quantum dots being the nanoscale crystals of a semiconductor material, such as cadmium selenide, can be used to measure levels of cancer markers such as breast cancer marker Her-2, actin, microfibril proteins and nuclear antigens [47].

Tumour treatment can be succeeded with nanoscale devices (such as dendrimers, silica-coated micelles, ceramic nanoparticles and liposomes). These devices can serve as targeted drug-delivery vehicles capable of carrying chemotherapeutic agents or therapeutic genes into malignant cells. As an example, a nanoparticle-based drug called "*Abraxane*", consisting of paclitaxel conjunctive to protein

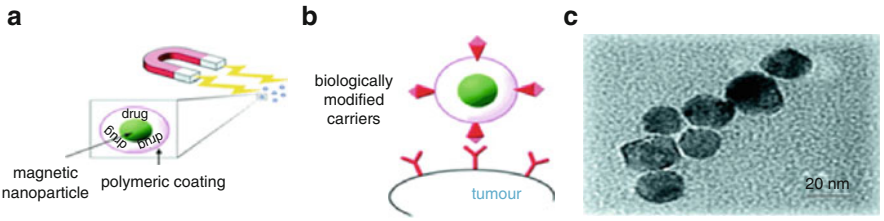

Fig. 1.13 (**a**) External magnetic field guiding the magnetic drug carriers near the tumour, (**b**) surface antigen recognition by attaching the carriers to the tumour for drug release on site, (**c**) electron microscopy image of magnetic nanoparticles

albumin particles, was approved by the Food and Drug Administration for breast cancer treatment few years ago [48].

It is worthwhile to mention that selective delivery and targeting of nanoparticles to tumours may overcome the problem of toxicity and may increase the effectiveness of drug delivery. The barriers involving this procedure and that should be under consideration are a variety of physical and anatomical characteristics of solid tumours, such as the necrotic core with the surrounding hypoxic area, the elevated local temperature and the interstitial liquid pressure [36, 49, 50].

Several approaches have been used to target nanoparticles to tumour-associated antigens, including direct conjugation of nanoparticles to monoclonal antibodies, modified plasma proteins or viral vectors. Recent progress has been made with targeted viral vectors for gene therapy applications. In addition to this, laser-induced thermal effects around nanoparticles attached to specific targets have recently been used for the treatment of cancer. The basic concept for this application of nanotechnology is the fact that nanoparticles of different properties (magnetic, optical, etc.), due to their size, can be delivered more easily to target cells than larger particles, via conjugation with antibodies, conjugation to viruses and physiological transportation as shown in Fig. 1.13 [36, 49–51].

After reaching target cells, these nanoparticles are then self-assembled into larger nanoclusters within cells. Afterwards, these nanoclusters can be activated by laser irradiation, microwaves or magnetic fields, depending on the nanoparticles' synthesis. By this process and its photothermal effects, destruction of cancer can be achieved.

More specifically, the nanoshell-assisted photo-thermal therapy (NAPT) is a non-invasive procedure for selective photo-thermal tumour destruction. It is based on nanoshells that absorb light in the NIR region, which is the wavelength that optimally penetrates tissues. These nanoshells have a core of silica coated with a metallic layer, usually of gold. The metal shell converts the absorbed light into heat with great efficacy. Further specificity can be engineered by attaching antigens on the nanoshells which are specifically recognized by the cancer cells. By supplying a light in NIR from a laser, the particles produce heat, which destroy the tumour. It has been found that the temperature within the nanoshell-treated tumours rose by about 40°C compared to a rise in 10°C in tissues treated with NIR light alone.

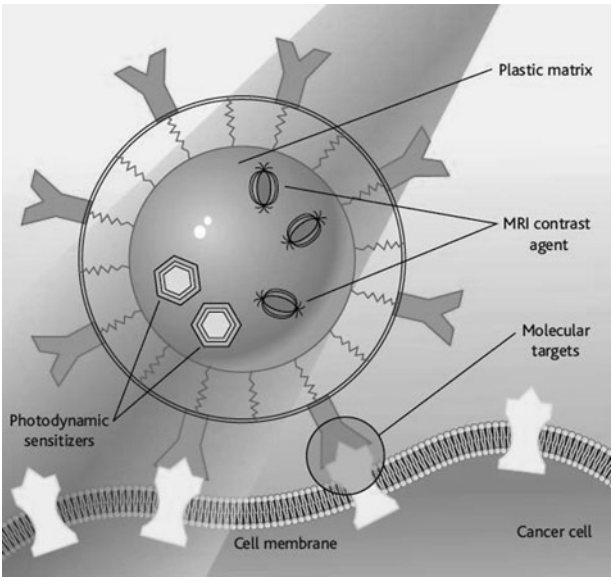

Fig. 1.14 Triple threat: Multifunctional nanoparticles can combine tumour-seeking sensors, imaging agents and toxins that kill cancer cells [34, 35]

The benefit of thermal therapeutics is that most procedures are non-invasive and have the potential to treat tumours that cannot be surgically treated [52, 53].

In vivo, Raoul Kopelman, Ann Arbor and colleagues, of the University of Michigan, have recently created three-component nanoparticles that target, image and destroy tumours in the brain of rats. The particles consist of an iron oxide core that serves as an MRI contrast agent. Attached to them are copies of a cancer-targeting peptide called F3, as well as a light-absorbing compound called photofrin that kills cells when hit with red light (Fig. 1.14). When Kopelman's team used their combination particles to treat rats previously injected with cancer cells inside their brains, animals receiving the combination particles survived more than twice as long as control animals receiving the non-targeted photofrin compound [34, 35].

1.4.7 "Theranostics"

Medical devices for in vitro diagnostics, such as gene-, protein- or lab-on-a-chip devices, do not have any of the safety concerns associated with nanoparticles introduced into the body. Numerous devices and systems for sequencing single molecules of DNA are feasible. Nanopores are finding use as new nanoscale technology for cancer detection, enabling ultra-rapid and real-time DNA sequencers. In general, developments in protein chips and lab-on-a-chip devices are more challenging compared to that in gene chips. These devices are anticipated to play

an important role in medicine of the future, as they will be personalized and will combine diagnostics with therapeutics into a new emerging medical area called "theranostics".

Over the next 10–20 years, nanotechnology may fundamentally transform science, technology and society, offering a significant opportunity to enhance human health in novel ways, especially by enabling early disease detection and diagnosis, as well as precise and effective therapy tailored to the patient [3]. Molecular diagnostics markets, for example, overlap with markets for non-molecular diagnostic technologies in the in vitro diagnostic market and are less well defined than those for pharmaceuticals [54]. The great influence that nanotechnology and pharmacogenomics can have in the further development of the field of personalized medicine is discussed in Chap. 7.

1.4.8 Prospects of Nanotechnology in Medicine and virtual Environments

Intelligent imaging, robotics and, in particular, nanotechnology developments will have key implications for the development of future biomaterials in the following areas:

- External applied surgery will require biomaterial properties to be controlled remotely, perhaps by self-assembly or development "in situ"
- Biomaterials that can be secured to anatomic structures using less invasive surgical procedures
- Improved accuracy in the placement of biomaterial devices
- Smart implants that react to implanted biosensor
- Implanted biomaterials will be used to control the delivery of some drugs and biologics [55]

Finally, applications of special relevance to improving health and enhancing human physical abilities include the use of virtual environments for training, education and interactive teaching. This will provide new ways for medical students to visualize, touch, enter, smell, and hear the human anatomy, physiological functions and medical procedures, as if they were either the physician or a microscopic blood cell travelling through the body. Similarly, impaired users, ordinary people, athletic coaches and a range of health-related professionals could train in these virtual environments.

1.5 Nanotoxicity: Possible Impact on Human Health

While products based on nanotechnology are actually reaching the market, sufficient knowledge on the associated toxicological risks is still lacking. The literature on toxicological risks of the application of nanotechnology in medical technology is scarce.

Reducing the size of structures to nanolevel results in distinctly different properties. In addition to the chemical composition, which largely dictates the intrinsic toxic properties, very small size appears to be a dominant indicator for drastic or toxic effects of particles. It is generally accepted that nanoparticles pose a separate problem within the area of toxicology, designated as nanotoxicology, which is the field of nanoscience that aims at the exploration of the background of the interactions that occur between the host organism and the nanostructured constructs while focusing on the impact that parameters of the above constructs including size, structure and chemical composition have on human health. Therefore, chemicals and materials in nanoformulation need to be evaluated for their activity and toxicity as nanoparticles. Chemical composition, which dictates the intrinsic toxic properties of the chemical, is of significant importance in determining the toxicity of particles.

It has been found that biodegradable substances are normally decomposed and their waste products excreted by the kidneys and intestines [48]. However, non-biodegradable nanoparticles have been studied and it seems that they accumulate in certain organs, especially the liver. It is not clarified what potential harm they may trigger, or at what dosage, but further investigation is needed [56]. Also some nanoparticles have been found to cause fatal damage to cell membranes and mitochondria, resulting in cell death via necrosis [57]. Furthermore, scientific evidence that shows that nanoparticles can interact with DNA molecules and destroy their integrity has appeared [58]. Finally, the impact that nanostructures can have on host immune system is described in Chap. 6.

Based on these conclusions, the development of specific guidance documents at a European level for the safety evaluation of nanotechnology products applied in medical technology is strongly recommended, and the need for further research in the field of nanotoxicology is clearly identified.

Ethical and moral concerns also need to be addressed in parallel with the new developments in some areas, for example neuroethics need to be investigated before brain and neural system research. Another key challenge is forecasting and addressing possible unexpected ethical, environmental and health consequences of the revolutionary science and engineering developments in nanobiosystems. Priority science and technology goals may be envisioned for international collaboration in nanoscale research and education: better comprehension of nature, increasing productivity, sustainable development and improving human performance [59].

1.6 Conclusions

It is believed that nanotechnology can greatly contribute to the evolution of modern medical approaches and practices. Nanoscale constructs are already used in therapeutical applications against cancer and pathogens, mostly by acting as drug carriers. Also, either in vivo or ex vivo engineered scaffolds and tissues are implanted in patients whose own organs and tissues are damaged or lost. Furthermore, specific nanostructures are widely used as imaging and detection

agents in diagnostic procedures. It is obvious that nanotechnology has improved the quality of human life by providing powerful tools that can benefit the patients faster and more effectively than traditional medical approaches. The emergence of nanotechnology has boosted the scientific research as well since new protocols and tools are designed which, in turn, gradually enhance the understanding of the way the biomolecules and cells interact either with living or nonliving systems. However promising the above facts may be, further research in the field of nanotoxicology is still required so as to reveal the nanotoxic effects that nanomedical devices may have on a host's health. The rapid and constant development of nanotechnology and nanomedicine will give birth to novel constructs that will change the groundings of modern medical science. The ideal goal is to use nanomedicine in order to improve health by enhancing the efficacy and safety of nanosystems and nanodevices and to provide inexpensive and simple solutions to incurable diseases.

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Chapter 2
Nanomedicine Pillars and Monitoring Nano–biointeractions

V. Karagkiozaki, S. Logothetidis, and E. Vavoulidis

Abstract The current revolution in medicine is strongly associated with the availability of new tools, methods and materials that enable the visualization and handling of molecules and even atoms in order to explore the etiology of many diseases and foster the insights within the biological nano-world. This chapter describes the main nanomedicine pillars that involve nanodiagnostics, targeted drug delievery and regenerative medicine. It gives an overview of key nanotechnologies that will advance the diagnosis and treatment of various diseases. Several experiments are employed to help the reader to understand how nanomedicine can advance mainly the study of mechanisms of bio and non- bio interactions for the design and development of highly performed implants. The hazards and risks for nanomedicines and the future challenges and perspectives of their application in clinical practice will also be discussed.

2.1 Introduction

> The principles of physics, as far as I can see, do not speak against the possibility of manoeuvring things atom by atom.

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> It is not an attempt to violate any laws; it is something, in principle, that can be done; but in practice, it has not been done because we are too big.
> There's Plenty of Room at the Bottom.
>
> Richard Feynman, Nobel Prize winner in physics, 1959

Nanomedicine refers to highly specific medical intervention at the molecular scale for the preservation and improvement of human health by curing diseases or repairing damaged tissues. The cells consist of nanomachines capable of carrying out a wide range of functions through specific and highly intricate interactions that drive and control the cellular biochemistry. The current revolution in life sciences is strongly associated with the availability of new experimental tools that enable the visualization of molecules and even atoms in order to explore the etiology of many diseases and foster the insights within the biological nano world.

Nanotechnology involves the design and synthesis of devices and biomaterials at the atomic level as well as the control of their properties and functionalities for favorable cellular responses to translate into addressing medical challenges. Understanding these nanoscale properties can lead to the development of a new class of biomaterials with unique set of properties different from those of original macroscale materials. The thorough analysis of bio/non-biointeractions achieved by versatile nanotools is essential for the design and development of highly performed medical implants.

Nanomedical developments range from nanoparticles for molecular diagnostics, imaging, and therapy to tissue engineering strategies for restoration of biological functions and regeneration of damaged tissues and even organs. Therefore, nanomedicine is comprised of the pillars of nanodiagnostics, targeted drug delivery, and regenerative medicine. Cardiovascular diseases, neurodegenerative diseases, diabetes mellitus, severe burns, wounds and cancer are a few of the paradigms that will be affected drastically by such technologies.

While the major focus of this chapter is to outline how nanomedicine can advance mainly the study of mechanisms of bio and non-biointeractions for the design and development of highly performed implants, in the following paragraphs, the main nanomedicine pillars and an overview of key nanotechnologies that will improve the diagnosis and treatment of various *diseases* will be described, together with some specific examples. The hazards and risks for nanomedicines and the future challenges and perspectives of their application in clinical practice will be the final part of the chapter.

2.2 Nanomedicine Main Pillars

2.2.1 Targeted Drug Delivery

Nanotechnology has already been occupied for quite a few years in medicine, particularly in oncology [1]. The ideal chemotherapeutic strategy is to navigate through the vasculature after intravenous administration, to reach the tumor site at

full concentration, to kill the cancer cells selectively, and to avoid all the systemical harmful side effects. Thus, nanotechnology can assist in this direction by engineering nanovectors with multifunctional activities such as drug payloads and/or contrast agents' release.

The most studied and commercially available drug delivery system is the *liposome*,with FDA having granted approval since 1996 for the use of liposomal doxorubicin against Kaposi's sarcoma. Later, it was approved for use in metastatic breast cancer and recurrent ovarian cancer [2].

Liposomes are lyotropic liquid crystals consisting of an aqueous core entrapped by one or more bilayers of natural and/or synthetic lipids. Drugs with a great range of lipophilic behavior can be encapsulated in three different regions of the liposomal molecules. More specifically, the drug cargos can be placed either into the phospholipid bilayer, into the inner core, or at the bilayer interface. Liposomes own the benefit of controllable biodistribution and drug release inside the host organism, making it possible to find the most suitable experimental conditions that enhance the therapeutic index of the entrapped drugs. Furthermore, they can be used to control the retention of loaded drugs in the presence of biological agents, to configure the vesicle concentration in the systemic circulation or other residues in the body, and to optimize the level of liposomal endocytosis conducted by the targeted cells [3].

Liposomes composed of natural lipids are biodegradable, weakly immunogenic, produce no antigenic reactions, and possess limited intrinsic toxicity. As an outcome, the drugs that are loaded into the lipid vesicles can be transferred with restricted degradation and limited side effects to the recipients. Moreover, efforts have been made to optimize or even alter the specificity of these drug-delivery systems, making it possible to target a variety of different organs and cell lines.

Entrapment of drugs into liposomes can result in (1) enhanced circulation lifetime, (2) increased deposition in the targeted organs and tissues, (3) protection from the host mechanisms of drug metabolism causing drug degradation or decrease in drug efficiency and (4) altered tissue biodistribution with high uptake in organs with increased levels of mononuclear phagocytic cells such as liver, spleen, and bone marrow, and at the same time, low uptake in the kidney, myocardium, and brain. However, sometimes the size of liposomes can act as a great drawback that limits their range of applications as drug carriers due to possible inability to enter into some targeted tissues such as specific tumors in high concentrations [4]. In addition, liposomes can be used as carriers not only for drugs but also for DNA molecules. In particular, cationic liposomes are shown to be a significant human gene delivery system with promising potential [5].

Nanoparticles (NPs) can be used as carriers for therapeutic and imaging contrast agents for site-specific targeting. They may have the same size as proteins, and this property makes them suitable for biotagging or labeling. However, the size of these vesicles is a limiting factor that must be taken into account in applications where nanoparticles are used as potential candidates for biotagging. Since the goal is to provoke an interaction between the nanoparticle and the targeted molecule, a biological coating or layer behaving as a bio-organic interface should be connected to the nanoparticle vesicle. For the above purpose, a wide variety of biological

coatings have been used, such as antibodies, biopolymers like collagen [6], or single layers of small molecules that make the nanoparticles obtain biocompatibility [7]. In addition, as optical detection techniques are widespread in biological research, NPs should either fluoresce or change their optical properties. For example, iron oxide particle provides a T2-mode negative contrast for magnetic resonance imaging (MRI), while gold nanoparticles can enhance the contrast in X-ray and computed tomography imaging.

Further functionalization of NPs can be made by linear reactive linker molecules, depending on the function required by the application [8]. Nanoparticles have been used in a great number of *drug-delivery* applications. Studies where paclitaxel (Taxol) was inserted into Vitamin E TPGS-emulsified poly (D,L-lactic-co-glycolic acid) (PLGA) nanoparticles showed an increased cytotoxicity for tumor cells of human colon adenocarcinoma in vitro and increased drug efficiency in vivo when paclitaxel nanoparticles were used instead of free drug [9]. In addition, gold nanoparticles loaded with methotrexate have been found to promote seven times higher cytotoxic effects compared to free drug against Lewis lung carcinoma cells [10]. Apart from antitumoral applications, NPs have been used in other medical applications as well. For example, poly (*n*-butylcyanoacrylate) nanoparticles coated with polysorbate 80 were used as carriers for rivastigmine through the blood–brain barrier in order to enter into the brain and treat Alzheimer's disease [11]. Finally, silver nanoparticles containing antibiotics including penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin resulted in higher levels of antibacterial activity compared to free drugs against *Staphylococcus aureus* and *Escherichia coli*, two very common bacterial strains that affect humans [12].

Besides the molecular recognition of the target tissue via antibodies and other biomolecules conjugating onto the NPs' surface, the NPs have the ability to circumvent the biological barriers because of their nanoscale dimensions. Currently, a variety of targeting moieties besides antibodies are under investigation, such as ligands, aptamers, and peptides binding to specific target cell-surface markers expressed in the disease microenvironment. An example of a multistage nanosystem as a carrier is the time-controlled release of multiple payloads of active NPs, with different subcellular targets by crossing different biological barriers [13].

The nanoparticles can enter into the cells via different pathways as presented in Fig. 2.1. They may actively be taken up by cells via phagocytosis (1), macropinocytosis (2), clathrin-mediated endocytosis (3), clathrin- and caveolae-independent endocytosis (4), or caveolae-mediated endocytosis (5). Apart from these mechanisms, a passive movement through the plasma membrane with subsequent access to all subcellular compartments, including nucleus and mitochondria, has been proposed (6).

Another class of applications being developed is chemically functionalized *dendrimers*, highly branched molecules with a "tree-like" branching structure that can be used as molecular building blocks for gene therapy agents or as MRI contrast agents [15].

More specifically, dendrimers are spherical nanostructures with a particular formation of three main regions (a) an inner core which is either a single atom

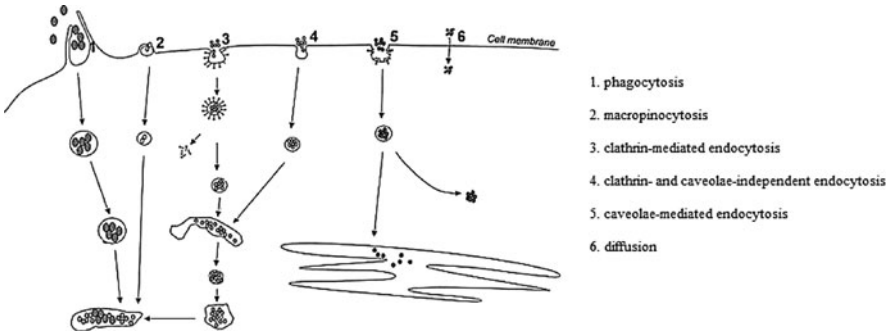


Fig. 2.1 Schematic presentation of the possible pathways by which nanoparticles can enter into the cell. Nanoparticles may be actively taken up by cells via phagocytosis (1), macropinocytosis (2), clathrin-mediated endocytosis (3), clathrin- and caveolae-independent endocytosis (4), or caveolae-mediated endocytosis (5). Apart from these mechanisms, a passive movement (diffusion) through the plasma membrane with access to all subcellular compartments, involving nucleus and mitochondria, has been proposed (6) [14]

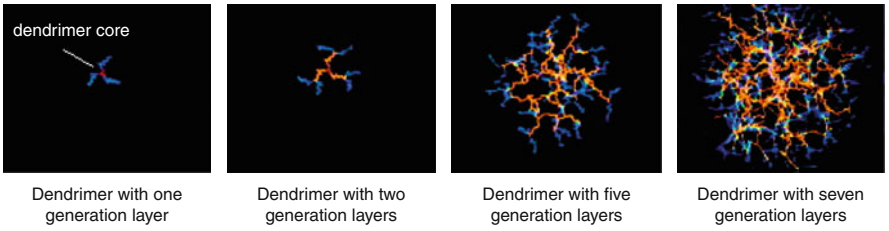


Fig. 2.2 Graphical presentation of dendrimers from core dendrimers with one generation layer to multibranched dendrimers with seven generation layers

or an atomic group, (b) mediate branches surrounding the surface of the core, consisting of units with at least one branch junction through well-defined repetition of which a series of concentric layers called generations are finally formed, and (c) many terminal groups, located in the outer shell of the dendrimer, which define the functionality of the dendrimers [16]. The structure of dendrimers varying from core dendrimers with one generation layer to multibranched dendrimers with seven generation layers is presented in Fig. 2.2.

Dendrimers are attractive molecules for drug-delivery and diagnostic applications due to the high level of control possible over their architectural design, including their size, shape, branching length, density, and surface functionality. Bioactive agents may be encapsulated into the interior of the dendrimers or chemically attached or physically adsorbed onto the dendrimer surface. In this regard, the high density of the surface bioactive groups allows further attachment of targeting groups or functionality that may modify the solution behavior or toxicity of dendrimers [17].

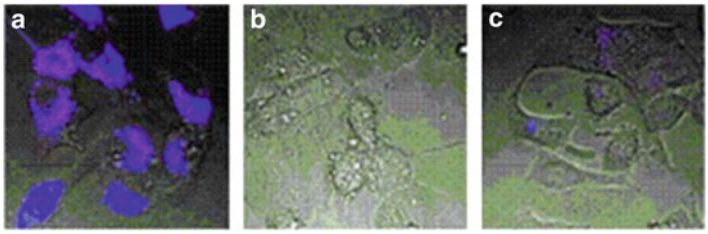


Fig. 2.3 Microscopy images showing the endocytic internalization of fluorescently labeled DNA–single wall CNT conjugates: (**a**) After incubation under normal experimental conditions, the conjugates were able to enter into the cells and (**b**, **c**) after incubation, under experimental conditions that block the endocytosis pathway, cellular uptake of conjugate molecules was significantly lower

Dendrimers may be used for gene therapies because they are nonviral delivery vehicles for DNA which tends to wrap itself in the dendrimer branches [18]. Dendrimer-based MRI contrast agents have been shown to produce increased relaxivity, which translates into higher contrast image. Remarkably, dendrimers have been studied as potential nanodrugs against tumors, bacteria, and viruses [19].

Within the family of nanomaterials, *carbon nanotubes* (CNT) have emerged as a new alternative and efficient tool for transporting and translocating therapeutic molecules. Their versatile physicochemical features enable the introduction of several pharmaceutically relevant entities and allow for rational design of novel candidate nanoscale constructs for drug development. CNT can be functionalized with bioactive peptides, proteins, nucleic acids, and drugs, and used to deliver their cargos to cells and organs for targeting, imaging, and therapy. Functionalized CNT have been shown in many studies to be able to cross cell membranes [20]. The ability of CNT to cross cell membranes has allowed them to become of particular high interest for drug-delivery strategies [21]. In targeted delivery of drugs to cells, drugs are first attached to the carrier by either covalent or noncovalent bonding. The cargo–carrier conjugates are then directed to the targeted cells. After reaching the site of interest (organs, tissues, or even single cells), there are two different routes that can be followed (a) only the drug enters the cells without internalization of the nanotubes or (b) both the drug and the nanotubes enter the cell and then the cellular mechanisms will degrade the drug–carrier conjugates, releasing drug molecules inside the cytoplasm (Fig. 2.3a–c). The internalization of the CNT can be achieved either via the endocytosis pathway [22] or via the diffusion mechanism [23].

2.2.2 Regenerative Medicine

Regenerative medicine appears to take as its patron the Prometheus, whose liver was able to regenerate daily, as the scientific field that aims at the restoration of cells and tissues that are lost or damaged. More specifically, the field of regenerative medicine

seeks to devise new therapies for patients with severe injuries or chronic diseases in which the body's own responses do not suffice to restore functional tissue. There is a wide array of major unmet medical needs which might be addressed by regenerative technologies. These include neurodegenerative diseases, congestive heart failure, diabetes mellitus, osteoporosis, and severe burns [24].

Normal wound healing and tissue regeneration exploit selected molecules from the biomolecule pool to direct events such as cell attachment, growth, extracellular matrix (ECM) formation, and phenotypic differentiation. For tissue engineering strategies, these same processes must be mimicked to encourage tissue development and regeneration within artificial scaffolds and gels [25]. Imitation of the above natural processes is insufficient since a tissue-engineered artificial construct should also be biodegradable, and at the same time, the degradation rate needs to be in line with the formation of new tissue to ideally serve the template purpose [26]. Furthermore, many tissues such as cardiac muscles and blood vessels own unique mechanical properties that are important for their functionality, and as an outcome, tissue-engineered scaffolds and gels for such purposes are required to have specific elastomeric potential in order to achieve the biomimetism of tissue mechanical properties [27, 28].

Generally, the artificial constructs provide the proper microenvironment for regenerative cells. However, since these constructs prepared ex vivo lack the multitude of cues present in the in vivo microenvironment, regenerative cells often need to be supplied with external biological and physical stimuli to coax them toward targeted tissue functions [29]. Recently, a new approach of in situ regenerative medicine has appeared, called endogenous regenerative technology that basically involves the use of patient's own scaffolds, growth factors, and proteins in order to achieve tissue healing and regeneration [30]. The therapeutic use of growth factors and cytokines to stimulate the production and/or function of endogenous cells represents the area of regenerative medicine that, arguably, has shown great clinical impact [31, 32].

Regenerative therapies comprising living cells have also entered into practice, at first, through the widespread adoption of both allogeneic and autologous bone marrow transplantation. The presence of hematopoietic progenitor and stem cells with great replicative capacity in vivo, and their ability to reenter the bone marrow niche from the circulation, enabled the successful tissue engineering [33].

However, *stem cell tissue engineering* is a direct gene cell therapy process that basically involves the in vivo transfer of natural nonmodified genes which activate molecular pathways that lead to formation and regeneration of the host tissues and cells. Nowadays, an improved version of the above procedure has been developed. More specifically, with the procedure of genetically engineered cell therapy that takes advantage of tissue-specific or therapeutic genes combined with primary cells that overexpress these genes, it is possible to produce specific therapeutic agents at the site of interest where regeneration is required, or to stimulate new cells to differentiate into desired cell lines and thus promote tissue regeneration [34–36].

In addition, a new approach of using cell lines alternative to stem cell has appeared. Research related to the molecular biology and gene expression of stem cells has shown that some particular transcription factors are responsible for the stem cell pluripotency by activating some specific signaling cascades and suppressing others [37]. So when a stem cell is differentiated into a distinct cell type, the expression profile of the above factors is altered to a great extent. Although this differentiation is generally an irreversible procedure under physiological conditions, scientists have designed a reverse procedure that basically reprograms somatic differentiated cells. This nuclear reprogramming refers to the change from the differentiated state of somatic cells to a less differentiated state similar to that of stem cells (stem cell-like state). These new cells are called induced pluripotent stem cells and are produced by a cocktail of pluripotent gene activators and self-renewal modulators [38].

Regenerative medicine is capable to restore the physiology of the patients, apart from designing scaffolds and gels that are developed into new tissue after their insertion into the host organism, by constructing completed alternatives to host organs and tissues that are developed in a laboratory prior to their insertion. Such tissue-engineered organ equivalents are developed ex vivo under controlled conditions in laboratory bioreactors. Ex vivo engineering of living tissues is an area with promising potential. Today, the clinical use of functional in vivo engineered tissues is restricted to an extent by the limited understanding of the important role that the natural interactions of host cells, ECM, growth factors, and other biomolecules with the scaffold or gel after its implantation into the patient. By enabling reproducible and controlled changes of specific environmental factors, bioreactor systems provide not only the technology to study fundamental mechanisms of cell functions and interactions in a 3D environment but also the potential to improve the quality of engineered constructs. In addition, by standardizing the experimental conditions of tissue formation in controlled and restricted systems, bioreactors could reduce production costs, thus facilitating a wider use of ex vivo engineered tissues [39].

The synthesis of new biomaterials and the development of improved fabrication techniques have led to significant enhancements in the complexity and biomimetism of the latest tissue engineering constructs. With the assistance of microscale and nanoscale technologies, it is possible to construct arrays of biomaterial to test the interaction of the regenerative cells with extracellular signals and the effects on cell shape and differentiation. As cells adhere onto the biomaterial arrays, they align themselves to the formation of the biomaterial substrate which has been shown to be a key factor that defines and alters the cell functionality and behavior. As a result, the use of microscale and nanoscale technologies in tissue engineering, either directly as transplantable constructs or indirectly as a tool for understanding the biology of organs and tissues, has pushed the field closer to clinical applications [40]

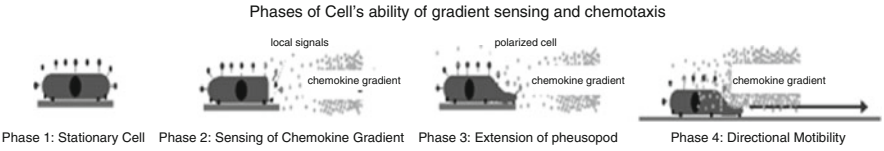


Fig. 2.4 Schematic presentation of stages of cell migration and chemotaxis, in response to chemokine gradient

2.3 Nanomedicine for Monitoring Bio/Non-Biointeractions

Whenever a biomedical application of a material is intended, the device surface and the response of the host to a foreign material remaining in the body for an extended period of time are issues of concern.

When an implant is inserted into the body, a biolayer consisting of water, proteins, and other biomolecules from the physiological liquid is formed on the implant surface. Cells' adhesion involving mainly platelets and leucocytes occurs in a later stage and it is mediated by the proteins initially adsorbed on the surface [41, 42].

This dynamic cascade ends via the activation of platelets and coagulation system to the formation of thrombus onto the biomaterial surface. The blood cells migration toward the implant is a remarkable behavior at the molecular level. The filamentous actin (F-actin), intermediate filament, and microtubule cytoskeleton systems are key mediators of cell morphology. Endogenous and exogenous chemical and mechanical signals control the precise arrangement of these dynamic polymers.

The cell crawling involves three basic steps (a) leading edge protrusion, (b) contraction of the F-actin polymer network, and (c) tail retraction. Net cell movement cannot occur unless the F-actin network is anchored to the substrate.

The basic steps of cell migration and chemotaxis are illustrated in the schematic diagram: (Phase 1) Nonmotile cells are attached to the underlying ECM, most likely through integrin receptors on the cell surface; (Phase 2) cells are exposed to a soluble gradient of growth factors or chemokines, which binds to and activates cell surface receptors (Fig. 2.4). As a cellular response (Phase 3), a localized actin polymerization leads to membrane protrusion (of a pseudopodium or lamellipodium) in the direction of the gradient. The establishment of a dominant leading pseudopodium and rear cell body compartment marks the first sign of morphological polarity. Once a dominant pseudopodium is formed, cell movement commences in the direction of the gradient as the cell undergoes repeated cycles of membrane extension at the front and retraction of the rear compartment (Phase 4) [43].

For example, a cut section of an artery is presented in Fig. 2.5: the endothelium is the green lining; the white blood cells have been activated by chemoattractants and are beginning to migrate through the endothelium.

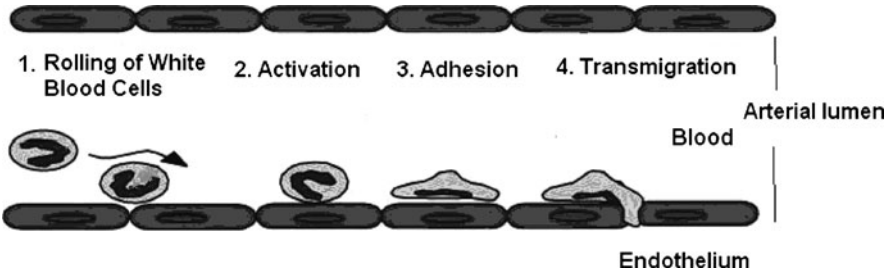


Fig. 2.5 Schematic presentation of white blood cells that migrate through the endothelium after activation by chemoattractants (tissue depicted in green, which covers the inside of arterial blood lumen), after activation by chemoattractants

2.3.1 Atomic Force Microscopy for Imaging of Proteins and Cells

Atomic force microscopy (AFM) is one of a family of scanning probe microscopes which has grown steadily since the invention of the scanning tunneling microscope by Binning and Rohrer in the early eighties for which they received the Nobel Price for Physics in 1986. It has become a well-established technique for imaging individual macromolecules at a spatial resolution of smaller than 1 nm. Although it was originally conceived to image nonconductive materials, due to its nanoscale resolution, it was quickly applied in biosciences. It was used for analysis of crystals of amino acids and organic monolayers, nucleic acids and their complexes with proteins, chromosomes, individual isolated proteins and peptides, membranes and membrane bound proteins, ligand–receptor binding, etc. [44, 45].

There are some significant advantages of AFM as an imaging tool in biology and physics when compared with complementary techniques such as electron microscopy. Not only does AFM achieve molecular resolution, but it can also be performed under fluids permitting samples to be imaged in near-native conditions. The fluid may be exchanged or modified during imaging and, therefore, there is the potential for observing biological processes in real time, something which electron microscopy is not currently able to achieve. Thus, it has also been applied to study living cells in their natural aqueous environment without their damage, although there are difficulties associated with imaging soft, living biological materials in situ, and with being a time-consuming task. The instrument is also capable of manipulating molecules and measuring the strength of molecular interactions with pico-newton sensitivity [46].

The main operation principles involve the scanning of the surface of the sample with a sharp tip having a contact area of only a few square nanometers, at the end of a microfabricated cantilever. A piezoelectric crystal is used to raise or lower the cantilever, to maintain a constant bending of the cantilever [47]. The interatomic forces between the tip and the sample are sensed by the cantilever, whose deflection

is measured (usually) by a laser. The reflected laser beam strikes a position-sensitive photodetector consisting of four side-by-side photodiodes. The difference between the four photodiode signals indicates the position of the laser spot on the detector and thus the angular deflection of the cantilever. If the tip is scanned over the sample surface, then the deflection of the cantilever can be recorded as an image which represents the three-dimensional shape of the sample surface (deflection image).

Atomic resolution is easily obtained on relatively robust and periodic samples. Soft samples – particularly biological samples – provide a more difficult surface to image because the forces exerted by the tip during imaging can cause deformation of the sample. The problem involved with imaging soft samples has been overcome to a large extent by the introduction of "tapping mode" AFM imaging. Instead of maintaining a constant tip–sample distance of a nanometer or so, the cantilever is oscillated in a direction normal to the sample, resulting in only intermittent contact with the surface. This greatly reduces the lateral forces being applied in the plane of the sample which are responsible for most of the damage as the tip is scanned. The AFM is capable of better than 1-nm lateral resolution on ideal samples and of 0.01-nm resolution in height measurement [48].

2.3.2 Implementation of AFM for Protein Adsorption Studies

In recent years, the remarkable improvement in understanding the pathology of numerous human diseases at the molecular level leads to the etiology of a disease to be traced in one or more key proteins [49]. It is known that protein conformations/modifications take place at the nanoscale. In order to detect and identify subtle differences, it is necessary to develop new tools for diagnostic, prognostic, and monitoring purposes based on nanotechnology. Imaging at cellular, and even subcellular and molecular level is still largely a domain of basic research. AFM and AFM-related techniques (e.g., scanning near-field optical microscopy – SNOM) have become sophisticated tools, not only to image surfaces of molecules but also to measure forces between molecules.

AFM has been used for the visualization of single protein molecules and for the comprehension of protein adsorption mechanisms on surfaces. It can provide detailed information about the biomolecules' conformation and size with nanoscale precision, the protein layer surface morphology, roughness, and can reveal protein adsorption mechanisms that determine the subsequent cellular behavior. Protein adsorption is a process that takes place in effect on the interface of liquid solution and solid substrate. In general, it is a quite multiparametric procedure as it is regulated by numerous factors like hydrophobicity, pH, surface roughness, chemical composition, etc., and this diversity of parameters makes it complex [50, 51].

Below, an overview of the AFM analysis related to the hemocompatibility (possibility of thrombus formation) of a surface is presented. In this context, fibrinogen (Fib) and human albumin (HSA) morphological characteristics during adsorption are analyzed. The selection of these proteins is based on the fact that

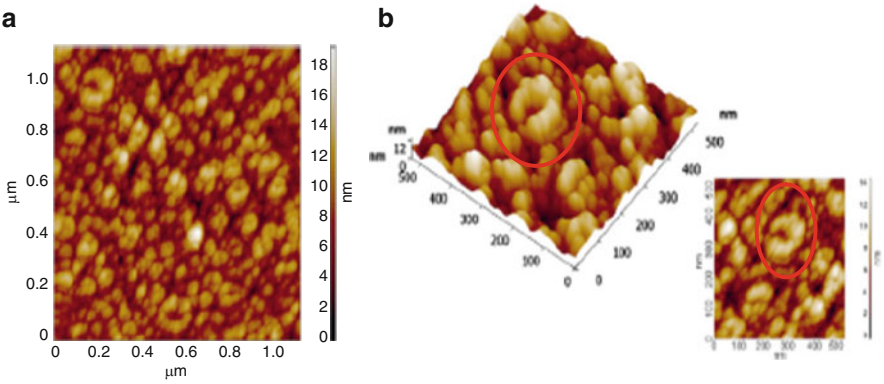


Fig. 2.6 AFM topography image of (**a**) Fib on a-C:H thin film after 5-min incubation time (scan size 1 mm x 1 mm) and (**b**) Fib on the same biomaterial after 5-min incubation time, with a 500 nm × 500 nm size focus on one of the fibrinogen cluster (Inset: the 2D equivalent image) [54]

these are two of the most significant human plasma proteins. More particularly, Fib takes part in blood coagulation, facilitates adhesion and aggregation of platelets, and is important in the processes of both hemostasis and thrombosis, whereas HSA is believed to act controversially to Fib, although its specific action is not yet clear [52, 53].

It is known that Fib is a *linear* blood plasma protein with size 48 × 6 × 9 nm with three globular domains. In Fig. 2.6, topography images of Fib on amorphous hydrogenated carbon (a-C:H) thin film are presented after 5-min. incubation time. The circles indicate the molecular cluster features of fibrinogen. Thus, it was revealed after 5 min. of incubation that the three-lobe structures that appear are, indeed, small clusters of few molecules of fibrinogen – forming either extended or V-shaped conformations – that still retain the shape of a single molecule.

Protein conformation changes are extremely important for the in-depth comprehension of the protein adsorption mechanisms [55]. Causing protein denaturation, for example, through heating or pH change of the protein solution used, is a way to explore protein conformation changes in detail. Proteins in their native conformations are highly oriented, and the three-dimensional shape of a protein is critical to its function. This shape is maintained because of mainly hydrogen bonds, which can be easily broken by chemicals or high temperature (protein denaturation). AFM technique provided information not only about the topography of protein layers formed on nanomaterials as already described, but it also enables the study of the effect of temperature on protein conformation. Previous work at our laboratory showed that by using the heating stage of AFM, the Fib molecules unravel and the three-dimensional structure of the protein is changed. For example, topography images of Fib on amorphous carbon thin film at 116°C (advanced stage of denaturation) compared to Fib adsorption at room temperature are depicted in

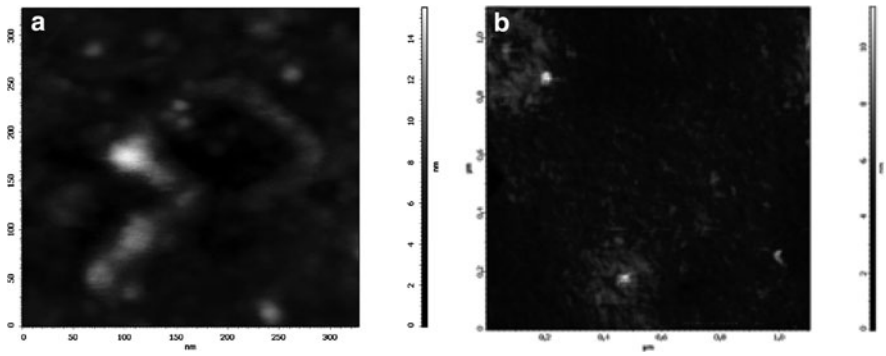


Fig. 2.7 AFM topography image of (**a**) FIB adsorbed on a-C:H thin film at room temperature and (**b**) at 116°C

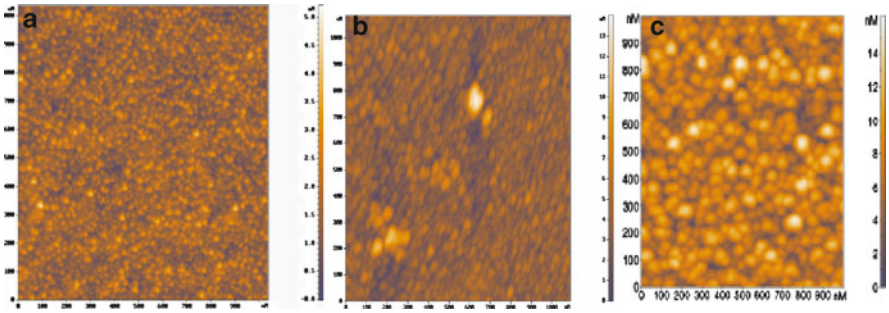


Fig. 2.8 AFM topography images of (**a**) a-C:H (floating) thin film as deposited (scan size:1 mm ×1 mm), (**b**) HSA on a-C:H (floating) thin film after 5-min incubation time, and (**c**) HSA on a-C:H (floating) thin film after 10-min incubation time

Fig. 2.7. It is obvious that the Fib molecule at this temperature possibly change from a linear to a folded conformation, due to the temperature effect [56].

In the case of human serum albumin, a heart-shaped blood plasma globular protein with dimensions 8 × 8 × 3 nm plays an important role in inhibiting the thrombus formation; it seems that in general, aggregates are formed which finally coalesce. More precisely, it was observed that initially, the protein molecules form clusters (5 min), the size of which increases with time (10 min), and the surface of the thin film is partially covered, as can be concluded by the topography images of AFM, shown in Fig. 2.8a–c. The surface of a-C:H film (floating type, without ion bombardment during deposition) without proteins appears to have grain-like surface features, the size of which is around 20–30 nm (Fig. 2.8a). In Fig. 2.8b (protein incubation time 5 min), it is clearly seen that protein aggregates exist. Typical height is around 8 nm (once or twice the height of one HSA molecule). The protein molecules tend to spread laterally, rather than forming "hills". Figure 2.8c

shows that in 10 min of incubation time, there are larger protein clusters and the surface is partially covered. Finally, the protein islands coalesce, and the surface of the thin films is totally covered by the proteins [54, 55].

2.3.3 AFM Implementation for Imaging Platelets and Erythrocytes

AFM for imaging platelets: Blood platelets are implicated in a series of cellular recognition and adhesion phenomena (adhesion to the subendothelial matrix and platelet aggregation), which are key events in the processes of hemostasis and thrombosis. They also play an important role in blood–material interactions as their activation leads to thrombus formation and on their surfaces take place essential steps of coagulation cascade such as the conversion of prothrombin to thrombin; the activation of coagulation factors VIII, V, and XIII; and subsequent formation of fibrin polymers synthesized by activated monomers. Platelets undergo a change in their shape which exposes a phospholipid surface for the coagulation factors that require it and they also release agonist compounds of dense and alpha (a)-granules to attract and activate additional platelets and leucocytes, promoting the growth of thrombus. During their *activation*, there is an increased membrane expression of receptors such as glycoprotein GPIIbIIIa, which is the receptor for fibrinogen and von Willebrand factor, leading to the linkage of adjacent platelets via fibrinogen. This receptor is a macromolecular complex, made of two transmembrane glycoproteins, GPIIb and GPIIIa, assembled into a heterodimer whose conformation depends upon the binding of calcium (Ca^{2+}) to GPIIb.

Platelet aggregation is a model of homotypic cellular adhesion. It is mediated by the binding of bifunctional molecules of fibrinogen to the plasma membrane of adjacent platelets, following stimulation of the platelets by agonists such as ADP, thrombin, or collagen, with the fibrinogen serving as an intercellular glue [57].

Therefore, one of the key aspects of hemocompatibility is the platelets' response to the biomaterial, and their morphological changes as index of activation are of crucial importance [58].

Platelet adhesion on biomaterials is a multiparametric process controlled by (1) surface properties of biomaterials such as topography, stoichiometry, electrical properties, wettability, and surface free energy [59]; (2) protein adsorption to interfaces determined by their structural properties like a-helices, b-sheets, and protein charge, and strength of intermolecular bonds; (3) conditions of the biological environment, e.g., pH, temperature, electrolytes, and ions concentration [60]; (4) the intermolecular interactions such as van der Waals, chemical, and electrostatic ones [61]; and (5) platelet bonding groups, specific cell receptors, cell surface charge, sialic acid content, active modification of cellular glycocalyx, and thermodynamic conditions [62].

Since proteins and cells range in size from nano- to micrometer, these relevant length scales should be observed by an imaging tool having the ability to observe

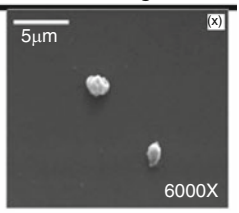
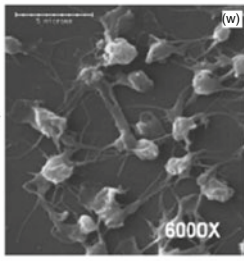
and manipulate molecular and atomic level features and can be used for high-resolution and real-time studies of bio and non-biointeractions. Until now, the platelet studies were based on conventional imaging methods, such as scanning electron microscopy which requires special preparation of the cells with gold and does not guarantee their viability. Going beyond the state-of-the-art techniques for cells' visualization, AFM enables platelet observation in a native, unlabeled state for several hours without damage [63, 64]. It is an important tool for probing both the structural and the kinetic properties of biological samples, and it can be used as a nondestructive, imaging nanoscale technique for platelets' response, and *thrombogenicity* assessment. The knowledge of basic cell–material interaction and an understanding of on-going processes at the cellular level can aid in the development of new biomaterials with prescribed and regulatable properties [65,66]. Therefore, there is a need for accumulation of data covering the interrelationship of physicochemical phenomena at biomaterial interfaces and their hemocompatible properties.

Preliminary studies on the development of a new methodology for AFM observations of human platelets adhering onto case study materials such as a-C:H and titanium nitride nanocoatings were made as presented in [67–69]. Platelets were derived from human platelet-rich plasma (PRP) after an appropriate centrifugation of whole blood drawn from healthy donors who did not take aspirin, clopidogrel, or other medicine that may affect platelet function. In these studies, the main characterization technique that was applied is AFM in semi-contact mode, to provide topographical information of carbon films at a resolution far superior to that of optical methods and visualization of platelets during adhesion.

Platelets in their resting stage are disc shaped with a size of 0.5–3 μm in order to resist blood pressure. When platelets first encounter a surface, their activation takes place involving the (a) flattering of their shape, (b) composition of pseudonucleus, due to the gathering of their granules into the center of the cell (transforming into egg-like type cell), and (c) polymerization of actin filaments and the formation of pseudopodia. Platelets then progress through a series of pseudopodial shapes characterized by an increase in area of contact with the substrate and the growing of extended, flattening, pseudopods [70]. This procedure normally ends in coagulation, thrombus formation, and embolization. In Table 2.1, the morphology description of platelets during activation is given, and in the corresponding SEM images of platelets, it can be noticed that first, platelets are in an unactivated stage when they come in contact with the biomaterial and second, the cells are in a highly activated stage, connecting with each other via pseudopodia.

All these morphological alterations of platelets during activation were taken into account for the analysis of AFM images. Stages of *platelet activation* are illustrated in Fig. 2.9a–c. Especially, in Fig. 2.9a, the platelet has been activated after 15-min adhesion on a-C: H thin film and it lost its discoid shape and became spherical [65], whereas in Fig. 2.9b more activated platelets have egg-like type structure forming filopods. Figure 2.9c illustrates AFM images of highly activated platelets with pseudopodia that connect each other forming a network (pre-thrombus state).

Table 2.1 Morphology description of platelets during activation with the corresponding SEM images

Type	Morphology description of platelets during activation	SEM images
I	Round and discoid – no pseudopodial	
II	Dendritic – early pseudopodial with no flattening	
III	Spread-dentritic – intermediate pseudopodial with one or more pseudopodia flattened, no hyaloplasm between pseudopodia	
IV	Spreading – late pseudopodial with hyaloplasm spreading	
V	Fully spread – hyaloplasm fully spread with no distinct pseudopodia	

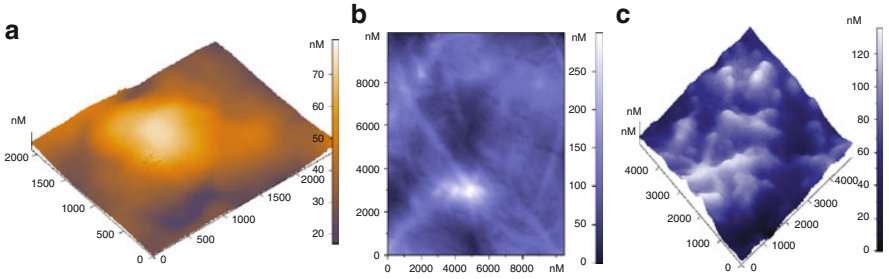


Fig. 2.9 AFM topography images of (**a**) inactivated platelet, (**b**) platelets at a later stage of activation forming filopods, and (**c**) highly activated platelets with pseudopodia connecting with each other, onto nanomaterials

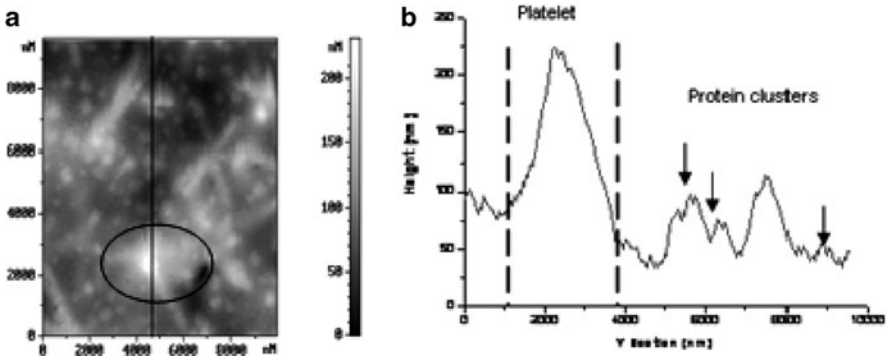


Fig. 2.10 (**a**) 2D AFM image of platelets on over-stoichiometric TiN_x thin film. An activated egg-like platelet with pseudopodia is illustrated in the circle and (**b**) Y section of Fig. 2.10a, showing in detail that the size of the activated platelet is 3,000 nm in length and approximately 150 nm in height

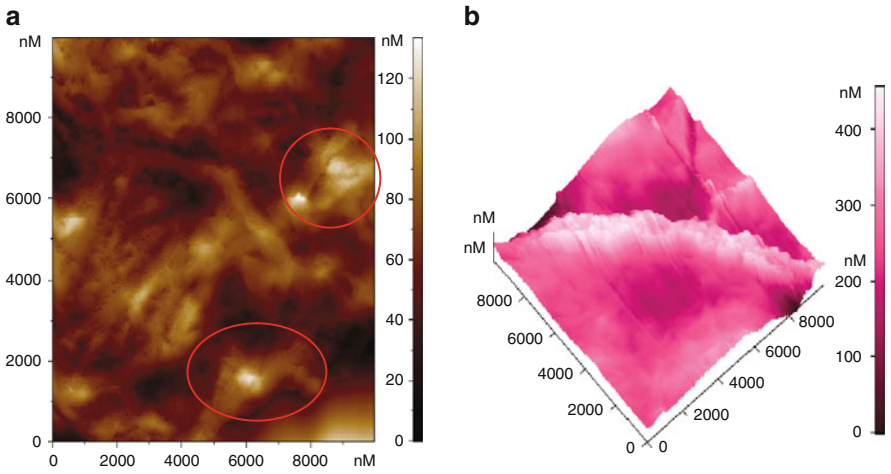


Fig. 2.11 (**a**) 2D AFM image of activated platelets (at a late pseudopodial stage with hyaloplasm spreading) on over-stoichiometric TiN_x thin film after 1-h incubation, (**b**) 3D AFM image of activated platelets forming cluster-like island after 2-h incubation on the same biomaterial [67]

For cell adhesion studies, AFM can provide information with nanoscale precision, as indicated, for instance, by performing a Y section crossing an activated platelet with pseudopodia and the plasma protein clusters (Fig. 2.10a–b).

An AFM image of activated platelets (encircled) in the late pseudopodial stage with hyaloplasm spreading adhered onto over-stoichiometric titanium nitride (TiN_x) thin films (1 h incubation) is shown in Fig. 2.11a.

After two hours of incubation, the platelets form clusters like island, as depicted in Fig. 2.11b.

By accumulation of quantitative and qualitative data (variations in time-dependent platelets surface roughness and AFM images, respectively), the platelets response toward biomedical nanocoatings can be in-depth analyzed and correlated with the surface properties of the biomaterials.

Previous studies at our laboratory showed that platelets response toward carbon- and titanium-based nanomaterials (developed by magnetron sputtering technique) is in strong correlation with their surface nanotopography, stoichiometry, electrical properties, surface free energy, and wettability [55, 59, 65, 67–69]. The growth variables can control and lead to favorable physicochemical surface properties of the nanomaterials for desirable thromboprotectivity [71–73]. Repulsion of platelets is an essential element for implants that come into contact with blood, such as stents, cardiac valves, and grafts.

AFM for imaging platelet-thrombospondin: *Thrombospondin* (TSP) is a multi-functional glycoprotein that has many features in common with ECM constituents located in the a-granules of human platelets. It is known to mediate platelet adhesion and upon thrombin stimulation, TSP is secreted from the a-granules and binds to the activated platelet surface in the presence of calcium. Recent studies strongly suggest that the specific binding of fibrinogen to the platelet membrane glycoprotein HIb/IIIa complex plays a crucial role in the platelet aggregation process. TSP serves to stabilize fibrinogen binding to the activated platelet surface and reinforces the strength of interplatelet interactions. It is proposed that platelet aggregation is a dynamic, multistep process, governed initially by the platelet membrane glycoprotein Ilb/Illa–fibrinogen interaction, with the TSP–fibrinogen interaction playing an important role in determining the size and reversibility of platelet aggregates [74]. TSP is also synthesized, secreted, and incorporated into the ECM by a variety of cell types, including endothelial cells and smooth muscle cells. Electron microscopy of tungsten replicas of thrombospondin reveals a tripartite structure resembling a "bola" which is about 60 nm across when fully extended [75].

In Fig. 2.12a, TSP clusters within the circle (with the size of 200–300 nm) on a-C:H thin films and the relevant X section crossing the cluster are shown at nanometer scale. After 1 h of PRP (from healthy donors) incubation, we can see the activated platelets with egg-like type structure and pseudopodia (Fig. 2.12b). After 2 h, as depicted in Fig. 2.12c, TSP enhances the formation of a fibrin network, which is essential for the development of a clot.

AFM for visualization of red blood cells: The human body's cells have a diversity of shapes highlighting the significance of physical properties in biological function. For example, human erythrocytes are discoid shape cells in order to avoid filtration in the spleen, whereas platelets are disc like in shape to assist their function of adhesion and rolling on vascular endothelium.

Under different osmotic states, for instance in hypoosmotic conditions, *AFM* shows that human red cell loses its discoid shape and becomes spherical and slightly swollen. In Fig. 2.13a, the AFM topography image shows that in the hypoosmotic state the enlarged human red cell has dimensions of 9.3 nm × 8.9 nm. As the lipid bilayer of the red cell membrane is permeable to water molecules, water diffuses into the cell, driven by the different osmolarity in and out of the cell, causing the

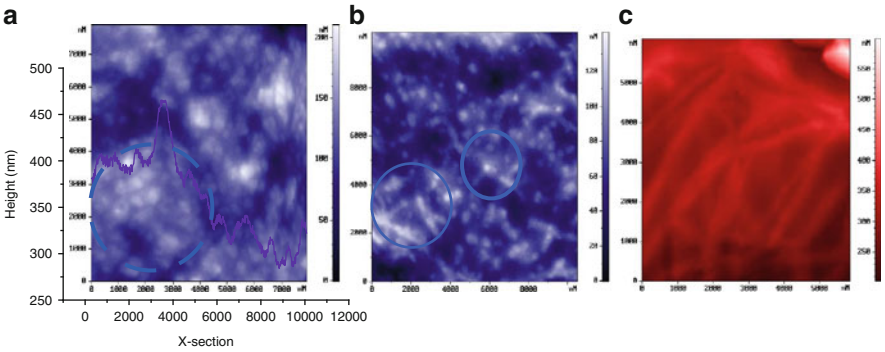


Fig. 2.12 AFM topography images of (**a**) thrombospondin cluster with the size of 200–300 nm, (denoted with circle) onto carbon thin film and the corresponding X section crossing the cluster, (**b**) activated platelets (encircled) with egg-like type structure and pseudopodia, after 1 h of platelet-rich plasma incubation, and (**c**) fibrin network provoked by thrombospondin in platelet-rich plasma onto carbon biomaterial

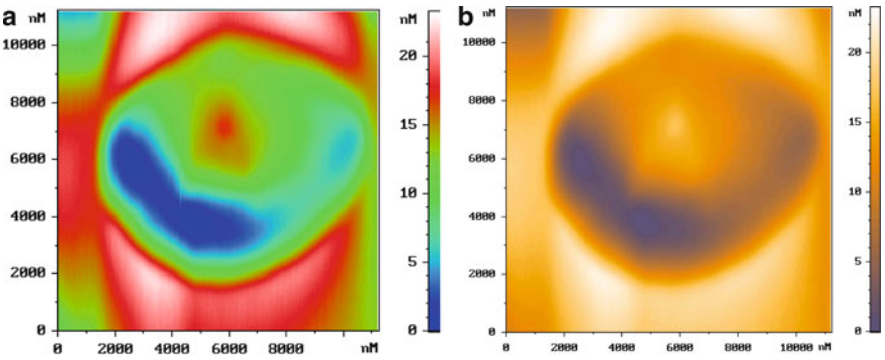


Fig. 2.13 (**a**) AFM topography of human red cell on carbon substrate, under hypoosmotic conditions. Its shape becomes spherical and it is enlarged due to the water diffuse into the cell, with dimensions of 9.38 nm × 8.9 nm, and (**b**) zooming into the swollen red cell that is presented in Fig. 2.13a

erythrocyte volume to be increased. This finding is consistent with an almost perfect adaptation of the erythrocyte cells to the *hypoosmolality*.

Thus, in Fig 2.13b, it can be observed that in the center of the nuclear red cell, there is a white area denoting the accumulation of water, in contrast to the darker surroundings which represent the cytoplasm filled with hemoglobulin.

Overall, AFM enables the systemic, in situ characterization of the mechanisms governing red cells' structural differentiation under different osmotic states and platelets morphological adaptations during intact contact with biomaterials, at or below the nanoscale. It is an important tool for elucidation of cellular interactions with the biomaterials and the provided data will aid in the development of highly biocompatible implants.

2.4 Nanotoxicity

While nanoscale materials possess more novel and unique physicochemical properties than bulk materials, they also have an unpredictable impact on human health.

Such unfavorable effects of nanomaterials on human physiology have triggered the genesis of a new scientific discipline known as "*nanotoxicity*," referring to the toxicity that is caused by the interaction between the host organism and the nanomaterials.

Nanotoxicity has been the reason for the establishment of *nanotoxicology* which is the scientific field related to the study of the interaction of nanostructures with the host organism with emphasis on understanding the relationship between the physical and chemical properties including size, formation, surface chemistry and aggregation of nanoscale constructs with the stimulation of toxic biological responses [76].

Nanoscale materials could have a toxic impact on host organs and cells mainly because of three reasons. First, studies have shown that bulk materials have specific electronic, optical and magnetic properties related to their physical dimensions and the reduction in size at nanometer scale may lead to different physicochemical properties and potential toxic effects [77]. Second, the surfaces of particular nanoconstructs are known to play a significant role in many catalytic and oxidative reactions [78]. The toxicity caused by the nanosurfaces would be much higher due to the fact that the surface to volume ratio for nanoscale materials is much greater and so are the toxic side effects [79]. Third, a number of nanomaterials contain metals or other chemical compounds with known toxicity and as an outcome, their breakdown could elicit similar toxic responses to the components themselves [80].

Nanomaterials can gain access to the blood stream via inhalation or ingestion [81]. At least a few nanomaterials can penetrate the skin [82], whereas a broken skin is an ineffective particle barrier [83]. Generally, the behavior of nanomaterials could be summarized as follows: The nanostructures can enter the host organism via six different access points including venous, dermal, subcutaneous, inhalation, intraperitoneal and oral [84]. Then the nanomaterials interact with biological components mainly cells and proteins and as an outcome their absorption occurs. Later, the absorbed structures can be transported to various host organs including the brain, heart, liver, kidneys, spleen, bone marrow and nervous system [85] and may retain their own formation and characteristics, or be modified, or even metabolized [77]. Finally, they cross the cellular membranes and move into the cytoplasm of the cells that form the organ and reside there for an uncertain period of time before leaving so as to infect other organs or it is reformed to be excreted out of the host organism.

The extremely small size of nanomaterials allows them to enter human cells. Different studies showed a fast uptake of nanomaterials into cells in vitro, and their uptake depends on the surface of the particles and is only limited by the *transport* of the material to the cell. Unlike larger particles, nanomaterials may be taken up by cell mitochondria [86] and the cell nucleus [87]. Studies demonstrate the

potential of nanomaterials to cause DNA damage [88]. Nanostructures could enter into the nucleus via transport through the nuclear pore complexes, diffusion through the nuclear membrane itself, or may be enclosed into the nucleus by chance as a result of the fact that the nuclear membrane and nucleus is degraded during cell division procedure then is reformed in each daughter cell. No matter which way the nanostructures use, they may enter the nucleus and damage DNA either directly or indirectly. Interacting directly with the DNA molecules or DNA-related proteins causes physical damage to the genome. Indeed, it has been shown that nanoparticles of titanium dioxide and silica can enter the nucleus [89, 90] where they cause protein aggregates that are responsible for the shutdown of replication, transcription, and cell proliferation procedures [91]. On the contrary, quantum dots have been found to interact with the pore of the nuclear membrane and the histone proteins. Indirectly, *DNA damage* may occur without requiring a physical interaction with the DNA molecule. What is required here is interaction with other cellular proteins like the ones involved in the cell division process. Furthermore, the nanomaterials are capable of stimulating other cellular responses that, in turn, lead to genotoxicity including oxidative stress, inflammation, and aberrant signaling responses [92].

In addition, a few nanoparticles induce major structural damage to mitochondria, even resulting in cell death [86, 89]. Such materials have proved toxic to human tissue and cell cultures, resulting in increased oxidative stress, inflammatory cytokine production, and cell death [93]. Furthermore, it seems that a sudden increase in the permeability of the inner mitochondrial membrane occurs, causing loss of mitochondrial functionality and finally promoting the cell necrosis procedure [94] (Fig 2.14). Thus, how exactly the nanoparticles behave in the cell and interact with its components is still a major question that needs to be resolved.

A number of studies have shown that nanostructures, especially particles, can strongly interact with cell membranes. This interaction is achieved either by adsorption of them onto the cell surface or by compromising the membrane integrity to result in the formation of holes, membrane thinning, and lipid peroxidation [86].

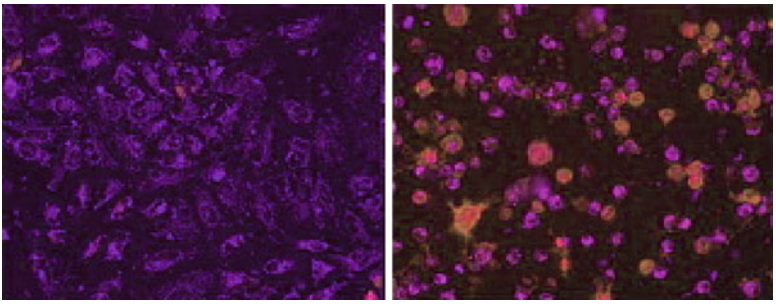


Fig. 2.14 Fluorescent microscopy images showing the mitochondrial damage that can be caused to cells after incubation with gold nanoparticles. (*Left*) Untreated cells, (*Right*) Cells after incubation with gold nanoparticle solution. The stain shows cells with increased mitochondrial membrane permeability and destabilized cellular membrane

Recent papers report in vivo and in vitro [87, 95] effects of *NPs* on membrane stability and pore forming. The term hole or pore can refer to a wide range of structural changes that could lead to enhanced permeability ranging from the formation of an actual hole in the membrane to more subtle changes in content of the membrane, leading to enhanced diffusion. Different mechanisms of permeabilization of cell membrane by nanoparticles have been proposed [14]. *Membrane permeability* could arise from a reduction in density of the plasma membrane. In this case, a hole or pore corresponds to a region of reduced material (e.g., lipid, protein, and cholesterol). Until recently, the biophysical behavior of lipid membranes was not of fundamental importance in toxicological studies. This is rapidly changing with the emergence of nanoparticles and questions regarding their safety. Concerns about cell plasma membrane disruption resulting in toxic effects are also paramount in the minds of nanoparticle designers focused on nanoparticle applications. A convenient system for the study of the effect of various substances on membranes is artificial phospholipid membranes, which can be readily obtained by forming phospholipid vesicles in water solution. As the phospholipid bilayer is the basic constituent of the cell membrane, it is believed that the cell membranes and phospholipid membranes share some important features, making it an ideal model for investigating the toxic effects caused to cell membranes due to interaction with nanostructures. In Fig. 2.15, fluorescent microscope images of hepatopancreatic cells after incubation with nanoparticle solution are presented. The nanoparticles caused changes in cell membrane integrity that resulted in differences in cellular permeability to the dye allowing its entrance to the cell and attachment to the nuclei [88].

According to Nel et al., the impact of *nanoparticle interactions* with the body is dependent not only on their size, but also on their chemical composition, surface structure, solubility, shape, and how the individual nanoparticles amass

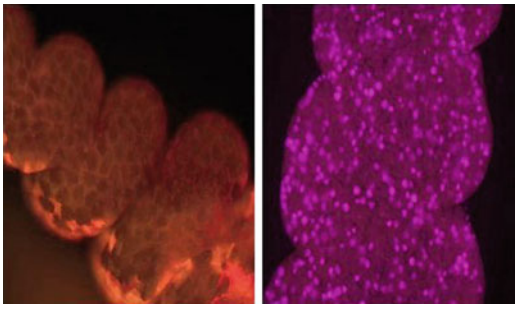


Fig. 2.15 Fluorescent microscope images of hepatopancreatic cells after incubation with nanoparticle solution. (*Left*) Untreated cells (*Right*) Cells after incubation with nanoparticle solution. The nanoparticles caused changes in cell membrane integrity that resulted in differences in permeability of cells to the dye. The stain shows cells with destabilized membranes through which the stain entered the cell and finally attached to the nuclei

together [96]. Nanoparticles may modify the way cells behave, and potential routes of exposure include the gastrointestinal tract, skin, and lungs.

In principle, a large number of particles due to their small size could overload the body's phagocytes, thereby triggering stress reactions that lead to inflammation and weaken the body's defense against other pathogens. Even more, if the sizes of NPs are too small (a few nanometers), they may not be able to be recognized by phagocytes, leading to unknown undesirable effects.

In addition to this, other questions that arise are about what happens if non-degradable or slowly degradable nanoparticles accumulate in human organs and cross biological barriers. *Nanoparticles* have much larger surface area to unit mass ratios which in some cases may lead to greater pro-inflammatory effects (in, for example, lung tissue). Maynard [97] reports that "certain nanoparticles may move easily into sensitive lung tissues after inhalation, and cause damage that can lead to chronic breathing problems."

Some nanoparticles seem to be able to translocate from their site of deposition (e.g., skin) to distant sites such as the blood and the brain [82]. The biodegradable NPs are dissolved and their excretion is made via kidneys or intestines, whereas the nonbiodegradable NPs may accumulate in the liver. This has resulted in nanoparticle toxicologists studying the brain, blood, liver, skin, and intestines, besides lungs.

Recently, efforts focused on the in vivo *biodistribution* of nanostructures have been made in order to estimate the quantity of nanostructures that aggregate at different time points and at different concentrations during specific medical applications. In studies where quantum dots were used as a probe in order to detect the distribution of single wall CNT (SWCNT), it was shown that the amount of SWCNT that accumulated in the liver was in relation with the modifications to which the surface of the nanotubes was subjected [85]. Organs such as spleen, lymph node, and bone marrow can absorb nanostructures as well. All of them contain high levels of macrophages that could possibly transfer the nanostructures into the cells via the phagocytic pathway.

In the context of risk assessment, novel size-dependent properties that influence nanoparticle reactivity are likely to affect nanoparticle hazard. Their greater chemical reactivity results in increased production of reactive oxygen species (ROS), including *free radicals* [98]. The presence of active sites of nanoparticles that are able to generate ROS and arise from size-dependent differences in atomic and electronic structure suggests one possible origin of size dependence in toxicity. Several authors have shown that ROS generation is involved in the toxicity of nanoparticles such as CeO_2, TiO_2, and Fe_3O_4 [99].

The ROS and free radical production is one of the primary mechanisms of nanoparticle toxicity; it may result in oxidative stress, inflammation, and consequent damage to proteins, membranes and DNA [99]

CNT are frequently linked to asbestos, due to their needle-like fiber shape. Poland et al. reported that CNT introduced into the abdominal cavity of mice demonstrated that long thin CNT showed the same effects as long thin asbestos fibers, raising concerns that exposure to CNT may lead to mesothelioma (cancer of the lining of the lungs caused by exposure to asbestos) [100].

Given these risks, understanding if and under what circumstances CNT may be harmful is of growing interest. Kostarelos et al. reported that functionalized CNT display low *toxicity* and are not immunogenic, and as a result such systems hold great potential in the field of nanobiotechnology and nanomedicine [101]. Recent findings suggest that the extent to which CNT are biodegraded may be a major determinant of the scale and severity of the associated inflammatory responses in exposed individuals [102].

Nanostructures can stimulate the *immune system* by inducing inflammation, immune response, and allergic reactions, or even affect the immune cells in a deleterious or beneficial way. Furthermore, nanoparticles can enter into the human body and interact with components of the immune system. This interaction leads to enhanced release of different biological agents including mainly pro-inflammatory and inflammatory cytokines. The use of different types of biomaterials may have a unique impact on the host immune system which, in turn, produces a different response in every case. For example, cobalt and nickel nanoparticles provoke inflammation, while crystals of hydroxyapatite stimulate tumor necrosis factor secretion from macrophages which leads to an increase in the number of activated phagocytes. Nanoparticles used in cosmetics and skin care products are found to be responsible for low systemic dermal toxicity. In addition, carbon nanoparticles stimulate allergic responses, while fullerene seems to mollify such responses. Thus, nanoparticles have the potential to cause either stimulatory or suppressive alterations to the profile and behavior of the host immune system. However, the above alterations are undesirable and as a result, successful nanoparticle medical applications are preferred to avoid them. The interaction of NPs with the immune system affects their biodistribution and increases the exposure time [103].

Another important issue in nanotoxicity is the *hemocompatibility* assessment of nanomaterials involving protein adsorption, blood cells, and cytotoxicity studies. Their combination with the evaluation of coagulation cascade activation is under rigorous research [104]. The surface nanotopography, stoichiometry, and chemistry of the nanomaterials for biomedical applications, as well as their electrical and other physicochemical properties, are found to affect their biocompatibility and fate as soon as they enter the human body [51, 55, 71].

More specifically, the first step in the interaction between blood and the biomaterial is the adsorption of blood plasma proteins with upcoming activation, denaturation, and/or desorption. This is not something unfavorable, but however this procedure could be followed by thrombogenesis due to platelet adhesion and activation with simultaneous release of cytokines and other proteins that finally stimulate platelet aggregation and formation of thrombus. Moreover, biomaterials could also cause hemolysis, leucocyte adhesion, spreading, and activation with further involvement of the inflammatory and complement mechanisms which could trigger the host immune system against the implanted biomaterial, which might result in the rejection of the implant itself. So it is important before using a material for medical applications to test its *hemocompatible* characteristics. This requires using a number of techniques in order to examine the interaction between the biomaterial and a model system with one or few proteins before attempting to study

whole plasma or blood interactions with biomaterials directly. Overall, the three key elements of the toxicity screening strategy should include the physical and chemical characterization of nanomaterials, tissue cellular assays, and animal studies. An understanding of nanotoxicity is essential as it could also lead to the harnessing of nanomaterials to be used for targeted therapeutic approaches.

2.5 Future Challenges and Perspectives

Modern medicine is rapidly reaching a crossroad where increased knowledge of how the human body and disease pathologies work at the molecular level is combined with the ability to manipulate materials at the nanoscale. The evolution of the concept of nanomedicine and its applications in clinical practice are described as the natural culmination of millennia of medical history.

By enabling nanotechnologies, such as nanoparticles, nanomaterials, CNT, and nanoscale tools and processes applied for targeted drug delivery, regenerative medicine, advanced diagnostic agents, and biomaterials, nanomedicine aims to treat degenerative and cardiovascular disorders, type-2 diabetes, and cancer. Undoubtedly, these technological advances will be fostered by the knowledge gained from the observations of natural events at nanoscale, leading to highly performed implants.

A few of *nanomedicine goals* are to provide much earlier and more accurate diagnosis, use far less invasive procedures, target smaller doses of drugs to specific delivery sites, and develop new paradigms of treatment where damaged tissues or even organs can be regenerated. Towards applying nanomedicine for patients' benefits, the efficacy of nanomaterials and their potential nanotoxicity should be addressed, to enhance their biological performance.

2.6 Summary

This chapter highlights some of the most recent nanomedicine-based approaches and tools that have been utilized to diagnose and treat human diseases, providing solutions applied in medical clinical practice.

It presents an overview of the main nanomedicine pillars and especially of nanovehicles for targeted drug delivery and diagnostic purposes as well as the principles of regenerative medicine for addressing unmet medical needs. The feasibility of nanotechnology applied for the monitoring of bio and non-biointeractions at nanoscale is described, via the utilization of nanotools for protein adsorption and blood cells visualization. Several experiments are employed to help the reader to understand the nano world of proteins and cells and their behavior towards biomaterials.

The extensive application of nanomaterials for human use poses a toxicity risk to human health and environment. Possible undesirable results of these capabilities and the drawbacks of nanomedicine are presented, mostly focusing on nanotoxicity.

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Chapter 3
Biofunctionalization of Surfaces with Peptides, Proteins, or Subcellular Organelles: Single-Molecule Studies and Nanomedical Approach

A. Katranidis and T. Choli-Papadopoulou

Abstract Immobilization of biologically active proteins and enzymes on surfaces is very important for the production of biofunctionalized surfaces for applications in medicine such as biosensors and in the diagnostics field. There are various approaches to immobilize and control the release of peptides/proteins from different surfaces. The identification of successful techniques to functionalize a particular material is a challenge. On the other hand, biomaterials are at the moment of great benefit for medicinal purposes and a lot of knowledge from different fields is required in order to design biomimetic scaffolds or biomimetic materials. The used methodologies are different for different materials and are mainly based on the special chemistry of the surfaces. Peptides with distinct properties are desired instead of entire proteins. However, in some cases, proteins cannot be replaced by peptide segments and therefore biochemical knowledge, such as in protein and/or genetic engineering is required.

3.1 Introduction

There are various approaches to immobilize and control the release of peptides/proteins from several surfaces. The identification of successful techniques to functionalize a particular material is a challenge and is of great significance in designing

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grafts to enhance, for example, endothelialization. Each protein/peptide attachment technique needs to be tailor made, considering the material, protein/peptide of interest, and also the anticipated interaction/delivery of the attached moiety via appropriate spatiotemporal kinetics. It is crucial to select suitable modification techniques, as unoptimized material surfaces can promote a number of undesirable effects, (e.g., free radical formation, inflammatory response, fibrous encapsulation, etc.) and lead to graft failure.

Immobilization of proteins and enzymes in their biologically active state on surfaces is very important for the production of biofunctionalized surfaces for bioapplications, biosensor chips [1], diagnostics [2], enzyme reactors [3–6], protein and peptide microarrays [7–9], and nanomedical applications [10].

Proteins, unlike DNA, are known to be susceptible to loss of activity upon immobilization on surfaces due to unfolding processes [11]. Therefore, knowledge of the conformation, orientation, and specific activity of proteins bound to surfaces is crucial for the development and optimization of highly specific and sensitive nanodevices.

Many investigations reported so far used unspecific adsorption or unspecific covalent coupling techniques, resulting in the analysis of ensembles of surface-bound proteins that are likely to exhibit a range of different conformations and orientations. When immobilizing proteins via functional groups that are not site specific, such as amino and carboxyl groups, the orientation of a protein molecule on the chip surface will be random. Furthermore, if the site of attachment is close to the active site of an enzyme molecule, its activity may be partially or totally lost due to steric effects. This is one of the reasons for decreased activity that is often observed when proteins are chemically immobilized on surfaces [12].

Efforts have been made toward oriented attachment of proteins on surfaces. Such an oriented immobilization strategy is believed to reduce steric hindrance and improve the accessibility of the active site of enzymes or binding site of receptors as well as the overall stability of proteins [13].

The key element to achieve controlled orientation is to attach the biomolecule selectively at a predetermined site on the protein surface. By protein engineering or genetic engineering methodologies, it is possible to modify the protein of interest on the DNA level to introduce a specific functionality. Beside point mutations at specific amino acids, the fusion of specific tags before the N-terminal or after the C-terminal region of the studied protein is also commonly used, in order to enhance structural stability or function. These additions endow the protein with “extra attachment possibilities” without disturbing its functional conformation.

3.2 Surface Treatment for Specific Immobilization of Proteins

In single-molecule experiments, immobilization of proteins on surfaces is essential for various biotechnological applications. To achieve specific binding of proteins, surfaces have to be modified toward specific biological recognition, whereas at

the same time avoiding uncontrolled adsorption that could lead to denaturation of proteins or unwanted activation of biological processes. The interaction between the protein under study and the surface environment should be minimal so that the results may reflect intrinsic properties of the protein, and not artifacts due to surface interactions [14].

Polyethylene oxide (PEO) surfaces have been especially recognized as biocompatible and resistant to protein adsorption due to the hydrophilic but uncharged nature of the polymer. The surfaces are glass slides, which are initially amino-functionalized with the use of an aminosilane. PEO solution is formed by mixing polyethylene glycol (PEG) with a small amount of biotinylated PEG and reacts covalently with the amino-functionalized surface via a *N*-hydroxysuccinimidyl ester (NHS ester). The surface is active only via the small amount of biotinylated PEG which can be bound to streptavidin.

Streptavidin is a tetrameric protein with four binding sites, which is structurally similar to avidin. The interaction between streptavidin and biotin is one of the strongest noncovalent bonds in nature ($Ka = 10^{14}$ instead of 10^{7}–10^{11} for antibody–antigen interaction). The strength of the bond as well as the small size of biotin (MW = 244.3) ensures an ideal system for affinity binding with numerous applications. Because streptavidin lacks any carbohydrate modification and has a near-neutral pI, it has the advantage of much lower nonspecific binding than avidin. After binding to the biotinylated PEG of the surface, streptavidin still has three binding sites, where site-specifically biotinylated proteins can bind under the same orientation.

3.3 In Vivo Protein Biotinylation

The numerous applications of the high-affinity interactions between biotin and streptavidin in biochemistry, immunology, cell biology, and biotechnology obscure the fact that biotin is a vitamin (vitamin H) essential for all life forms. It is used as a coenzyme and it is covalently attached at the active site of certain enzymes (carboxylases) that transfer carbon dioxide from bicarbonate to organic acids to form cellular metabolites. The reaction that attaches biotin with these enzymes is extremely specific [15]. Biotinylation is a posttranslational modification accomplished by the BirA enzyme in *E. coli* [16]. The attachment of biotin is a two-step reaction that results in the formation of an amide bond between the carboxylic group of biotin and the ε-amino group of a lysine. The shortest substrate that can be in vivo biotinylated by BirA is a 14-mere (G-L-N-D-I-F-E-A-Q-K-I-E-W-H) [17] (Fig. 3.1).

Nowadays, there are available systems for in vivo protein biotinylation (Fig. 3.2). *E. coli* strain AVB101 carries a plasmid with the gene of BirA (pBIRAcm) and can also be transformed with a second plasmid (pAN5), which carries as a tag the nucleotide sequence of the 14-mere that can be in vivo biotinylated. The gene of

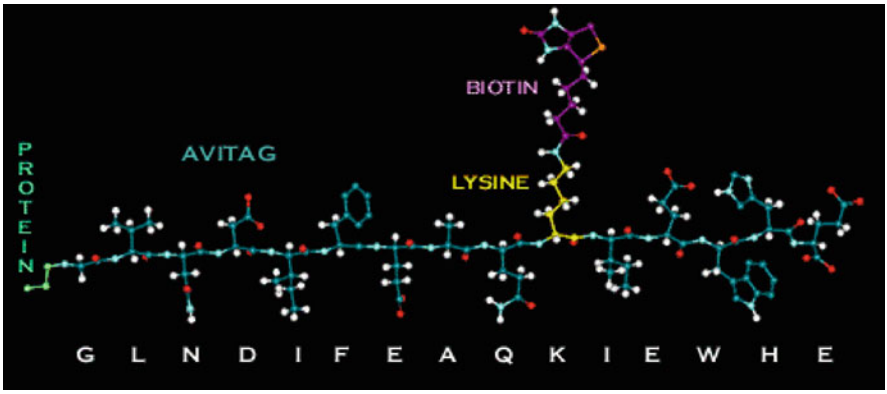

Fig. 3.1 14-mere that can be in vivo biotinylated by BirA and is used as a fusion tag by in vivo protein biotinylation systems

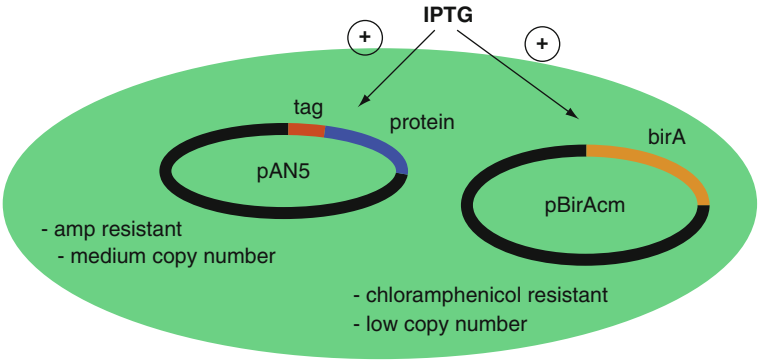

Fig. 3.2 Strain AVB101 carrying the two plasmids. Gene of BirA is shown in *orange*, gene of protein to be biotinylated in *blue*, and the tag in *red* (http://www.avidity.com)

the protein to be biotinylated can be cloned directly after the tag. Thus, the fusion protein is in vivo biotinylated in the N-terminus by the BirA enzyme (http://www.avidity.com).

3.4 Single-Molecule Studies of Protein Folding

After biosynthesis, proteins fold into a three-dimensional structure in order to become biologically active. Protein folding is a heterogeneous process, where a large number of possible pathways can lead from the unfolded to the native folded conformation. Single-molecule studies can provide direct evidence on the folding pathways of a protein, but in order to study folding intermediates, it is necessary to immobilize the proteins.

Reversible folding and unfolding of the protein Ribonuclease H (RNase H) were demonstrated after immobilization of the protein on PEO surfaces. Biotinylated RNase H was labeled with a fluorescence resonance energy transfer (FRET) pair of fluorescent dyes and tethered to the surface by a biotin–streptavidin linkage [14].

3.5 Cotranslational Protein Folding Vs. Refolding

Cotranslational protein folding has been also studied intensively in the past [18, 19]. The major difference of cotranslational folding with respect to refolding of full-length polypeptide chains is the vectorial appearance of the nascent polypeptide chain, which in principle leads to a subsequent vectorial folding process. In addition, the growing nascent polypeptide chain remains bound to the ribosome during the folding process [20]. Significant differences have been observed between both types of folding with respect to folding rates, to the appearance of folding intermediates, and to the yields [21].

An important question is how cotranslational folding ensures more effective and productive folding than refolding from full-length polypeptides [20, 22]. First, during synthesis and protein folding, the elongating nascent chain remains anchored to the ribosome and has less rotational and translational freedom. This restriction can significantly reduce intermolecular collisions with other (in particular, also not folded) proteins and thereby suppress aggregation. Second, in contrast to refolding, folding of N-terminal and C-terminal regions is separated spatially and temporally during sequential synthesis. Most probably, this prevents nonnative and unwanted interactions that often lead to off-pathway folding intermediates or aggregates. Third, several chaperones (e.g., the trigger factor in bacteria) act already during translation on the nascent chain and prevent misfolding or aggregation. Although this process is only poorly understood, it is assumed that this type of chaperones is most efficient only in cotranslational folding [23].

In order to study cotranslational folding at a single-molecule level, it is necessary to immobilize ribosomes and synthesize a single protein that remains attached on the ribosome after synthesis. Single ribosomes, in vivo biotinylated on the L4 protein and labeled with the fluorescent dye Atto655, were tethered on a PEO surface via a biotin–streptavidin linkage. By using a cell-free in vitro transcription–translation system, the protein GFP Emerald (GFPem) was synthesized and remained attached on the surface-tethered ribosomes (Fig. 3.3).

The plasmid carrying the gene of GFPem was linearized prior to use in order to remove the stop codon. The sequence of GFPem was elongated by 31 additional amino acids at the C-terminus (spanning the full tunnel length) in order to ensure proper folding of the full-length protein outside the ribosomal tunnel, which hinders the formation of tertiary structures [24]. Suppression of protein release after synthesis keeps the synthesized GFP attached to the ribosome and allows imaging of GFPem fluorescence for extended observation times.

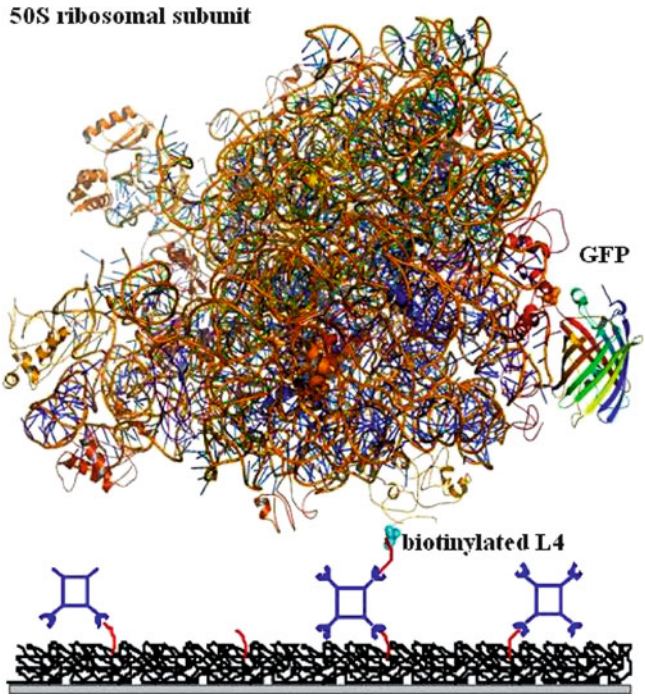

Fig. 3.3 Surface-tethered ribosome with synthesized GFP attached even after its synthesis. Biotin is shown in *red* and streptavidin in *blue*

In cases where the stop codon is missing, the molecule tmRNA initiates its function and induces protein release from the ribosome. Initially, it acts as tRNA and occupies the A-site of the ribosome, which is already attached to an mRNA, and forms a peptide bond with the polypeptide chain. Then it starts to act as an mRNA and codes for ten additional amino acids and a stop codon [25]. This way the protein is elongated by a sequence of ten amino acids and is released from the ribosomes because of the stop codon [26, 27]. Suppression of release was achieved by adding a short oligonucleotide with complementary sequence to the tmRNA, which anneals with it and blocks its function (Fig. 3.4).

Imaging of fluorescently labeled ribosomes (with Atto655) and emerging GFPem molecules was accomplished with the use of a dual color fluorescence wide-field microscope [28] (Fig. 3.5). Upon acquiring its final conformation, GFPem forms its chromophore, which consists of amino acids Ser 65-Tyr 66-Gly 67, and starts to fluoresce [30]. Studies in a time-resolved manner showed that the synthesis rate is close to that observed under in vivo conditions and that the co-translational folding and maturation occur relatively fast [31]. High rates of folding and maturation are assumed to play a crucial role in reducing unwanted side reactions, such as misfolding and aggregation, and thereby improving the efficiency of protein biosynthesis in the cell.

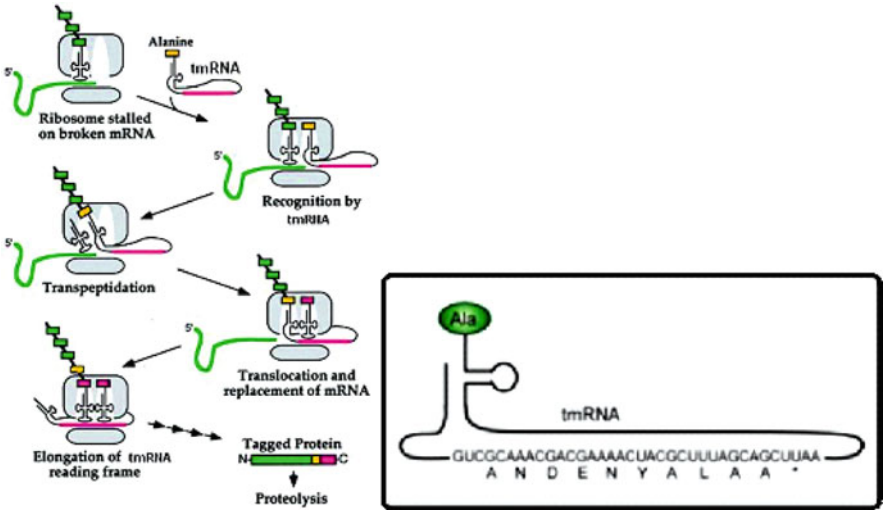

Fig. 3.4 Protein release from ribosome by tmRNA. It acts initially as tRNA and later as mRNA, replacing the damaged mRNA and inserting a sequence of ten amino acids (AANDENYALAA) and a stop codon (*inlet*) [26]

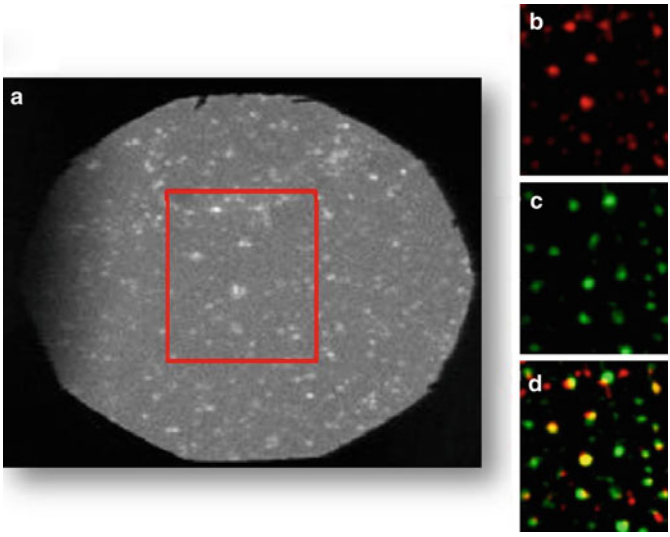

Fig. 3.5 Fluorescence wide-field images from single surface-tethered ribosomes. (**a**) The full screen of the red emission channel showing Atto655-labeled ribosomes (laser excitation at 640 nm with an exposure time of 2 s). (**b**) The red emission of ribosomes of a small selected area of image (**a**). (**c**) For the same area, GFP fluorescence emission is shown which was measured in the green channel after the transcription–translation reaction (laser excitation at 488 nm for 2 s). (**d**) The overlay of the red (ribosomes) and the green (GFP) channel demonstrates that single surface-tethered ribosomes synthesized GFP molecules which become mature (i.e., fluorescent) while bound to the ribosome. The *yellow* colored peaks in this figure localize the coexistence of single ribosomes and single GFP molecules bound to their synthesizing ribosomes [29]

3.6 Biofunctionalization for Nanomedical Applications

A large number of synthetic polymeric materials with various different properties are available for medical applications such as prostheses, implants, and tissue engineering matrices. Most of the common materials have sufficient mechanical stability and elasticity as well as desired stability toward degradation and are nontoxic. One important remaining problem is the interaction between polymer and cells.

Approaches to improve biomaterials include reduction of unspecific protein adsorption, enhancement of adsorption of specific proteins, and material modification by immobilization of cell recognition motives to obtain controlled interaction between cells and synthetic substrates [32–35].

3.7 The Biology of Cell–Cell or Cell–ECM Interactions

Cells are connected through a network known as the extracellular matrix (ECM). Many cellular processes involve interactions between the ECM and the cell. The ECM not only connects cells together in tissues, but also guides their movement during wound healing and embryonic development. Furthermore, the ECM relays environmental signals to cells. ECM proteins with great importance for cell adherence are collagens, laminins, and fibronectin (Fig. 3.6).

In multicellular organisms, contacts of cells with neighboring cells and the surrounding ECM are mediated by cell adhesion receptors. Among them, the *integrin* family comprises the most numerous and versatile group [41–45]. Integrins are receptors that mediate attachment between a cell and the tissues surrounding it,

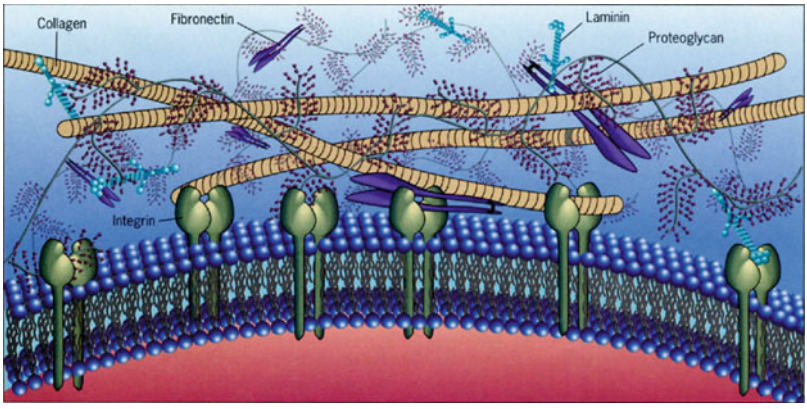

Fig. 3.6 The extracellular matrix (ECM) is composed of a variety of proteins and polysaccharides that are secreted locally and assembled into an organized network in close association with the surface of the cell that produced them. The glycocalyx (cell coat) is formed by carbohydrates projecting from membrane lipids and proteins

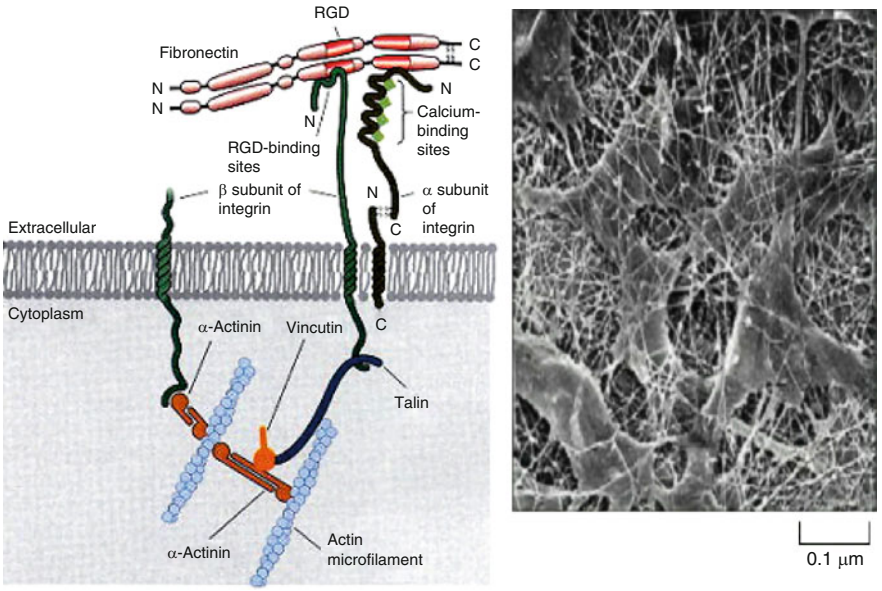

Fig. 3.7 (**a**) The extracellular matrix (ECM) is composed of a variety of proteins and polysaccharides that are secreted locally and assembled into an organized network in close association with the surface of the cell that produced them. The glycocalyx (cell coat) is formed by carbohydrates projecting from membrane lipids and proteins. (**b**) This scanning electron micrograph shows tissue from the cornea of a rat. The extracellular matrix surrounding the fibroblasts is composed largely of collagen fibrils (there are no elastic fibers in the cornea). The glycoproteins, hyaluronan, and proteoglycans, which normally form a hydrated gel filling the interstices of the fibrous network, have been removed by enzyme and acid treatment. (From T. Nishida et al., Invest. Ophthalmol. Vis. Sci. **29**, 1887–1890 (1988). © Association for Research in Vision and Opthalmology.)

which may be other cells or the ECM. They also play a role in cell signaling and thereby define cellular shape and mobility, and regulate the cell cycle. Typically, receptors inform a cell of the molecules in its environment and the cell evokes a response. Not only do integrins perform this outside-in signaling, but they also operate an inside-out mode. Thus, they transduce information from the ECM to the cell as well as reveal the status of the cell to the outside, allowing rapid and flexible responses to changes in the environment, for example, to allow blood coagulation by platelets. Integrins work alongside other proteins such as cadherins, cell adhesion molecules, and selectins to mediate cell–cell and cell–matrix interaction and communication. Integrins bind cell surface and ECM components such as fibronectin, vitronectin, collagen, and laminin. The β subunits of integrins contribute to both ligand binding and transduction of intracellular signals. The major integrin binding site is an Arg-Gly-Asp (RGD) tripeptide, present in a variety of integrin ligands (Fig. 3.7).

Coating of surfaces with constituents of ECM promotes cell attachment and monolayer formation.

3.8 Protein/Peptide Surface Immobilization

In the beginning of several medicinal attempts to functionalize surfaces for obtaining cell surface interactions, the studied materials were coated with cell adhesive proteins, mainly fibronectin, collagen, or laminin. The use of proteins, however, bears some disadvantages in the view of medical applications. First of all, proteins have to be isolated, purified, and immobilized, bearing their functional conformation. However, proteins are the object of proteolytic degradation and need to be refreshed continuously. Long-time applications of these materials would be impossible. Inflammation and infection can even accelerate protein degradation. Furthermore, only a part of the proteins have proper orientation for cell adhesion due to their stochastic orientation on the surface. In addition, the texture of the surface, determined by charge, wetability and topography, may influence the conformation and/or the orientation of the proteins. On hydrophobic surfaces, they tend to maximize interaction with hydrophobic amino acid side chains. This causes denaturation of the proteins or at least a different presentation of cell-binding motifs. On the contrary, most of the problems discussed above can be overcome by presenting cell recognition motifs as small immobilized peptides. They exhibit higher stability toward sterilization conditions, heat treatment, storage, and conformational shifting as well as easier characterization. Because of lower space requirement, peptides can be packed with higher density on surfaces.

Since 1984, RGD peptides have been known to enhance cell attachment on biomaterial surfaces. This attachment property is due to the presence of integrin receptors implied in the cellular adhesion phenomena (Fig. 3.7a) [36]. Today, a number of studies have proven that immobilization of adhesive peptides on materials enhances endothelial [37] and osteoblastic [38] cell adhesion. However, the surface chemistry of such engineered materials, characterized by the type of cell-binding ligands (peptides, proteins, etc.), their surface density [39, 40], and spatial distribution as well as their conformation, has been demonstrated to be important surface cues. Cell adhesive RGD sites were identified in many ECM proteins, including vitronectin, fibrinogen, von Willebrand factor, collagen, laminin, osteopontin, tenascin, and bone sialoprotein, as well as in membrane proteins, in viral and bacterial proteins, and in snake venoms.

3.9 Conclusion

Biofunctionalization of surfaces for biomedical uses is an emerging scientific field with many promises. The biomaterials are at the moment of great benefit for medicinal purposes, and a lot of knowledge from different fields is required in order to design biomimetic scaffolds or biomimetic materials. The used methodologies are different for different materials and are mainly based on the special chemistry of the surfaces. Peptides with distinct properties are desired instead of entire proteins.

However, in some cases, proteins cannot be replaced by peptide segments and, therefore, biochemical knowledge concerning their engineering is needed.

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Chapter 4
Imaging the Human Body: Micro- and Nanostructure of Human Tissues

Georg Schulz, Hans Deyhle, and Bert Müller

Abstract Computed tomography based on X-rays is known to provide the best spatial resolution of all clinical three-dimensional imaging facilities and currently reaches a fraction of a millimeter. Better spatial and density resolution is obtained by means of micro computed tomography well established in the field of materials science. It is also very supportive imaging human tissues down to the level of individual cells (Lareida et al. J. Microsc. 234:95, 2009). The article demonstrates the power of micro computed tomography for imaging parts of the human body such as teeth, inner ear, cerebellum, tumors, and urethral tissue with conventional X-ray sources and synchrotron radiation facilities in absorption and phase contrast modes. The second part of the chapter relies on scanning X-ray scattering of tooth slices (Müller et al. Eur. J. Clin. Nanomed. 3:30, 2010) to uncover the presence of nanostructures including their anisotropy and orientation. This imaging technique gives unrivalled insights for medical experts, which will have a major influence on fields such as dental and incontinence treatments.

4.1 Introduction

Physicists fully understand the handling of hydrogen atoms and molecules. Molecules such as carbon dioxide, however, are already too big to be treated by physicists. Dealing with organic molecules already needs additional experts (chemists). Biomolecules such as proteins are even more complex and biological matter as an individual cell is already another subject.

Medical doctors have different approaches. They do handle the human body as a whole and take decisions within very short periods of time mainly on their

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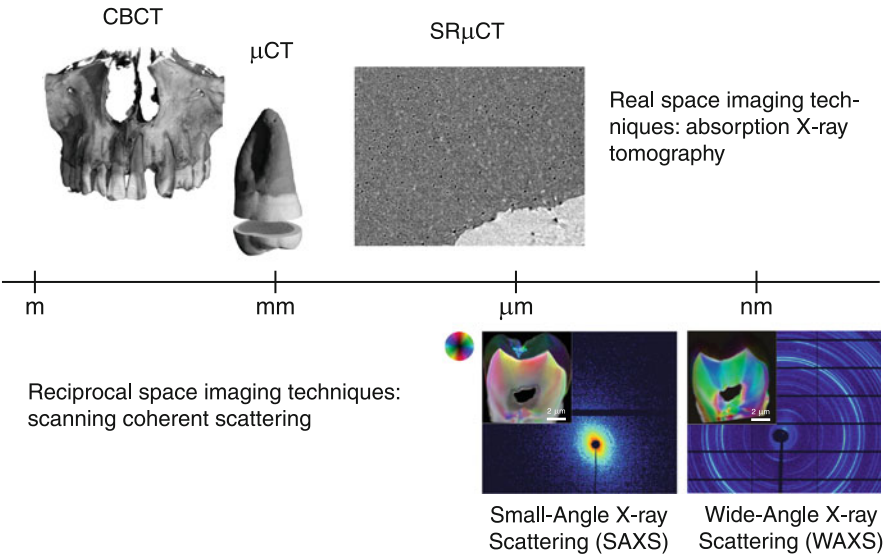

Fig. 4.1 X-ray imaging techniques and their corresponding length scale regimes

strong experience. Sometimes, however, they take benefit from radiology to get insight into the body. The three-dimensional imaging facilities of radiologists reach submillimeter resolution as indicated by the cone beam computed tomography cone beam tomography (CBCT) image in Fig. 4.1. Although these imaging facilities are extremely helpful in diagnosis and therapy and often yield a huge amount of data, their spatial resolution is far from the atomic scale.

This chapter is an attempt to bridge the gap between advanced radiology and the atomic world of solid-state physicists. The path is depicted in Fig. 4.1. Micro computed tomography (μCT) as well introduced in materials science allows mapping the microstructures within human tissues, if the contrast (density resolution) is reasonable. Using monochromatic X-ray beams, as usually done in synchrotron radiation-based μCT (SRμCT), improves the contrast of the three-dimensional data so that tiny microstructures such as the dentinal tubules (see Fig. 4.1) come to light. This, however, is the actual limit of hard X-ray tomography, if the application of X-ray optics is avoided. Nanostructures between 1 and 100 nm known from nanotechnology cannot be visualized.

X-ray diffraction using photon energies of about 10 keV, however, permits the characterization of these nanostructures as the average value obtained from the illuminated volume. The focusing of the X-ray beam to true micrometer diameters combined with scanning provides spatially resolved information on all kinds of nanostructures present. The related techniques are termed scanning small-angle X-ray scattering (SAXS) and scanning wide-angle X-ray scattering (WAXS), respectively (see Fig. 4.1). Spatially resolved SAXS and related images from human tissue will be discussed in detail below.

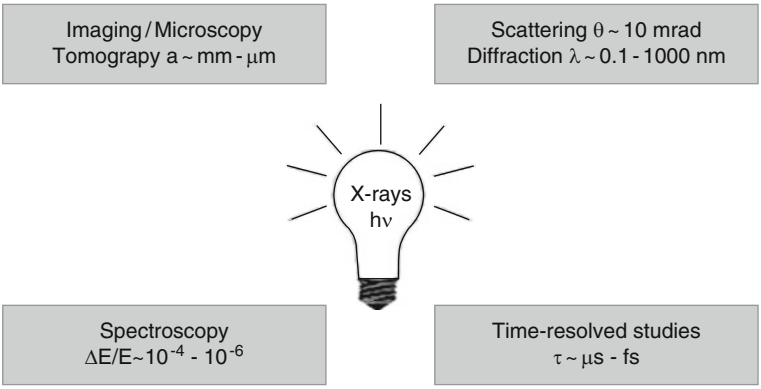

Fig. 4.2 Categories of methods to study human tissues with X-rays

The different X-ray-based techniques can be divided into four categories as shown in Fig. 4.2. Tomography (imaging) and scattering are often employed to characterize biological matter. For spectroscopy and time-resolved studies, only a very few examples are known and, therefore, these methods are not covered in this chapter. There are also methods, which belong to more than one of the categories and thus represent a combination [1].

4.2 Results and Discussion

4.2.1 X-Rays from Synchrotron Facilities

Synchrotron radiation sources provide such a high intensity that by using monochromators, one can easily build a tunable X-ray source. These developments allowed improving the imaging techniques in different manner. The main components of a synchrotron radiation facility, besides the storage ring, are the insertion device, the monochromator and the so-called endstation (Fig. 4.3). Electrons or positrons are injected into the synchrotron ring at high energies by either a linear accelerator or by an intermediate synchrotron which again is fed by a linear accelerator. Synchrotron radiation arises from magnetic deflection of the particle beam by the insertion device. The nowadays existing insertion devices are bending magnet, wiggler, undulator, and free-electron laser (Fig. 4.4). Whereas the X-rays induced by the first and second generation insertion devices interfere incoherently, a coherent interference appears for the third and fourth generation devices. Besides the different principles, the insertion devices vary in the intensity distribution and the brilliance of the generated X-rays. The main advantage of synchrotron light is its high intensity. This permits to select one photon energy from the polychromatic spectrum

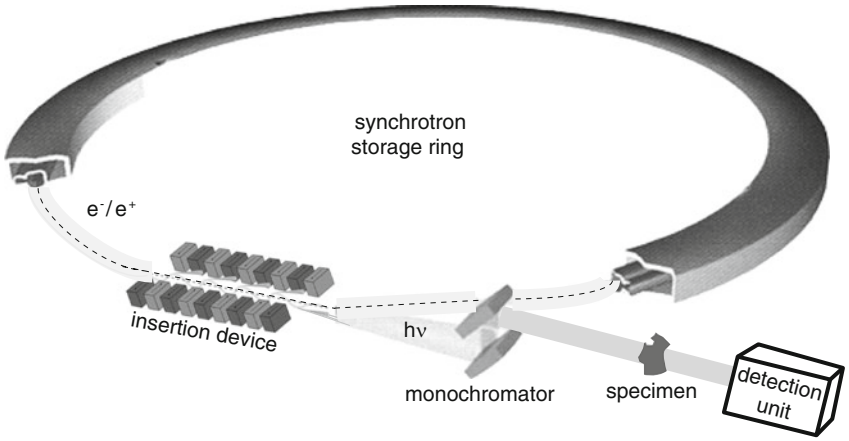

Fig. 4.3 Layout of a synchrotron radiation facility

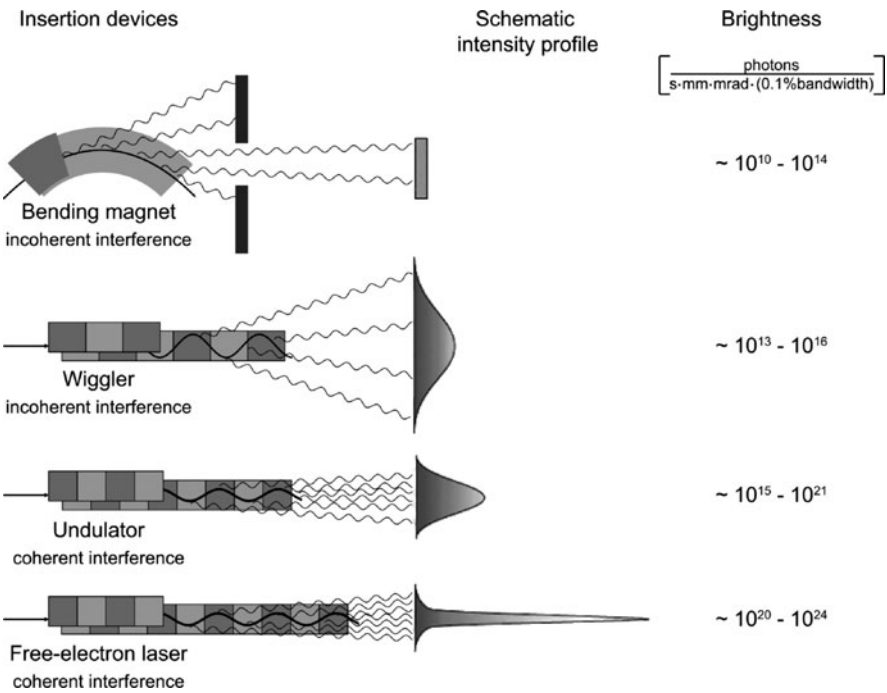

Fig. 4.4 Generation of X-rays with different insertion devices

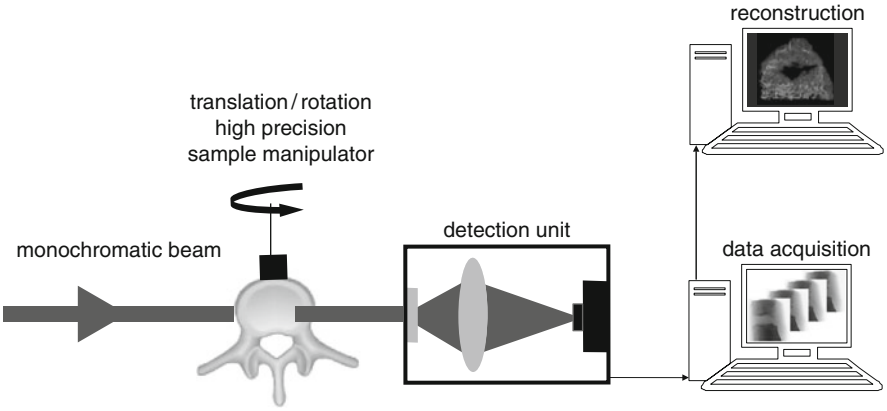

Fig. 4.5 Tomographic data acquisition and processing

generated in the insertion devices by means of a monochromator. X-ray energies used at synchrotron facilities range from several hundred eV to a few hundred keV.

4.2.2 Principles of Computed Tomography

The discovery of X-rays by Röntgen in 1895 [2] involved new applications in medicine both for diagnosis and therapy. Unfortunately, the information obtained by X-ray radiography does not include the whole three-dimensional information of the object. This three-dimensional information can be achieved by tomography. The principles of a tomographic data acquisition can be seen in Fig. 4.5. Using a high precision sample manipulator (translation and rotation), several hundreds of projections of the specimen can be acquired by the detection unit. The mathematical theory for the reconstruction of an object from the projections was proposed for the first time by Radon in 1917 [43]. Today, the three-dimensional information of the object results from creating sinograms from the projections and filtered backprojection reconstruction (Fig. 4.6) [3]. The additional advantage of tomography compared to transmission radiography, besides the three-dimensional information, is the much higher density resolution.

4.2.3 Absorption Contrast CT

For the investigation of human hard tissue, CT in absorption contrast mode is a widely used technique. The reconstructed dataset contains information about the three-dimensional distribution of the attenuation coefficient $\mu(x, y, z, E, Z, \rho)$ with the photon energy E, the atomic number Z, and the mass density of the specimen ρ.

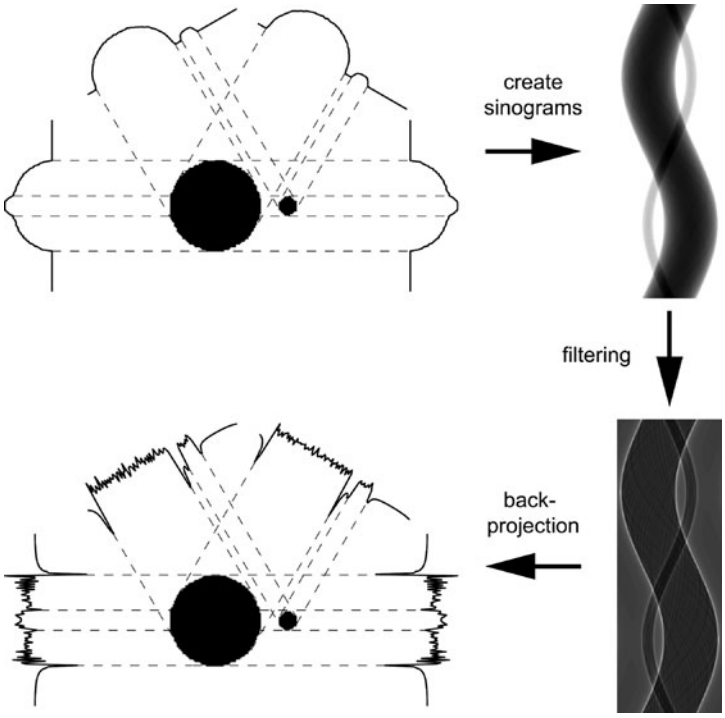

Fig. 4.6 Principle of tomographic data reconstruction by filtered backprojection algorithm

The approximate dependence between the attenuation coefficient and the photon energy of the incoming X-rays

$$\mu \sim \left(\frac{Z}{E}\right)^m \quad \text{with} \quad 2.5 < m < 3.5 \tag{4.1}$$

is valid in the region between two absorption edges [4]. The optimal energy for the scan can be estimated by the equation

$$\mu(Z, E) \cdot D = 2, \tag{4.2}$$

where D is the diameter of the specimen [5].

4.2.3.1 Visualization of Tumor Vessels

The formation of blood vessels in the human body, the so-called angiogenesis, is a crucial step for the initiation, survival, and metastases formation of malignant tumors. For the understanding of the biological regulation of it and in order

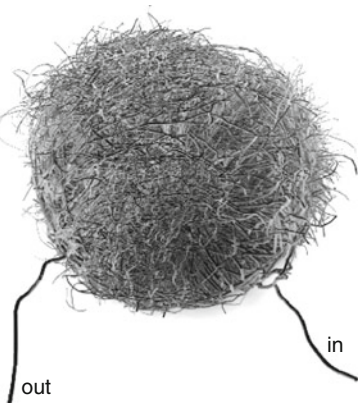

Fig. 4.7 Computational simulation of the vascular network in a tumor

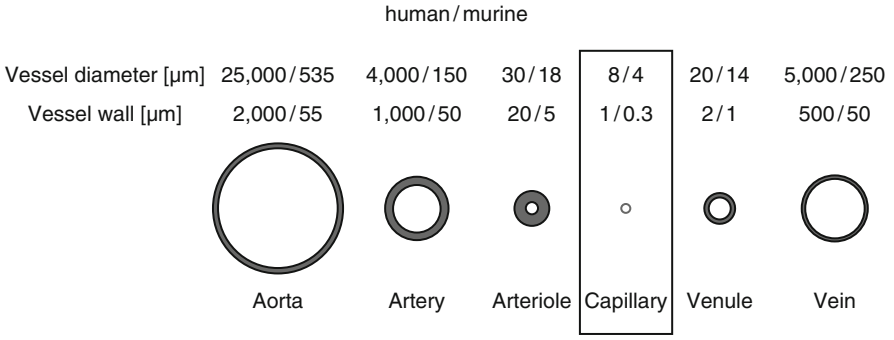

Fig. 4.8 Dimensions of blood vessels in humans resp. mice

to support the development of strategies against cancer, computer models were developed and related simulations performed [6]. Figure 4.7 shows such a model of the vascular network inside cancerous tissue.

However, for the validation of the theoretical network, a comparison between these models and the real blood vessel formation should be performed. Figure 4.8 shows typical dimensions of the different blood vessels of a human and for comparison of mice. In order to uncover even the smallest capillaries, an imaging technique with a spatial resolution down to the micrometer level is required. For such soft tissue, the density resolution of absorption contrast CT is not high enough for the differentiation of the blood vessels and the surrounded tissue. One possibility to enhance the contrast would be the staining of the blood vessels using a highly absorbing contrast agent [7–9]; another possibility would be phase contrast CT (see Sect. 4.2.4) which yields enough contrast even for aqueous specimens [8] where human derived mouse tumors were investigated.

In this section, we will deal with a third possibility to visualize the vascular network. Before the CT in absorption contrast of the tumor, a corrosion cast of

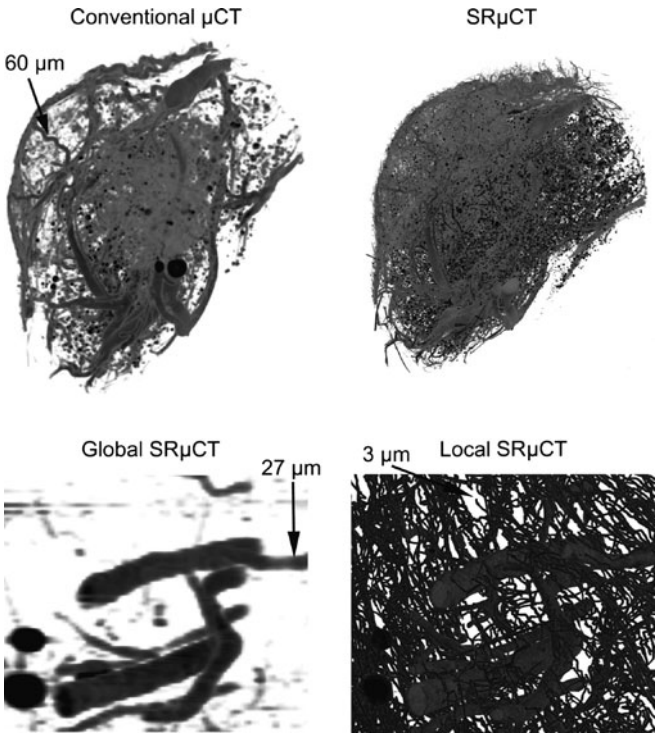

Fig. 4.9 SRμCT Three-dimensional representation of a cast of blood vessels inside a tumor acquired with conventional and synchrotron radiation-based microcomputed tomography in absorption contrast mode

the circulatory system of the mouse has to be fabricated [10]. In order to improve the contrast, the hardened polyurethane cast can be stained by OsO_4. The resulting three-dimensional renderings obtained by conventional CT and SRμCT can be seen in Figs. 4.9 and 4.14. First, the extracted tumor cast was scanned by means of the SkyScan 1174^{TM} system (SkyScan, Kontich, Belgium) at the selected acceleration voltage of 30 kV and the pixel size of 12 µm. The examination of this dataset showed that it is almost impossible to resolve blood vessels with diameters smaller than 50 µm. The SRμCT measurements were carried out at TOMCAT beamline at the Swiss Light Source [11] (Paul Scherrer Institute, Switzerland) with the selected energy of 15 keV. A total of 1,500 projections in equidistant angular steps of 0.12° between 0° and 180° were acquired. With the resulting detector pixel size induced by the first objective of 5.92 µm and the field of view of 12 mm, the whole specimen could be scanned (global tomography). However, these results only allowed to resolve vessel diameters larger than 20 µm. In order to visualize smaller capillaries, a second scan with another objective with a magnification of 10 and the resulting pixel size of 0.74 µm was arranged. These adjustments involved a field of view of

1.5 mm so that only a part of the specimen could be scanned (local tomography). Using this method, it was possible to resolve blood vessel diameters of around 3 μm.

4.2.3.2 Microstructure of Human Tooth

Human teeth are composed by two hard tissues, i.e. enamel and dentin. Enamel is the hard, brittle upper part of the tooth. It consists mainly of inorganic carbonated calcium phosphates with as little as 4% weight of organic material. Dentin is softer and tougher, and is composed of roughly 20 wt% of organic material, mainly collagen type I.

Teeth have rather limited reparation and regeneration capacities. While dentin can to some extent repair damages by formation of tertiary dentin, no self-repairing processes to counteract carious lesions or mechanically induced cracks or material loss is known in enamel. When the functionality of teeth is severely reduced by carious infection or mechanical action, it often becomes necessary to replace part of the damaged tissue or even the whole tooth with dental biomaterials or protheses.

Even though the quality of such replacements has constantly improved, their performance does not reach that of sound teeth, resulting in a limited lifespan that can render necessary further clinical interventions [12, 13]. It is, therefore, desirable to avoid the removal of tooth hard tissue whenever possible. Therefore, mechanisms that can restore the functionality of damaged teeth are matter of research. For example, induced repair mechanisms by means of bioactive glass have been proposed [14, 15]. A knowledge of the tooth microstructure will help to understand such processes as well as uncover to what extent the microarchitecture of teeth influences the formidable performance of human teeth.

Figure 4.10 shows three orthogonal slices through the three-dimensional tomographic dataset of a 700-μm-thin rod extracted from a human premolar, acquired at the TOMCAT beamline at the Swiss Light Source (Paul Scherrer Institute, Switzerland) [11], with a photon energy of 15 keV, exposure time of 170 ms, and isotropic pixel size of 0.37 μm. Dentin (dark gray) and enamel (light gray) can be clearly distinguished due to their different X-ray absorption. The circular arc on the right of the top left slice is an artifact caused by the fact that the tooth specimen was larger than the field of view.

In the dentin, tubular structures are visible as dark spots and streaks. These structures are known as dentinal tubules and serve the purpose of transportation of organic material and information between the pulp and the dentin. Diameter and density of the tubules vary over the specimen. Although the tubules are usually parallel, their orientation can change to become perpendicular to the dentin–enamel interface. Some of the tubules even spread across the interface and continue through the enamel.

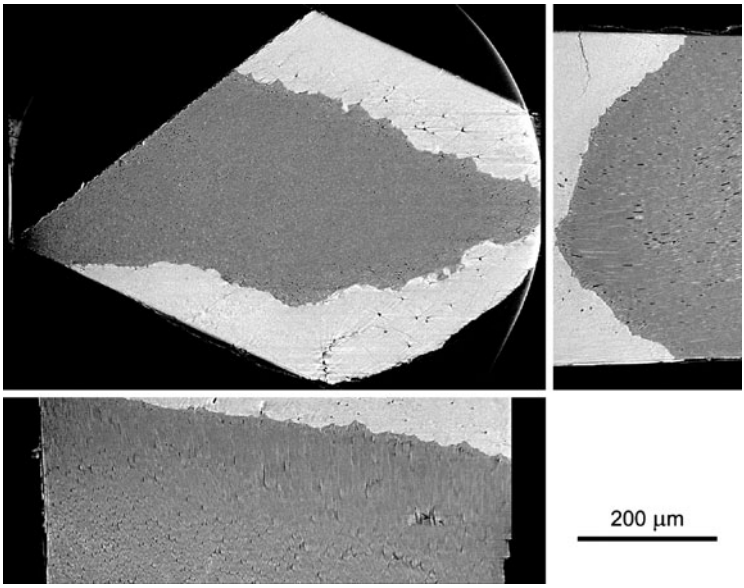

Fig. 4.10 Three orthogonal slices through a high resolution absorption contrast tomographic scan of a part of human tooth acquired at the TOMCAT beamline at the Swiss Light Source (Paul Scherrer Institute, Switzerland)

4.2.3.3 Imaging the Human Inner Ear

The morphology of the human hearing organ, which belongs to the most complex anatomical structures in the human body, is essential to achieve a better understanding of the inner ear pathologies (malformations) to improve the design and the insertion procedures of adapted cochlear implants as well as the treatment of hearing diseases.

Figure 4.11 shows a selected virtual cut of the sensory organ in the apical turn of the human cochlea and two schemes which should help identifying the morphological features. These SRμCT measurements were performed at the beamline BW2 (DESY, Germany) [16] at the selected photon energy of 10.8 keV, the resulting pixel size of 2.1 µm, and the spatial resolution of 4.3 µm characterized by the 10% value of the modulation transfer function (MTF) [17]. During the data acquisition, 720 projections over 180° were recorded. Before the SRμCT experiment, the specimen was osmium stained in order to increase the contrast of the tissue [18, 19]. The osmium stain served for the visualization of the Reissner membrane and permitted the thickness measurement with reasonable precision. It corresponds to two cell layers with a thickness of (10 ± 2) µm.

Another dataset even shows the visualization of individual ganglion cells [18] within the human tissue which can be seen in Fig. 4.12. The measurements were carried out at the TOMCAT beamline of the Swiss Light Source (Paul Scherrer

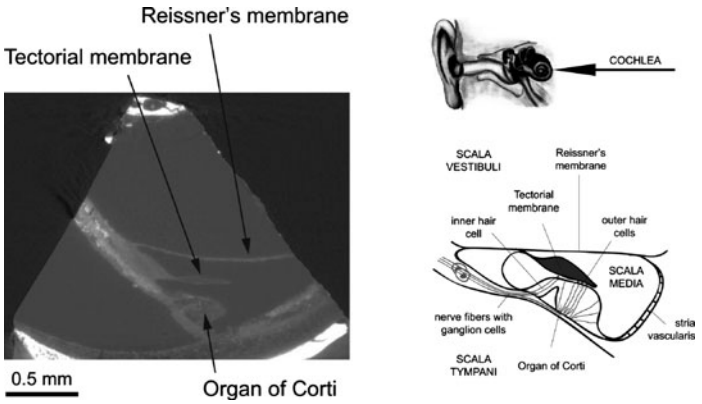

Fig. 4.11 Reconstructed slice of an absorption contrast SRμCT dataset (*left*) and a schematic representation (*right*) of a human inner ear

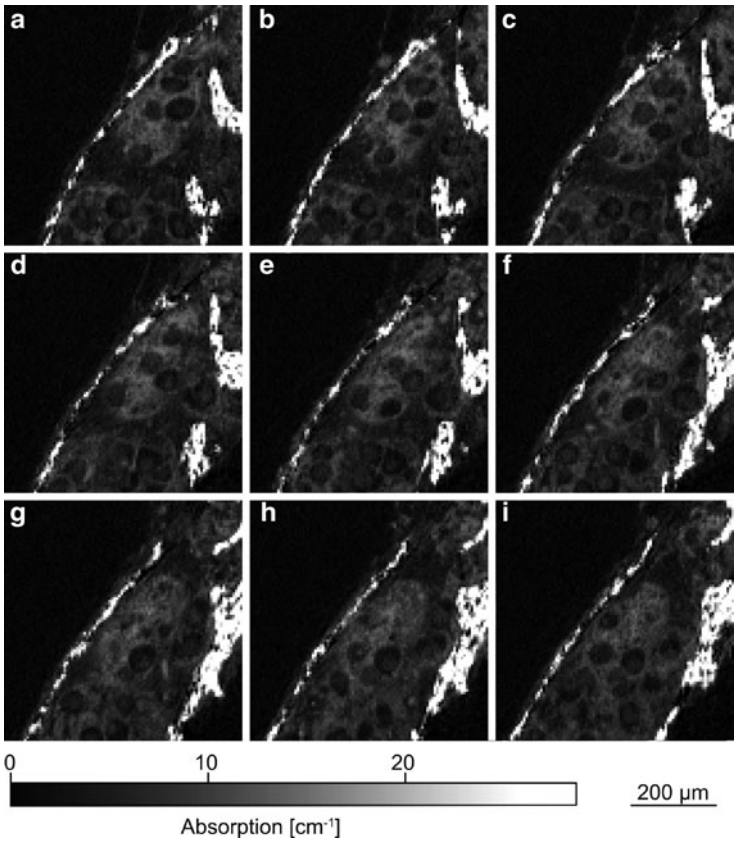

Fig. 4.12 Series of slices of absorption contrast SRμCT. Osmium-stained ganglion cells can be identified

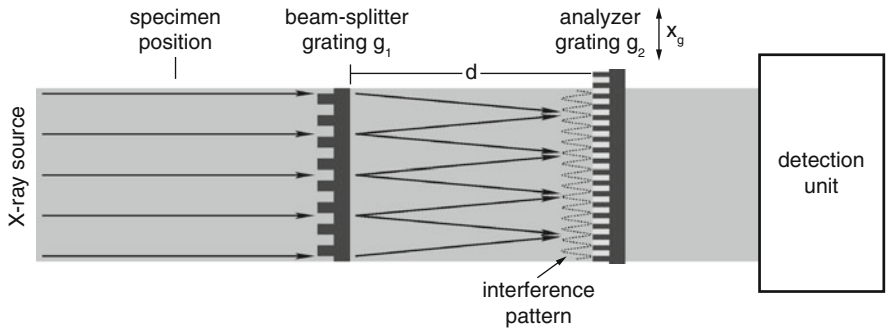

Fig. 4.13 Top view of the grating-based phase contrast setup

Institute, Switzerland) [11]. The data were acquired at the photon energy of 12 keV and an effective pixel size of 1.75 μm. A total of 1,501 projections between 0° and 180° were recorded. The neuron cell counting in a selected volume of 125 μm × 800 μm × 600 μm = 0.06 mm^3 gives rise to the estimate that 2,000 ganglion cells are present along 1 mm Organ of Corti.

4.2.4 Grating-Based X-Ray Phase Contrast CT

4.2.4.1 Instrumentation

Contrary to the absorption contrast, where the local X-ray absorption coefficient distribution $\mu(x, y, z)$ can be measured, phase-contrast CT provides the real part of the refractive index, often expressed in terms of its decrement from unity $\delta(x, y, z)$. This method is more advantageous than absorption contrast for specimens with small atomic numbers, particularly soft tissue. For X-ray energies far away from the absorption edges, it is related to the electron density distribution $\rho_e(x, y, z)$ via the equation

$$\delta(x, y, z) = \frac{r_e \lambda^2}{2\pi} \rho_e(x, y, z), \tag{4.3}$$

where r_e is the classical electron radius [20]. The detection of $\delta(x, y, z)$ can be achieved by a grating interferometer as schematically illustrated in Fig. 4.13, which is composed of a beamsplitter and an analyzer grating. The beam-splitter grating g_1 consists of silicon stripes with the periodicity p_1. It induces fringe patterns in the X-ray intensity distribution. At so-called Talbot distances from the beam-splitter grating of

$$d_n = \frac{n p_1^2}{8\lambda}, \tag{4.4}$$

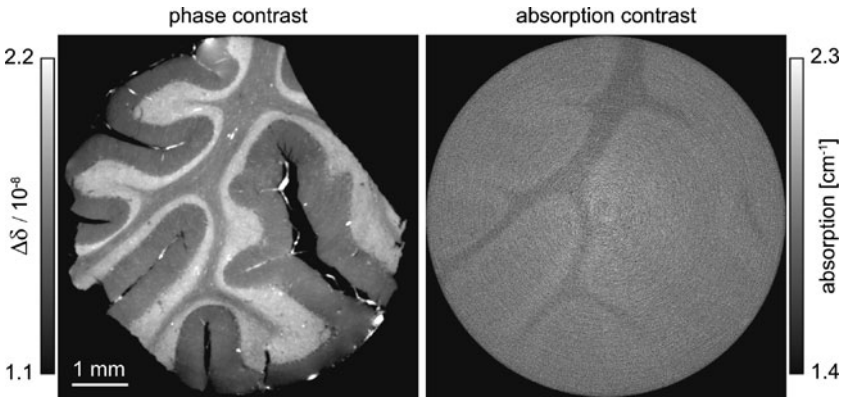

Fig. 4.14 Grating interferometry phase-contrast reconstruction of a human cerebellum acquired at the beamline ID19 (European Synchrotron Radiation Facility, France) plus the accordant slice obtained in absorption contrast mode at the beamline BW2 (HASYLAB at DESY, Germany)

the intensity fringe contrast takes extreme values at odd Talbot orders $n = 1, 3, 5, \ldots$, and vanishing contrast for even values of n [21]. For a phase contrast CT experiment, the distance between the grating g_1 and the analyzer grating g_2 equals the Talbot distances with odd Talbot orders. A phase object causes slight deflection of the incoming X-rays. The grating g_2 with a periodicity $p_2 = p_1/2$ has strongly absorbing stripes, here made of gold. This grating allows the detection of the disturbance of the interference pattern by using a phase stepping method where several images at different positions x_g of the grating g_2 are recorded [20]. The equation

$$\alpha(y,z) = \frac{\lambda}{2\pi}\frac{\partial \Phi(y,z)}{\partial y} = \int_{-\infty}^{\infty} \frac{\partial \delta(x,y,z)}{\partial y} \mathrm{d}x \tag{4.5}$$

describes the quantitative relation between the local beam propagation direction $\alpha(y,z)$, the wave phase shift $\Phi(y,z)$, and the decrement of X-ray refractive index $\delta(x,y,z)$.

4.2.4.2 Comparison Between Phase- and Absorption Contrast CT and Magnetic Resonance Microscopy

The power of phase contrast CT for aqueous specimens becomes apparent by comparing it with the other well-established techniques namely absorption contrast and magnetic resonance microscopy. Figure 4.14 shows one reconstructed slice of a human cerebellum piece in phase contrast and absorption contrast mode. The specimen was fixated in 4% formalin solution.

The grating-based phase contrast experiment was carried out at the beamline ID19 (European Synchrotron Radiation Facility, France) [22] with a selected photon energy of 23 keV using a double-crystal Si(111) monochromator in Bragg geometry.

The X-rays were taken from an undulator. The period of the beam-splitter grating was $p_1 = 4.785\,\mu\text{m}$ and of the analyzer grating $p_2 = 2.400\,\mu\text{m}$. As the experiment was carried out at the ninth Talbot distance, a distance of $d = 479.4\,\text{mm}$ between the gratings was adjusted. The Eppendorf container with the cerebellum in formalin solution was fixed at the high precision rotation stage and immersed in a water tank with parallel polymethylmethacrylat plates for the measurements in order to minimize artifacts owing to X-ray phase curvature induced by the container surface. For the detection, a lens-coupled scintillator and charge-coupled device (CCD) system using a FReLoN 2K (Fast-Readout, Low-Noise, ESRF Grenoble, France) CCD with $2{,}048 \times 2{,}048$ pixels with the effective pixel size of $5.1\,\mu\text{m}$ was used. Four phase-stepping images were taken over one period of the interferometer fringe pattern at each projection angle. With an exposure time of 1 s for each image, 1,501 radiographs were recorded over a range of 360°. An estimation of the spatial resolution of the experimental data was obtained by means of Fourier analysis of the processed projections resulted in $(16.5 \pm 0.5)\mu\text{m}$ and of the reconstructed tomograms $(20 \pm 1)\mu\text{m}$.

The SRμCT experiments in absorption contrast mode were carried out at the beamline BW2 (HASYLAB at DESY, Germany) [16] using a monochromatic beam of 14 keV generated by a wiggler. Using a detector with a resulting pixel size of $3.0\,\mu\text{m}$ 1,440 projections were acquired during the rotation of the specimen by 360°. The spatial resolution of the entire setup was determined by 10% of the MTF [17] corresponding to $6.48\,\mu\text{m}$. The projections were binned twofold before the reconstruction in order to improve the density resolution [23]. The twofold binning of the projected highly X-ray absorbing edge led to a reduced spatial resolution of 8.77 mm again determined by 10% of the MTF.

Whereas the reconstructed absorption contrast slice only shows marginal contrast between white (dark region) and gray matter (bright region), the phase contrast also allows, besides a clear differentiation between white and gray matter, a distinction within the gray tissue in the stratum moleculare (dark region of the gray mater) and the stratum granulosum (bright region of the gray matter).

For the visualization of human brain tissue or generally soft tissue, magnetic resonance tomography is a widely used technique. Today, it is irreplaceable in medicine because it delivers superb contrast between white and gray matter and it has no radiation damages for the patients. Therefore, a comparison between grating-based phase contrast and magnetic resonance tomography with better spatial resolution than the conventional medical scanners was done. The results acquired by high field magnetic resonance microscopy (Fig. 4.15) with a voxel size of $100\,\mu\text{m}$ show a better contrast between the white and gray matter compared to the absorption contrast CT but the contrast is not good enough to differentiate between the two strata of the gray matter.

The three-dimensional rendering of the grating interferometry results clarifies the power of this technique. Figure 4.16 shows an intensity-based segmentation of the stratum granulosum. Besides the stratum granulosum, many blood vessels can be resolved without the use of any contrast agent. The most important finding of this dataset is that the high spatial resolution and the high sensitivity of this

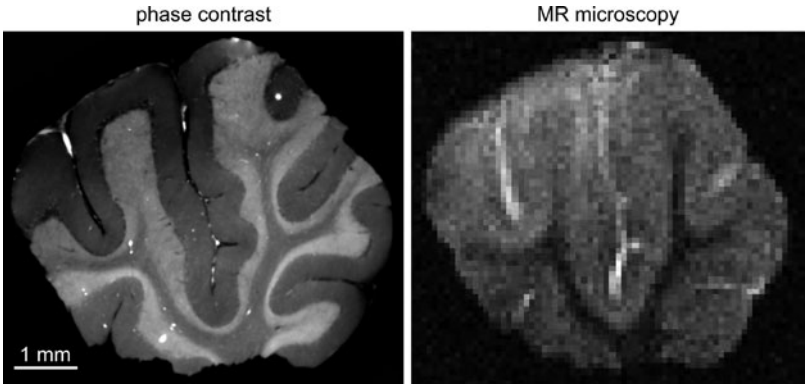

Fig. 4.15 Slice from the dataset of Fig. 4.14 and the accordant magnetic resonance microscopy image recorded at the Technische Universität München, Germany

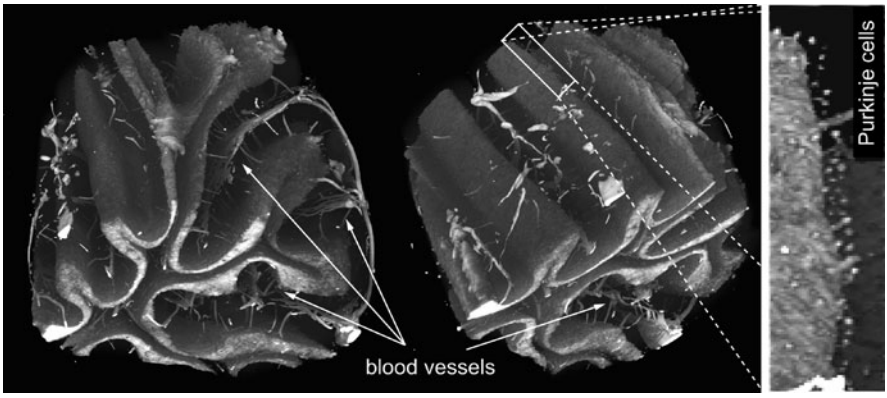

Fig. 4.16 Phase-contrast three-dimensional rendering of the human cerebellum block (cp. Fig. 4.14 *left*) showing various blood vessels and even individual Purkinje cells

imaging technique allows the detection of individual nonstained cells surrounded by soft tissue. The detection of individual Purkinje cells with spherical diameters of 40–70 μm without the application of any stain or contrast agent is a novelty in the field of computerized tomography [24].

4.2.4.3 Morphology of Human Urethra

Another application of the grating-based phase contrast is the morphological characterization of human urethra. As the number of incontinent patients is steadily increasing, the function of the urethra under static and, more important, under stress conditions has to be uncovered.

Figure 4.17 shows orthogonal cuts through a human urethra acquired by grating interferometry and clarifies the nonperfect symmetry along the opening.

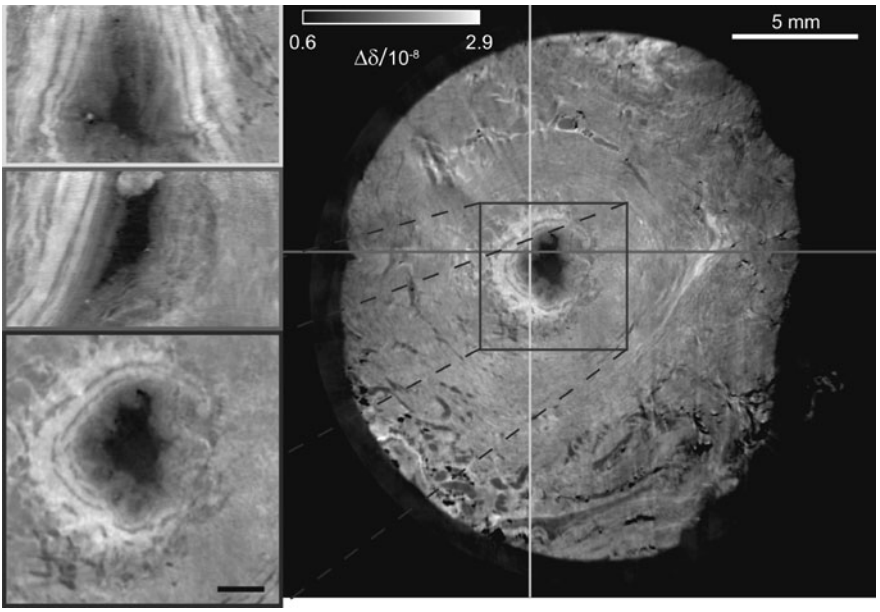

Fig. 4.17 Virtual cuts though a grating-based tomography data set of a human urethra acquired at the W2 beamline (DESY, Germany)

The measurements were performed at the beamline W2 (HASYLAB at DESY, Germany) with the recently established grating interferometer setup [25]. At the selected energy of 24 keV and a resulting pixel size of 10 μm and a spatial resolution of 37 μm 1,101 projections over 360° were recorded. The spatial resolution of the experimental setup was determined by the 10% of the MTF of a processed projection of a silicon wedge.

More information on the experimental details are given elsewhere [26]. The motivation of this study can be found in the recent message [27].

4.2.5 Small-Angle X-Ray Scattering

4.2.5.1 Instrumentation

Small-angle X-ray scattering (SAXS) belongs to the reciprocal space imaging techniques. It is characterized by a reciprocity law, which gives an inverse relationship between the size of the inspected particles and scattering angle. It occurs when an inspected specimen presents electron density inhomogeneities on the nanometer scale, thus resulting in scattering angles in the range of few mrad. SAXS is, therefore, of particular interest in fields where inhomogeneities at such length scales

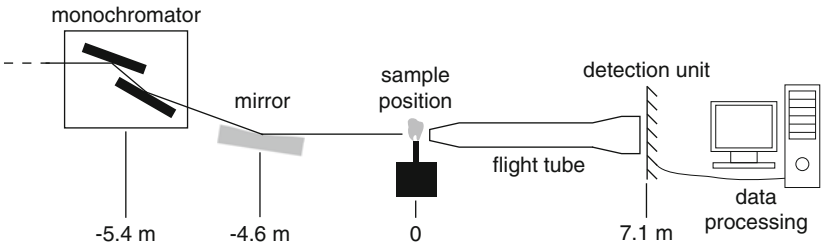

Fig. 4.18 Schematic representation of the cSAXS end-station

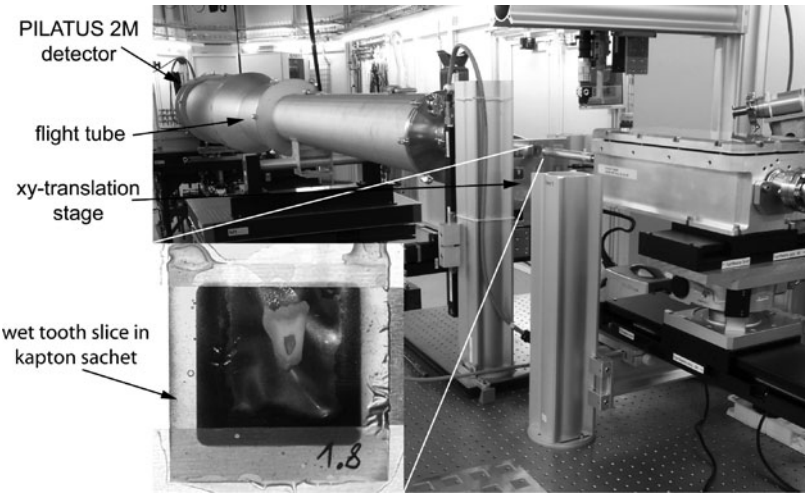

Fig. 4.19 The end-station at the cSAXS beamline at the Swiss Light Source (Paul Scherrer Institute, Switzerland)

occur, e.g., for the characterization of polymers [28], protein structures [29], or inspection of human tissues, e.g., cancerous and noncancerous tissue [30,44] myelin sheaths of neurons [31], dentin [32,33], or bone and cartilage [34,35].

In this section, a typical SAXS instrumentation on the example of the cSAXS beamline at the Swiss Light Source (Paul Scherrer Institute, Switzerland) [36] is given. Figure 4.18 gives a schematic representation of the endstation at the cSAXS beamline. Coming from the left, the X-rays pass through a fixed exit Si(111) monochromator, which allows to choose energies in the range between 4 and 19 keV. The second mirror crystal can be bent to allow for horizontal focussing of the X-ray beam, while a quartz glass mirror at 4.5 m from the sample allows for vertical focussing. The beam is normally focussed at the sample position to a spot size of approximately $5 \times 20\,\mu m^2$ (vertical $\times$ horizontal). A 7-m-long evacuated flight tube is placed between the sample and the PILATUS 2M detector [37] to minimize noise from air scattering. Dedicated data processing hardware and software is required for data evaluation. Figure 4.19 shows a photograph of the cSAXS endstation.

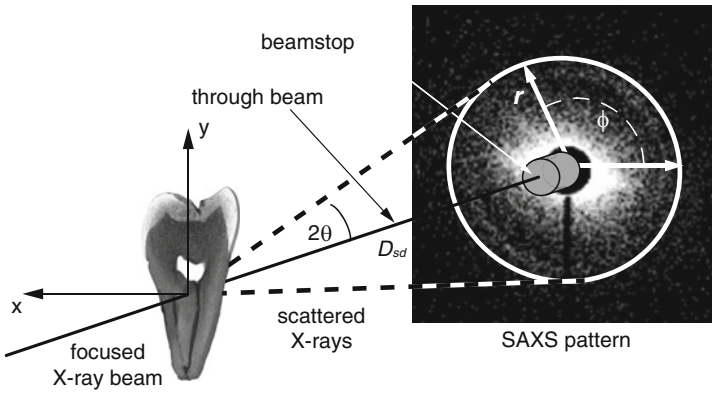

Fig. 4.20 Scanning SAXS data acquisition

Figure 4.20 depicts a typical experimental setup. The X-ray beam hits the sample perpendicularly. Only a fraction of the photons is scattered at the sample position, while the large part is transmitted through the sample. A sufficiently thin specimen is required to avoid excessive X-ray absorption in order to obtain sufficient photon statistics for data evaluation.

With the high-brilliance source combined with the fast detector technology available at cSAXS, imaging rates of up to 30 Hz are possible. These fast acquisition times allow to scan the sample through the beam in x and y directions while continuously recording images. In this setup, samples with areas in the cm^2 range can be scanned within 1 h or even less. The exact total acquisition time for the scanning X-ray scattering measurements depends on the number of spots in the raster scan and the exposure time used. The latter in turn will depend on the signal-to-noise ratio of the data, the X-ray absorption, and the scattering contrast in the sample.

To process the high amount of information generated, automated analysis routines are needed. The first step in SAXS data analysis consists in a radial integration of the scattering patterns over 16 radial segments in a specific q-range interval (Fig. 4.21, on the right). The intensity in each circular segment is then plotted as a function of its angular position θ (Fig. 4.21 left). If the scattering pattern presents a moderate asymmetric intensity distribution, the plot is well approximated by a cosine curve. The mean scattered intensity, indicated in Fig. 4.21 with I_{sym} relates to the abundance of scattering centers in the selected q-range, while the amplitude of the cosine I_{asym} relates to their orientation. The phase ϕ yields the mean orientation of the scattering signal.

As fitting, however, is time consuming and the results can significantly vary depending on the initial conditions and the goodness of the data, instead of a cosine-fit, the data are approximated with a Fourier transform

$$I(\theta) \cong I_{\text{sym}} + I_{\text{asym}} \cdot \cos(\theta + \phi). \tag{4.6}$$

For specimens with strong orientation on the nanometer scale however, the intensity distribution can differ from an ideal cosine assumed by the Fourier analysis. The

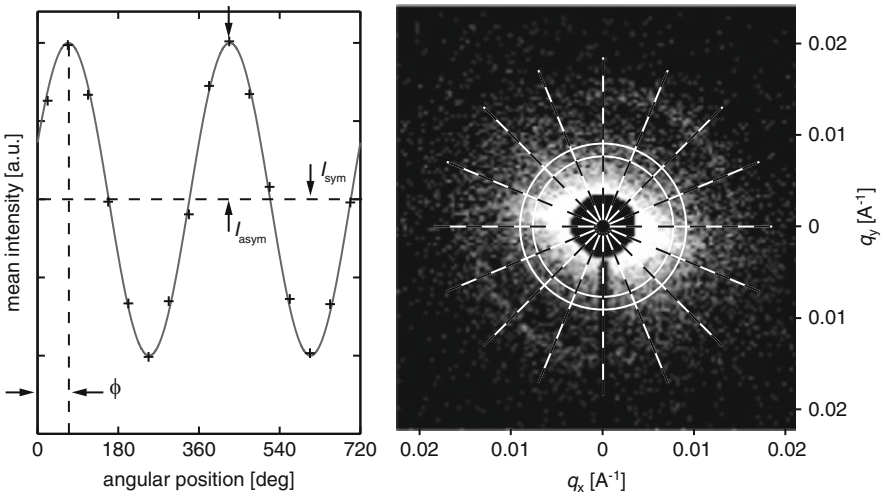

Fig. 4.21 Processing of a SAXS pattern

deviation from the cosine is expected to be significant in strongly oriented areas and indicates that for highly ordered materials, different analysis schemes could be worth trying.

Additional information can be extracted from the scattering patterns and from the radially integrated intensity. The exponent of the intensity curve $I(q)$ contains information about the geometrical shape of the scattering centers in the sample. Spherical scatterers will exhibit a q^{-4} intensity fall off, whereas flat lamellar or disc-like structures will have an exponent closer to -2 and elongated, rod-, or needle-like features an exponent of -1. As the scattering patterns possess an inversion symmetry around the beam center, one can look at asymmetries of the scattered intensity with respect to this inversion center. This signal contains information similar to that obtained by grating interferometry (cp. Sect. 4.2.4.1). Like it is the case for a true grating interferometry signal, topographical features like edges and scratches become clearly visible.

4.2.5.2 Nanostructures of the Human Tooth

Human tooth hard tissues present strong preferential orientations on the nanometer scale. Figure 4.22 shows a collection of SAXS data of a 400-μm-thin slice extracted from human third molar in the q-range between 0.021 and 0.016 Å^{-1}, corresponding to the range between 30 and 40 nm in real space, acquired at the cSAXS beamline [36] at 18.58 keV photon energy with 180 ms exposure time per frame and a step size between the raster scan points of 50 μm in both x and y directions.

The symmetric intensity (top-left image), related to the density of scatterers in this range, shows a strong increase near the dentin–enamel junction (DEJ), indicating a high abundance of nanostructures. The orientation of the scattering

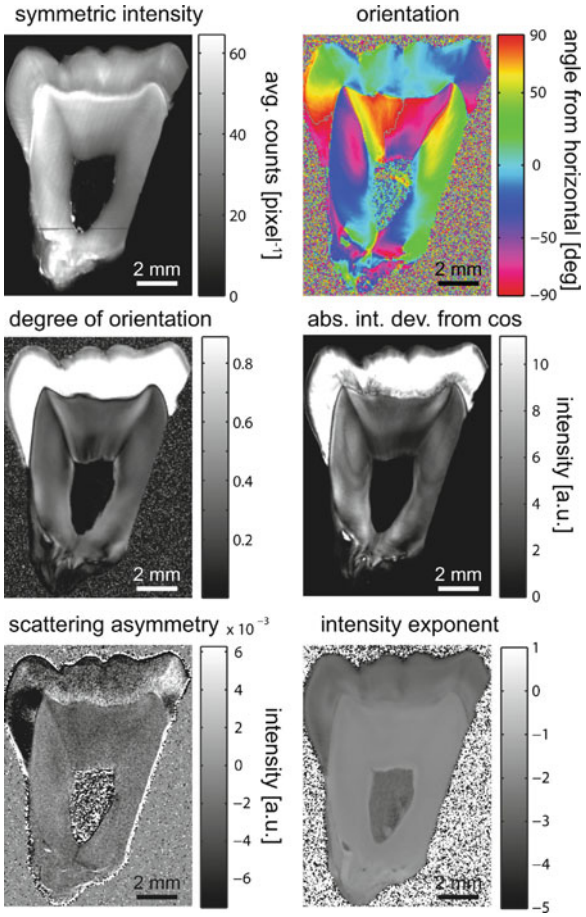

Fig. 4.22 Different information routinely extracted from scanning SAXS data at the cSAXS beamline illustrated on the basis of a human tooth slice

signal (top-right image), which is perpendicular to the orientation of the scattering nanostructures, shows clear differences between enamel and dentin. While the signal is mostly oriented parallel to the DEJ through the whole of the enamel, it is mainly oriented parallel to the DEJ in the upper part of the dentin. Sharp changes in dentin orientation can be observed at lines connecting the tooth cusps and the pulp. The degree of orientation (middle left image), defined as the ratio of oriented to unoriented scattered intensity $I_{\mathrm{sym}}/I_{\mathrm{asym}}$, relates to the degree of anisotropy in the plane perpendicular to the incident X-ray beams. Much higher directional organization is found in the enamel, while the degree of orientation in the dentin slightly decreases from the DEJ toward the pulp. As expected, the deviation of the integrated intensity from an ideal cosine (middle right image) strongly resembles the orientation pattern, with high values in the strongly oriented enamel and reduced intensity in the dentin. The scattering asymmetry signal (bottom left image) yields a rather homogeneous signal in both enamel and dentin, with strong shifts at the edges of the specimen, indicating the absence of sharp edges or cracks inside the

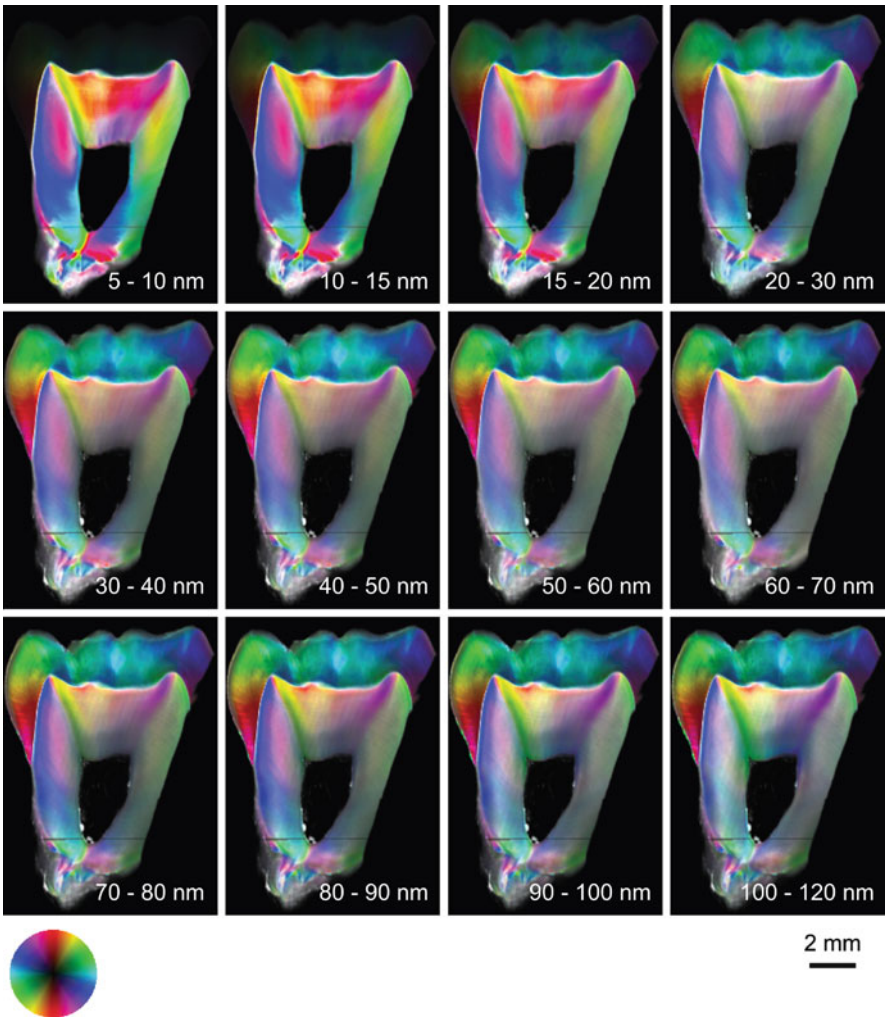

Fig. 4.23 Orientation, abundance, and degree of orientation of different sizes of nanostructures of a human tooth slice (cp. Fig. 4.22). The main orientation of the scattering signal is color-coded (see color wheel). The color intensity codes the scattered intensity, while the color saturation codes the degree of orientation of the scattering signal

tooth slice. The intensity exponent, related to the shape of the scatterers, is given in the bottom right image. In the investigated range, the dentin contains more needle- or rod-like scatterers, thus giving raise to an intensity decay proportional to q^{-1}, whereas the signal in the enamel indicates the presence of two-dimensional scatterers with an intensity profile proportional to q^{-2}.

Figure 4.23 shows the combined information of total scattered intensity I_{sym}, orientation ϕ and degree of orientation $I_{\mathrm{asym}}/I_{\mathrm{sym}}$ for different q-ranges of the same

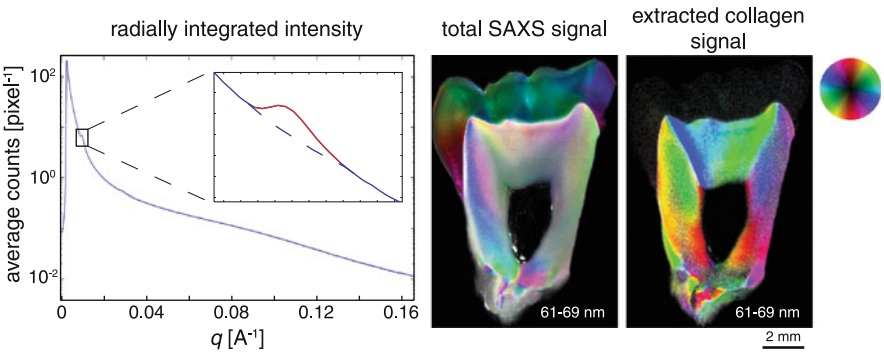

Fig. 4.24 Radially integrated intensity of a typical SAXS pattern of dentin exhibiting a characteristic peak related to collagen. The collagen signal can be extracted and processed separately

tooth slice. The orientation of the scattering material is according to the color wheel, showing which color represents which orientation. Bright colors mean more total scattering intensity than dim colors. Effectively oriented material shows up in color whereas unoriented material shows up white. Gray shades mean less unoriented material than in white areas.

The scattering signal in the enamel disappears below 10 nm, indicating that the smallest extension of the nanocrystallites composing the enamel lies above 10 nm [38]. For all length scales, the main orientation in enamel and dentin is comparable, with the scattering signal parallel to the DEJ in the enamel and perpendicular in the dentin. However, the degree of orientation diminishes toward larger length ranges, and higher anisotropies are found toward the enamel compared to the region surrounding the pulp. Abrupt changes in dentin orientation can be seen along lines connecting the tooth cusps and the pulp over all ranges. The changes are sharper toward low and high ranges, while they are blurred out between 30 and 70 nm.

Dentin is composed to 20 wt% of organic material, mainly collagen-I fibrils [39]. The building blocks of collagen arrange themselves along the collagen fibril with a main periodicity of 67 nm, as determined, for example, by means of atomic force microscopy and SAXS [40]. This periodicity gives rise to a distinct peak in scattered intensity at the corresponding q range between about 0.009 and 0.01 Å^{-1}. Due to the disposition of the building blocks inside the collagen fibrils, this peak-like scattering signal is parallel to the collagen fibrils.

The plot in Fig. 4.24 shows a typical intensity profile $I(q)$ of dentin after radial integration and a magnification of the region containing the collagen peak. The background intensity below the peak can be fitted with a power-law exponent close to -2. After subtracting the background from the total signal, the remaining intensity is solely associated to the scattering signal from the collagen. The two images in Fig. 4.24 show the orientation, degree of orientation, and total scattering intensity both before and after background subtraction for the range between 61 and 69 nm. As expected, no significant signal can be seen in the enamel after the

background subtraction, due to its anorganic nature. The scattering signal associated with the collagen shows a perpendicular orientation to the total scattering signal in the dentin, indicating that collagen fibrils are oriented parallel to the dentin crystallites, which make up for the largest part of the total SAXS signal.

4.3 Conclusion

Since the discovery in 1895 by Röntgen [2], X-rays have played a dominant role in our everyday life. For example, they make the bones in our body visible, serve for the nondestructive testing of a wide variety of materials including human tissues postmortem, and enhance our security in air travel.

Some time later, M. von Laue and others discovered and interpreted X-ray diffraction in crystals [41], which provided a wealth of information about solid states including the arrangement of atoms.

X-ray spectroscopy techniques made it possible to nondestructively determine the chemical composition of solids simply by analyzing the X-ray fluorescent spectra when the atoms were excited. With a focused X-ray source realized at different synchrotron radiation facilities, one can not only determine the chemical composition with reasonable spatial resolution but also get access to nanostructures in the entire nanometer range.

Today, highly intense X-ray sources as the free electron lasers allow measurements within such a small time interval that the molecule of interest does not have enough time to become destroyed.

In medicine, X-ray imaging attracted the greatest interest, since the penetrating power of X-rays reveals the bulk properties of the body rather than the surface shape. We mainly see the bones because of the strong dependence of attenuation on the atomic number. The limitation in spatial resolution is mainly given by the detection unit and not by the X-ray source or the stability of manipulator for rotation. The availability of powerful computing resources has permitted the tomographic reconstruction of the three-dimensional microstructure of the specimen or the entire human body. High spatial resolution, however, requires a related photon density and dose, so that a spatial resolution below 50 μm cannot be reached for in vivo studies.

Synchrotron radiation facilities have a restricted availability and therefore require a different working regime. The scientists have to carefully prepare their experiments, much more carefully than in a conventional laboratory. They have to organize their work during the 24 h per day experiment in an efficient manner and have finally plenty of time for the data treatment and the publication of the results. The main advantage of the synchrotron radiation sources, however, is their intensity and brightness, which are numerous orders of magnitudes higher than conventional bremsstrahlung sources. It allows incorporating a monochromator and still having a sufficiently high intensity for the experiment to be performed in reasonable time frames.

The microstructures within the human tissues can be made visible postmortem by means of SRμCT. These microstructures include the vessel tree of healthy and cancerous tissues, which enable a direct comparison also with simulations to validate them. Gathering the smallest capillaries is here of upmost interest, because the vascularization of tumors is often much better, although they can also contain a necrotic core. Dentinal tubules play a major role in the supply of the dentin. Their density and orientation reflect the history of the tooth. The complete characterization of the dentin's morphology is a vital step toward the quantitative understanding on why restorations can fail even if the clinical procedures are maintained constant. Only a very few research teams worldwide have been able to uncover the tubules using hard X-ray tomography [33, 42].

It is a dream of the researchers to build a human out of individual cells. Hence, the visualization of the individual cells within the human body is an interesting challenge. The membranes of the inner ear (double layer of cells) belong to the first structures, which were imaged in its three-dimensional shape. This is very important, as in histological sections the membranes relax and usually appear as straight line. The human cochlea, however, has two and a half turns so that the membranes have to exhibit a curved shape. The first example of identification of individual cells in humans by hard X-ray tomography is the successful visualization of hundreds of ganglion cells of the inner ear, which were osmium stained. Very recently, using the grating-based phase contrast tomography, even unstained Purkinje cells came to light. It will take another decade until about hundred cell types within the human body can be imaged with the necessary resolution. Here, the phase contrast will play a more and more important role, since the contrast especially is orders of magnitudes better than in the conventional absorption contrast.

Scanning SAXS uncovers a wealth of information on the nanostructures in human tissues over macroscopic areas in real space. The identification of the specific features from the scattering patterns needs additional information, which requires detailed medical knowledge. So far data of human tissues are available from healthy and carious teeth, bone and cartilage, healthy and malign breast biopsies, as well as male and female urethras. In all cases, one finds a high degree of organization along the entire nanometer range for both hard and soft tissues in humans. Human tissues exhibits a strong anisotropy often related to the direction of mechanical loading.

We do expect that scanning SAXS will play a dominant role in the further development of nanomedicine and related fields. One example, namely bioinspired dental fillings that should have different orientations on the nanometer scale within the dentin and the enamel has been already published and will help to further understand the unique mechanical properties of our denture. The actual realization of the nanoscopic anisotropy of the fillings, however, is still a challenge for the researchers in the field of nanodentistry.

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Chapter 5
Nanodentistry

Simone E. Hieber and Bert Müller

Abstract Nanodentistry is defined as the application of nanotechnology to diagnose, treat, and prevent oral and dental disease. These approaches intend to preserve and improve the dental health. Nanotechnology deals with structures in the range of 1–100 nm and focuses on the development of materials with novel properties often not present in nature. As a result, it is considered as a key technology of the twenty-first century and promised to deliver innovative methods to medicine in general and to dentistry in particular. Clinical studies already deal with nanotechnology-based tooth treatments and innovative nanocontainers for local drug delivery for more efficient treatments. Nanotechnology has already started to have a significant impact in dentistry namely in periodontology, implantology, prosthetic dentistry, orthodontics, and endodontics. Nanotechnology will offer sophisticated methods for diagnosis, therapy, and prevention, so that a new era in medicine becomes reality, often termed nanomedicine. These tools will also create the field of nanodentistry, which finally results in an interdisciplinary challenge to efficiently educate and train all specialists in dentistry and related materials science.

5.1 Introduction

Nanotechnology is based on structures that are ten thousand times smaller than the diameter of a human hair. It allows not only the fundamental investigation on the molecular level, but also the development of nanostructured materials with novel fascinating properties. As a result, nanotechnology is considered as a key technology of the twenty-first century, where innovative solutions can be expected for many

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medical and dental problems. Nanocontainers for local drug delivery offer powerful tools for an efficient treatment with less negative side effects. Intelligent implants and devices based on nanotechnology will conquer the market and change dental treatments significantly as it already occurred to the field of materials science and medical imaging.

5.2 Nanodentistry

"Nanodentistry" can be defined as the science and technology of diagnosing, treating, and preventing oral and dental disease, relieving pain, and of preserving and improving dental health, using nanoscale-structured materials [1]. The nanotechnology considers generally scales between 1 and 100 nm leading to new properties and functionality of materials that differ fundamentally from what is known from meter, millimeter, and micrometer scales. Nanostructures behave often differently than macroscopic or microscopic ones. The surface of the nanoparticles dominates the materials properties, which are usually given by the bulk. For example, the color of a polymer depends on the size of the components. The nanomaterials are not only promised to improve the properties and functionality of medical products but also to lead to completely new products including drugs. In particular, nanomaterials will be part of engineered products, such as implants, devices, and diagnosis tools.

The term nanodentistry was introduced to a larger community by the cover story of Freitas Jr. in the Journal of the American Dental Association more than a decade ago [2]. He developed his vision to use dental nanorobots for orthodontic realignments in a single office visit, for dentition regeneration and oral health maintenance. He also elucidated the role of nanomaterials and tissue engineering. Finally, he pointed out that properly configured dentifrobots will identify and destroy pathogenic bacteria residing in the plaque and elsewhere. He launched the new era in dental medicine, termed nanodentistry.

5.3 Imaging

The fundamental knowledge of the human tissues on the nanometer scale is required to take advantage of these innovative technologies for patients in an efficient manner. Radiologists can make visible the human body down to a spatial resolution of a fraction of a millimeter using computed tomography. The resulting datasets are so huge that the data processing cannot be performed manually and requires a handling by means of appropriate software.

A higher spatial resolution can only be achieved by means of postmortem methods. Microtomography is an established method in materials science and reaches a spatial resolution down to the submicrometer range. It allows the nondestructive

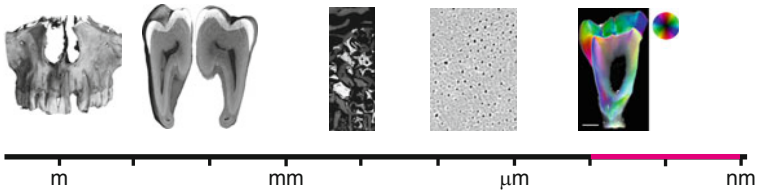

Fig. 5.1 The scales ranging from the size of a human down to the molecular level in the field of dental medicine (nanotechnology length scale given in *red*)

three-dimensional imaging of human tissues as recently demonstrated by the visualization of individual ganglion cells [3] or Purkinje cells [4] leading to complementary information to conventional histology. The resulting three-dimensional datasets are several orders of magnitude larger than the typical clinical tomograms and require the handling by an expert. As a result, the nanometer-sized structures are invisible for the conventional X-ray tomography methods.

Electron microscopy offers an alternative to image nanostructures. However, it can only reproduce surfaces of the specimens and requires vacuum conditions. The wet tissues cannot be investigated in physiological state. In general, however, electron microscopy techniques deliver valuable images particularly for the qualitative analysis.

Scanning probe microscopy techniques enable imaging in fluids, but only provide two-dimensional images of small surface areas. Reasonable areas on human tissues require long scanning times.

X-ray scattering and diffraction methods allow experiments in fluids. The resulting data are exact mean values of the illuminated area. These methods are very useful to quantify nanostructures in human tissues such as collagen fibers or apatite crystallites. Using a focused X-ray beam of about 10 or 100 µm in diameter, which can be scanned along a tissue specimen, one can combine the scattering data uncovering the nanometer range with the less detailed real-space raster. In this way, one obtains fascinating colorful images yielding the spatial distribution of abundance and orientation of all present nanostructures as exemplarily indicated in Fig. 5.1 by a 300-µm-thick tooth slice.

Figure 5.1 shows a logarithmic scale from 1 m down to 1 nm. The incorporated images are characteristic for the length scale. Nanomedicine can significantly influence the entire human body, but is based on phenomena in the red-colored range between 1 and 100 nm. As a result, the methods, such as small-angle X-ray scattering (SAXS) [5], will play an important role for the characterization of human tissues and the future development of biomimetic, nature analogue implants [6]. The images incorporated in Fig. 5.1 show a dataset from clinical CT representing the hard tissues of a part of the human head, conventional micro computed tomography data of a tooth, two virtual cuts through tomograms obtained by synchrotron radiation-based micro computed tomography of a biopsy from bone augmentation treatments [7,8] and a dentin specimen with resolved dentinal tubules [9], and finally the scanning SAXS pattern mentioned above. More details are given in another chapter in the present book [10].

To illustrate the Herculean task to build a human being atom by atom, we estimate the number of atoms within our body. Assuming that the body basically consists of water (molar mass 18 g/mol), we obtain for a human body of 90 kg a number of 3×10^{27} atoms. This huge number cannot be captured by our imagination. Restricting the consideration to the number of atoms within an individual biological cell or the number of cells within our body, one obtains numbers of the order of 10^{14}, which is still an unimaginable number. Note, it is thousand times larger than the number of stars in the Milky Way. You can find more analogies on the nanometer scale given for dentists in [11]. Conclusively, the three-dimensional visualization of the human tissues has progressed significantly and will face demanding challenges for interdisciplinary research teams in future.

5.4 Roadmap

Figure 5.2 shows a roadmap illustrating the likely progression of successful research activities in the field of dentistry applying nanotechnology. Nowadays, implant surfaces are micro- and nanostructured to improve the bone-implant interface. One uses procedures such as etching and sandblasting without knowing the best conditions. The recipes are based on experience of engineers and medical specialists. Especially the tailoring of the nanostructure could significantly improve the inflammatory behavior of the bone implants [12]. In bone, we do find apatite crystallites, which are assumed to exhibit a similar functionality. Multifunctionality of biomaterials is already observed in several cases. A degradable poly(D,L-lactic-co-glycolic acid) membrane, for example, covers the wound after tooth extraction and the degradation products act antibacterial and, therefore, avoid infections. The full potential of multifunctionality, however, is not exhausted yet. Therefore, numerous examples will be developed during the next decade.

One of the forthcoming challenges is associated with the realization of organs or essential parts of them. Maybe the first prototypes are extracorporal devices, but with increasing miniaturization, implantation becomes more and more likely.

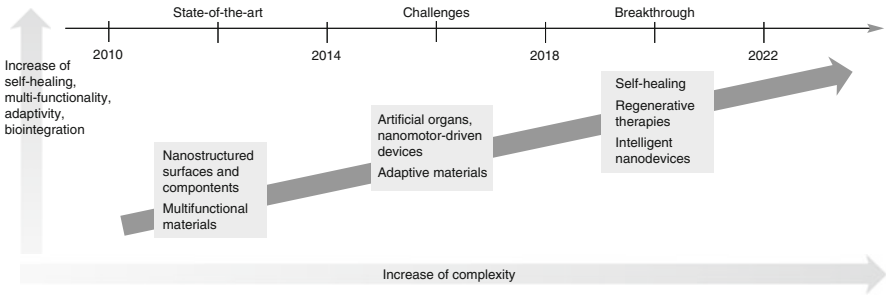

Fig. 5.2 Roadmap of possible nanotechnology approaches toward breakthrough in nanodentistry

Nanomotors may drive orthodontic treatments and distractors or extraction tools with superior precision. In a next step, regenerative therapies should be established to support self-healing for the wide variety of patients avoiding negative side effects.

Biomedical engineers will improve the multifunctional biomaterials to more complex and adaptive ones, so that potential stress-shielding and other nondesired phenomena are kept away from the affected parts of the body. Along with these demanding research tasks, engineers will develop intelligent nanoscale systems promoting the self-healing potential of the human body. Regarding the current state-of-the-art, we expect a breakthrough around the year 2020 associated with a substantial increase of complexity. After the breakthrough, simplifications are expected to lower the degree of complexity and to reduce the costs of nanotechnology-based devices. The application of intelligent nanodevices or nanorobots [1] will not only optimize the multifunctionality, the adaptation and the integration of implants, but also enable the desired self-healing capabilities guided by well-educated and well-trained surgeons and dentists.

5.5 Biomaterials Science

The common biomaterials are ceramics, metals, and polymers or any kind of combination (see Fig. 5.3). Using nanotechnology, one can integrate patterns on the nanometer scale at the surfaces and within the volume of the materials to accomplish the dedicated functionality. Nanostructures can optimize the biocompability of implants or carry drugs towards the target. Reactive nanostructures will emerge in diagnosis, monitoring, and therapy to improve the patient treatments. Numerous activities will fertilize the basic research of bio-nanomaterials.

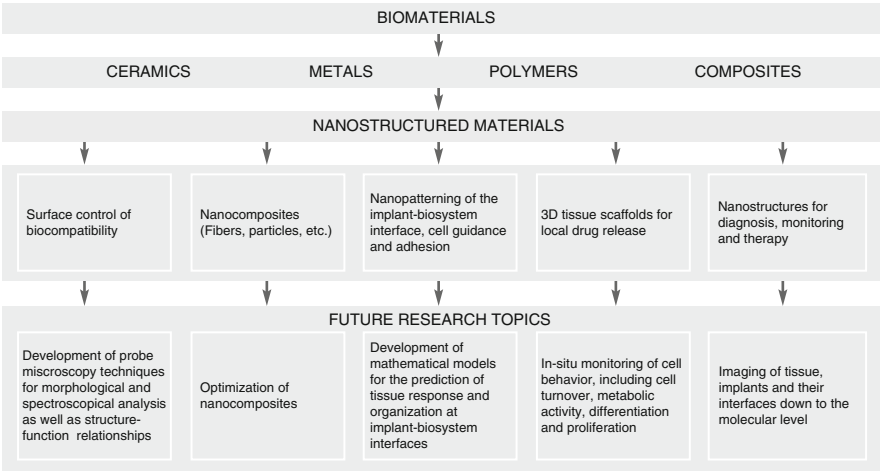

Fig. 5.3 Materials science for nanodentistry

For example, scanning probe microscopy techniques will enable the morphological and spectroscopic analyses of tissues to understand the structure–function relationship on the atomic scale. To improve the properties of the individual materials and their combinations sophisticated mathematical simulations will be expanded to predict the tissue response and the self-organization at the implant–biosystem interfaces down to the molecular level. Moreover, physics-based in vivo measurements supporting cell biologists will reveal the cell behavior with respect to metabolism, cell differentiation, and proliferation especially at the interface between the man-made material and the biosystem. For this purpose, high-resolution imaging facilities, as discussed above, will become more and more important to reveal the nanostructures of tissues, implants, and their interfaces with the necessary precision.

5.6 Major Research Topics

The German BMBF program in nanotechnology involved grants of in total 24 million Euros for German research projects in 2005. The 7th Research Program of the EU supports projects in nanomedicine with 100 million Euros during the period 2007–2013. More than a third of this amount was granted for cancer research, followed by regenerative medicine and imaging. Consequently, one expects that especially the nanotechnology will become a cross section and key technology in diagnosis and therapy of all kinds of cancer including those in the oral cavity. Researchers work for designated nanodevices that allow diagnosing cancer already in the early stage and deliver the drugs directly to the affected cells. Nanodevices will detect cancer-specific biomolecules in vivo faster and more precise than nowadays in committed laboratories.

Moreover, the research will focus on the development of biosensors for monitoring purposes, which includes different quantities related to the oral cavity such as halitosis. The techniques require an improvement of the micro- and nanoscopic methods as shown in Fig. 5.4. These methods play a predominant role for the three-dimensional visualization of nanoscale features, the development of nanocomposites as biomimetic, anisotropic implants [6], and the monitoring of biological processes in real time.

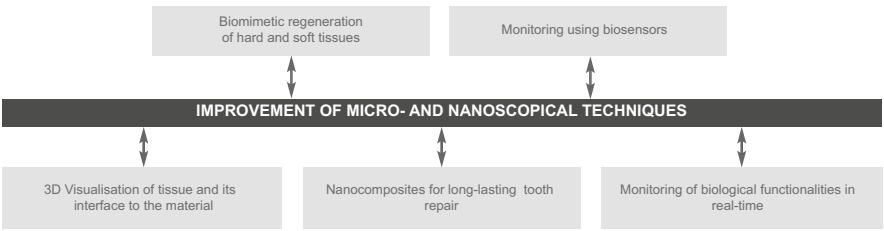

Fig. 5.4 Major research topics in nanotechnology for Cancel dental dentistry

5.7 Applications of Nanotechnology in Dentistry

Figure 5.5 shows selected applications of nanotechnology in the field of dentistry. Filling materials for reconstructions, dental root implants, bone augmentation, and dentin remineralization already take advantage of nanotechnology today, but have increasing growth potential. Today's dental materials will be replaced by nature analogue, anisotropic tooth restorations. It is, however, still unclear how the anisotropic biomaterials can be oriented. The nanostructures in dentin are orthogonally oriented to the ones of the same size in the enamel. In this way, the dentin–enamel junction acts as crack barrier [13]. The calcium phosphate phases for bone augmentation gain more and more importance along with the increase in age of the population. The resorbable calcium phosphate phases or bioglasses support the growth of the natural bone being applied to larger and larger defects. The materials have to be optimized on the micro- and nanometer scales to tweak the biocompability, the bioactivity, and the osteoconductivity promoting tissue regeneration and resisting the mechanical loads.

The tooth implants of established manufacturers are inserted with a high success rate. A broader distribution is hindered by their high costs, peri-implantitis, and time-consuming osteointegration until first loading. We expect products of even

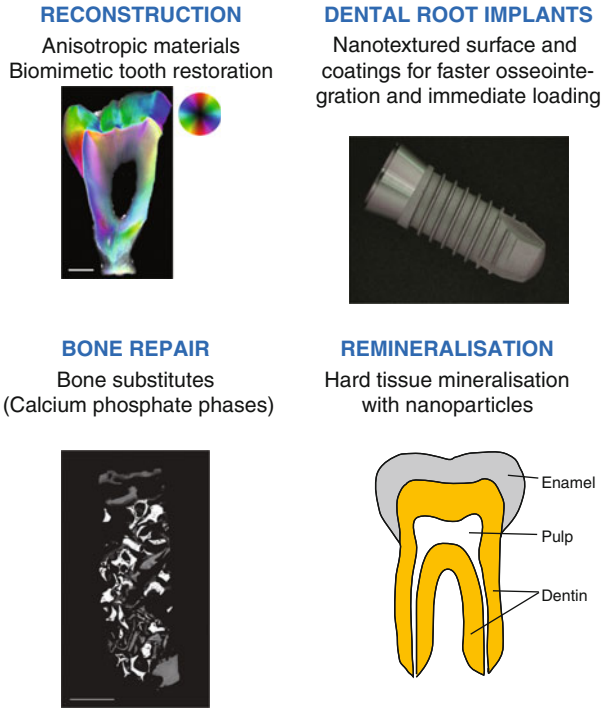

Fig. 5.5 Applications of nanotechnology in dentistry

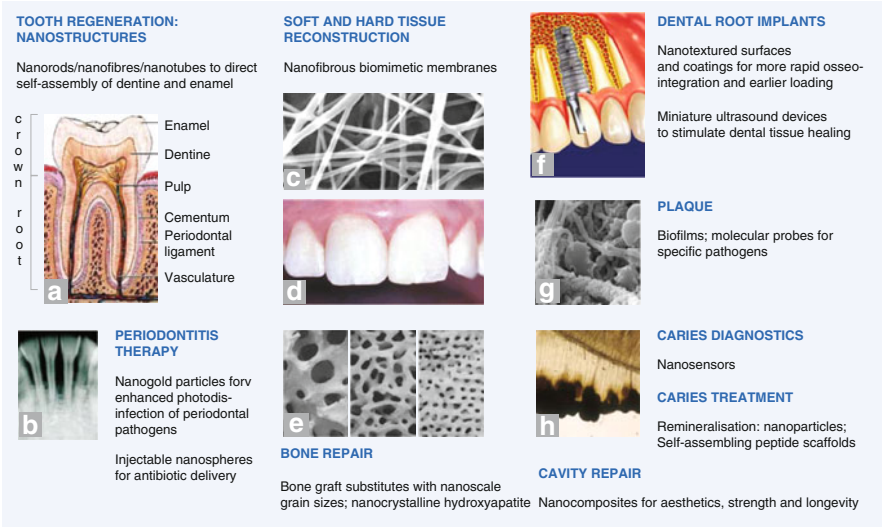

Fig. 5.6 Overview of several applications of nanotechnology in dentistry. (**a**) Anatomy of the tooth; (**b**) Radiograph showing bone loss in periodontal disease; (**c**) A nanofibrous membrane for soft tissue repair to mimic the natural extracellular matrix; (**d**) Composite fillings must match as closely as possible the color and translucency of natural teeth; (**e**) Hydroxyapatite scaffolds for bone repair; (**f**) Diagramme (DENTSPLY Friadent), of a dental implant showing osseointegration; (**g**) Scanning electron microscope image of dental plaque showing mixed bacterial biofilm and cells. (**h**) Tooth section showing caries developing in the enamel (copyright [1])

higher quality standards and significantly lower costs due to the fabrication progress in the coming years. This especially comprises the micro- and nanostructures surfaces that guarantee the osteointegration.

The remineralization of tooth hard tissues will be one of the main tasks of nanotechnology in the aging industrial societies. Nanoparticles are already used in "sensitive" toothpastes. In the near future, damaged teeth (by caries) will be remineralized applying mature ceramic nanoparticles.

Figure 5.6 contains further applications: more or less complex nanostructures for self-assembly of tooth hard tissues, periodontitis therapies by means of nanoparticles/nanophotonics, plaque monitoring, and caries diagnosis and treatment. Note caries is the most frequent dental disease known to damage the enamel, the dentin, and the cementum through the production of acidic species that dissolve the ceramic tooth components. Regrettably, the destroyed tooth structures almost do not regenerate, although remineralization of slight carious lesions occurs under optimized dental hygiene conditions.

This fundamental knowledge of the tooth's nanostructure should be applied for introducing biologically inspired dental fillings. It is the aim to realize dental materials with a nanometer-scale architecture similar to the ones in enamel and dentin [9]. After caries significantly damaged the tooth's hard tissues, the dentist removes the damaged part and rebuilds the crown using conventional isotropic

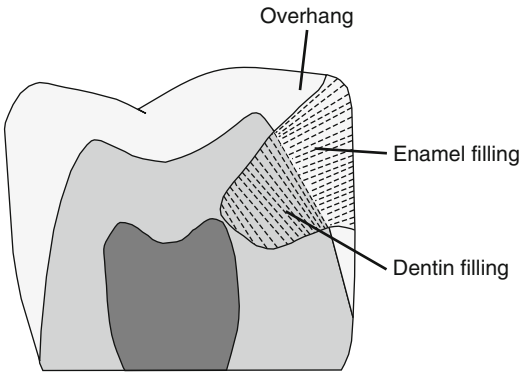

Fig. 5.7 Bioinspired, nature analogue dental fillings

composites that do not resemble the anisotropic nanostructure of human teeth. The use of such shrinking material is the reason, why the dentist regularly removes more enamel than necessary. Overhangs, as shown in Fig. 5.7, are avoided to prevent crack formation. Therefore, nonshrinking nature analogue fillings are highly desirable to achieve better restoration quality by less invasive treatments. The key challenge in achieving such nanotechnology-based fillings lies in the arrangement of the nanometer-sized building units. Here, the following approaches to mimic the hard tissues of the human teeth can be foreseen. Crystalline growth, which take place at nonequilibrium conditions with temperature or concentration gradients, result in strongly anisotropic nanostructures [12]. Unfortunately, such processes are demanding and seem to be impractical for patient treatments. Alternatively, nanofiber composites as already successfully built-in in dental posts can be applied. The appropriate orientation of the fibers, however, is not solved. Nanometer-sized arrangements of charged or dipolar units such as dedicated nanorods or nanotubes could give rise to parallel arrangements of the anisotropic nanostructures. The dashed lines in Fig. 5.7 symbolize the potential arrangements of charged nanorods. Because of the positive or negative charges, the nanorods show repulsive interactions leading to more or less parallel equidistant alignments [14]. Such dental restorations will have excellent resistance to thermal and mechanical shocks as recognized from the hardest substance in our body – the nanostructured enamel.

5.8 Challenges

Figure 5.8 shows the main challenges in nanomedicine/nanodentistry from current point of view. Assessment tools for nano-biomaterials are important to judge the toxicity and the biocompability and to pursue the personalized medicine with respect to material incompatibilities. Nanomarkers are developed to deliver more accurate test results for diagnosis down to the cellular level and below. Nanocontainers will enable local drug delivery for therapies preventing undesired side effects. Nanomaterials have already been used to stimulate the healing as

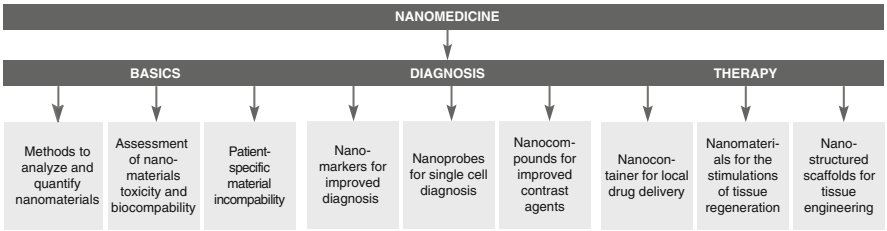

Fig. 5.8 Selection of challenges in nanomedicine/nanodentistry

demonstrated in first clinical trials. Nanostructured scaffolds for cell guidance are the basis in contemporary tissue engineering. Likewise, these scaffolds can be applied to stimulate tissue formation in vivo. As a result, nanomedicine in general and nanodentistry in particular will affect the basic dental science, the diagnosis and the therapy of oral diseases as well as maintain the oral health.

5.9 Future Risks

Nanotechnology involves not only benefits, but also serious risks that are not completely investigated. The toxicity of nanoparticles has to be assessed. The size of the particles leading to the interesting properties can also be a risk for human health. Nanoparticles undermine the human immune system and have almost unlimited access to the entire body [15]. Sensitive organs such as the brain can suffer from them. Moreover, the interactions with the immune system have to be investigated in detail. An open question exists concerning the final stage of the particles: Do they drop out or do they accumulate in specific organs? Biodegradable substances should decompose and leave the body. Nonbiodegradable particles often accumulate in organs as liver [15]. Up to now, we cannot estimate the long-term effects of nanotechnology. The impact on our environment has also to be uncovered in a similar manner as we do it today for drugs. The nanoparticles can contaminate water, soil, and air. The environment is faced with man-made particles becoming a new class of pollution [15]. Besides the investigation of the side effects, its report to the public will be important to achieve the acceptance of nanomedicine and nanodentistry.

5.10 Tailoring Biocompatibility of Implants by Nanostructures: Benefitting Patients

At first glance, one may expect that biocompatibility is considered as a specific material property, such as color or heat conductivity. Biocompatibility, however, is a strange term, not as well defined and standardized as typical material properties.

Nonetheless, we do have a certain understanding of biocompatibility. Consulting a dictionary, one finds: "Biocompatibility is the ability of a material to perform with an appropriate host response in a specific application" [16]. Using such a definition, however, the engineers and natural scientists are lost since biocompatibility not only depends on the specific solid-state material but also on the surrounding tissue. How one deals with a term such as biocompatibility, which does not even have a dedicated unit? One of the related questions concerns the roughness of titanium bone implants. This roughness generated by sandblasting and etching guarantees the osteointegration experimentally proven for million times. The details on the preparation procedures belong to the secrets of the implant suppliers. The measurement of the roughness, however, is more than challenging, since one cannot simultaneously determine it on the micro- and nanometer scales. Certainly, different techniques do exist, suitable for length scales of interest, but there is no unique parameter for surface roughness – with one exception – the Wenzel ratio, which is the actual surface divided by the projected one. In fact, nobody had measured this on the atomic level until it was realized that Ge nanopyramids on Si(100) with their well-defined facets, termed hut and dome clusters, can be counted to derive the Wenzel ratio [17]. This Wenzel ratio was correlated to biocompatibility measurements. First, the dynamic contact angles were measured on the surfaces with different nanopyramid densities, but the effect was only marginal. Second, absorption studies with the most important proteins (albumin and globulin) as well as in vitro cell experiments were carried out. Here, huge effects were uncovered. While the monocytes were exclusively damaged on the surfaces without nanopyramids, the rough surfaces (with many nanopyramids) had a very positive effect on inflammatory reactions [18]. This observation gave evidence that biocompatibility of medical implants can be tailored using nanostructures. Therefore, we obtained not only a positive message for the patients but also for the engineers and natural scientist.

Several parameters (see Fig. 5.9) are identified to intentionally manipulate the biocompatibility of bone implants. Each of the parameters (surface morphology

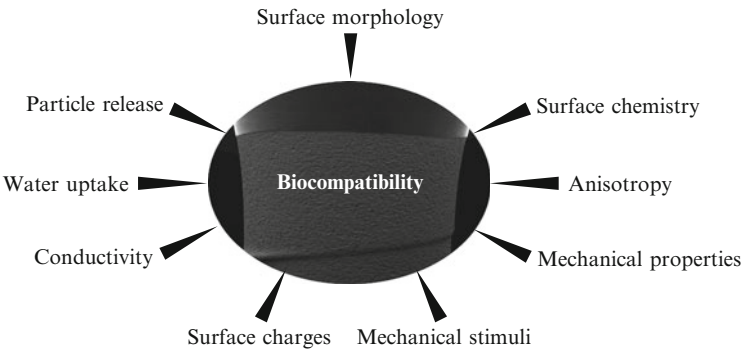

Fig. 5.9 Many parameters are used to tailor the biocompatibility of medical implants

and chemistry, mechanics, local and global electrical charges, uptake and release of atoms, molecules, and particles) can be used to improve the implant's functionality and also depends on the host tissue or the implantation site, as stated in the definition mentioned above – a lot of fascinating challenges for future generations of nanoscientists and nanoengineers [19].

5.11 Conclusions

Nanotechnology will lead to a new era of dental medicine that will change the current methods in diagnosis, treatment, and prevention of the different patients. As medicine advances and people live longer, nanodentistry will play an increasing role in enabling people to keep their natural teeth and oral tissues healthy and functioning forever. The scientists will understand in detail how the teeth grow, develop, and heal. The medical experts will understand the assembly of nanostructures in dentin and enamel to enable the development of biomimetic tooth repair and regeneration. Dentists will be able to reconstruct hard and soft periodontal tissues as well as to treat caries including biomimetic remineralization and repair of diseased teeth. In this manner, the role of well-educated and well-trained dentists will become more and more important.

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Chapter 6
Complement Activation-Related Pseudoallergy Caused by Nanomedicines and its Testing In Vitro and In Vivo

Janos Szebeni and Rudolf Urbanics

6.1 Introduction

Nanotechnology has has been giving birth to a variety of therapeutic and diagnostic products, referred to as nanomedicines (NM), whose successes are based on improved efficacy and/or diminished toxicity. However, these benefits are not without a price. The introduction into the clinics of many NM revealed the presence of an acute immune response to the particles, manifested in hypersensitivity reactions (HSR). The phenomenon is often due to the structural similarity of reactogenic NM to viruses, which may trigger the nonspecific arm of humoral immunity, the complement (C) system to an immediate eliminatory response. The clinical manifestations of this reaction, called C activation-related pseudoallergy (CARPA), include cardiopulmonary distress, which is a safety risk for NM, particularly in the case of cardiac patients with atopic constitution. Thus, understanding CARPA and ways of its prediction and prevention represents an important challenge in NM R&D.

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6.2 Pseudoallergy as Adverse Effects of Drugs

According to the FDA Adverse Event Reporting System, during a 5-year period, for 610 drugs, the rate of adverse immune effects was 14.5% [1]. Symptoms are manifested in almost every organ systems (Table 6.1), varying from mild skin and respiratory changes to severe cardiopulmonary distress or even lethal anaphylaxis.

Importantly, more than three-fourth of these adverse immune reactions cannot be attributed to classical IgE-mediated drug allergy, qualifying them as pseudoallergy [2]. Furthermore, many of these pseudoallergic reactions have been shown to be due to complement (C) activation, rationalizing the distinction of CARPA as a novel subcategory of type I allergic reactions (Fig. 6.1) [2]. The symptoms of CARPA mostly overlap with those observed in classic allergy; however, there are unique features as well (Table 6.2), which clearly distinguishes CARPA from classic type I reactions [6, 7].

Nanoparticle forming medicines causing CARPA include liposomal and micellar drugs (Doxil, Ambisome, and Taxol), but monoclonal antibody therapies also elicit similar reactions (Table 6.3).

6.3 Mechanism of CARPA

As illustrated in Fig. 6.2, during CARPA, C is activated at several levels by various NM (shown are liposomes, micelles, and IgM), leading to the liberation of anaphylatoxins (C3a, C5a) and C5b-9, i.e., C cleavage products that can trigger mast

Table 6.1 Symptoms of CARPA

Cardiovascular	Bronchopulmonary	Mucocutaneous	Neuro-psychosomatic	Vegetative	Vital
Angioedema	Apnea	Cyanosis	Back pain	Chills	Death
Arrhythmia	Bronchospasm	Erythema	Chest pain	Diaphoresis	Loss of consciousness
Cardiogenic shock	Coughing	Flushing	Chest tightness	Diarrhea	Syncope
Hypertension	Dyspnea	Rash	Headache	Dizziness	
Hypotension	Hyperventilation	Rhinitis	Feeling of imminent death	Fever	
Hypoxia	Laryngospasm	Swelling	Fright	Nausea	
Myocardial infarction	Pulmonary infiltrates	Urticaria	Panic	Sweating	
Tachycardia	Respiratory distress		Rigors	Vomiting	
Ventricular fibrillation	Shortness of breath		Tightness in chest, throat	Wheezing	

Those most frequently caused by NM are shown in red

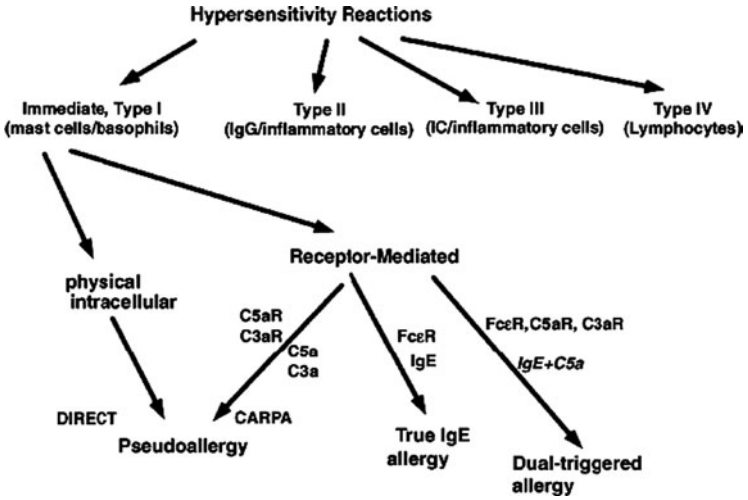

Fig. 6.1 Proposed new scheme of immediate hypersensitivity reactions [2]

Table 6.2 Symptoms of CARPA distinguishing it from type I allergic reactions

- Reaction arises at first treatment (no prior exposure to allergen)
- Reaction is milder or absent upon repeated exposures
- Spontaneous resolution
- High reaction rate (up to 45%), average 7%, severe 2%

cells, basophils, and leukocytes, via specific receptors, for the secretion of a large number of mediators, collectively called allergomedins. This redundant triggering, complex chain of molecular interactions and signaling, and numerous control steps, together with the patient's genetic and health status, may explain the substantial individual variation of CARPA symptoms.

Regulatory agencies recognize the critical importance of adverse immune reactions to NM, as reflected in their various publications, for example, a 2002 Guidance for Industry entitled "Immunotoxicological Evaluation of Investigational New Drugs." A recent guideline (ICH Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals, CHMP/ICH/302/95) states: "*If signs of anaphylaxis are observed in animal studies, follow-up studies should be considered.*" However, at present, there is no gold standard, or validated animal model of NM-induced anaphylaxis, or in vitro assays that could predict anaphylactic reactions, or CARPA, to NM. The in vitro and in vivo methods described below, which have been applied in our laboratory(ies) for some 15 years, highlight some possible approaches to fill this gap.

Table 6.3 Nanomedicines and other agents causing pseudoallergy

Liposomal drugs and diagnostics	Micellar drug formulations	Radio and ultrasound contrast agents	Antibody-based therapeutics & diagnostics	Enzymes proteins peptides	Miscellaneous other
Doxyl (Caelix)	Taxol	Diatrizoate	Avastin	Avonex	Cancidas
Ambisome	Taxotere	Iodixanol	Enbrel	Actimmune	Copaxone
Amphocyl	Cyclosporine	Iohexol	Herceptin	Abbokinase	Orencia
Myocet	Etoposide	Iopamidol	Humira	Aldurazyme	Eloxatin
DaunoXome		Iopromide	Raptiva	Activase	Salicilates
Tc^{99-}-HYNIC-PEG		Iothalamate	Synagis	Zevalin	
		Ioversol	Xolair	Neupogen	
		Ioxaglate	Compath	Neulasta	
		Ioxilan	Erbitux	Fasturtec	
		SonoVue	Mylotarg	Plenaxis	
		Magnevist	Remicade		
			Rituxan		
			Vectibix		
			Tysabri		

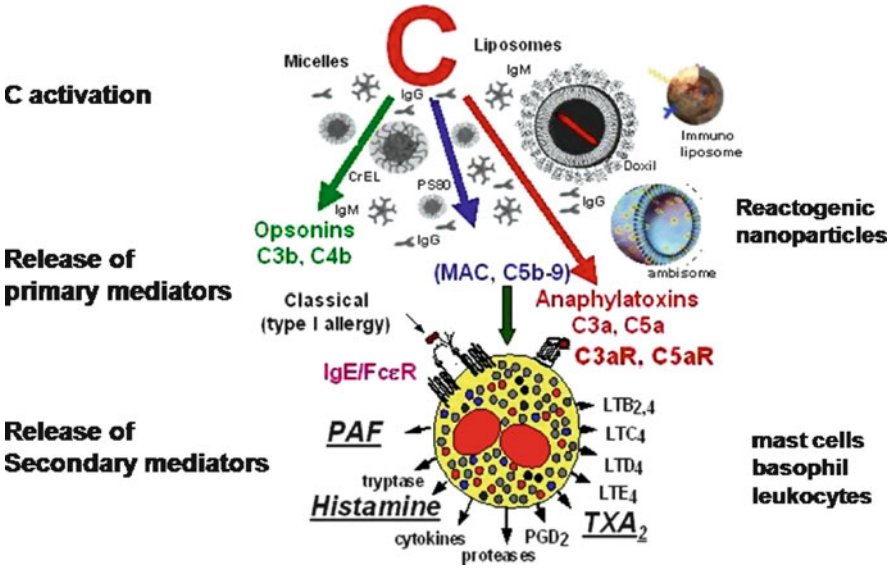

Fig. 6.2 Mechanism of CARPA

6.4 In Vitro and in Vivo Assays of CARPA

The following tests are available for assessing C activation by NM in vitro [3]: (1) ELISA of C5a, C3a, SC5b-9, C4d, and Bb; (2) C consumption assay (sheep red

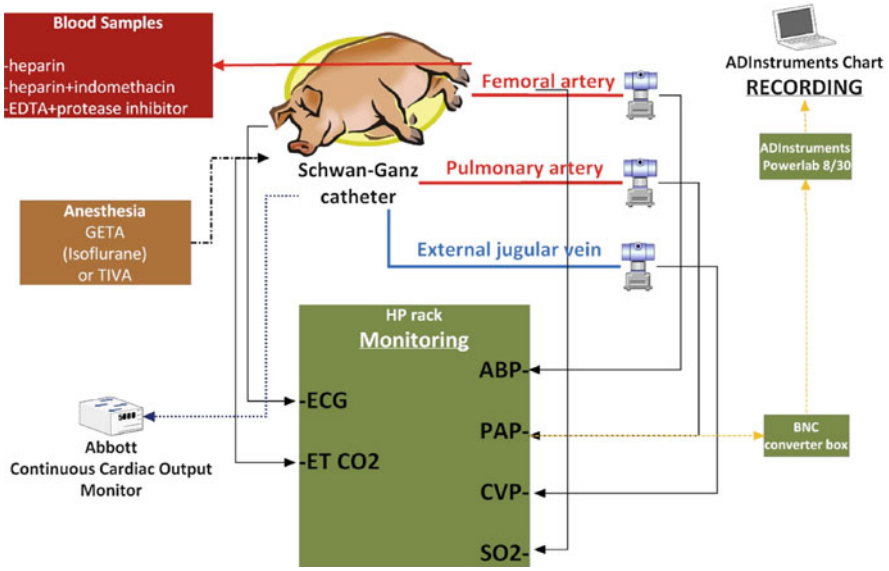

Fig. 6.3 Porcine bioassay of nanomedicine-induced CARPA

blood cell hemolysis, CH_{50}); and (3) basophil leukocyte activation in human blood, for example, FACS analysis of CD203c upregulation.

For the C ELISA and the CH_{50} assay, the test agents are incubated with whole (or diluted) human (or animal) serum, for example, for 30–45 h at 37°C with shaking (e.g., at 80 rpm), and C activation is assessed by measuring the C split products using the listed ELISA or assessing C consumption using the CH_{50} assay. For negative and positive controls, serum is incubated with PBS, pH 7.4, and 5 mg/ml zymosan, respectively. In the basophil assay, the test agents are incubated with whole blood [3].

The most sensitive and biologically relevant animal model of severe human HSR is the porcine cardiopulmonary response to iv-injected NM. Pigs are particularly sensitive to intrapulmonary injection of nanoparticles, such as liposomes [2–5], as they develop a major hemodynamic changes following iv injection of minute (milligram) amounts of particulate NM. In particular, intravenous injection of certain liposomes was shown to cause massive rises in pulmonary arterial pressure (PAP), rises or falls in systemic arterial pressure (SAP) and heart rate, and falls in cardiac output (CO) [4]. The ECG changes include arrhythmia with ventricular fibrillation and cardiac arrest, the latter being lethal unless the animal is resuscitated with epinephrine with or without electroconversion.

A scheme of the porcine CARPA model is shown in Fig. 6.3, to illustrate the various connections and instruments used to monitor the cardiopulmonary and hematologic changes that follow iv injection of NM, such as liposomes.

Adolescent Yorkshire swine in this model are sedated with im ketamine and anesthesized with isoflurane, using an anesthesia machine, or with nembutal iv. A pulmonary artery catheter is advanced via the right internal jugular vein through the right atrium into the pulmonary artery to measure pulmonary artery pre-wedge pressure (PAP). Systemic arterial pressure (SAP) is measured in the femoral artery. The test materials are diluted in PBS and injected into the pulmonary artery as bolus, via the pulmonary arterial catheter.

6.5 Summary

Hypersensitivity reactions to NM can be caused by activation of the C system, a phenomenon called CARPA. The CARPA reactogenic potential of NM in man can be assessed by measuring C activation and/or consumption in human sera, and basophil leukocyte activation in whole blood, and by monitoring the cardiopulmonary changes in pigs following i.v. administration of NM. These diagnostic approaches are likely to gain increasing use in cases when freedom from HSR is critical for bringing novel NM into clinical use.

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Chapter 7
Pharmacogenomics and Nanotechnology Toward Advancing Personalized Medicine

Ioannis S. Vizirianakis and Elsa P. Amanatiadou

Abstract The target of personalized medicine to achieve major benefits for all patients in terms of diagnosis and drug delivery can be facilitated by creating a sincere multidisciplinary information-based infrastructure in health care. To this end, nanotechnology, pharmacogenomics, and informatics can advance the utility of personalized medicine, enable clinical translation of genomic knowledge, empower healthcare environment, and finally improve clinical outcomes.

7.1 Introduction

Advancements in molecular pharmacology, genomics, bioinformatics, and nanotechnology are changing the health and pharmaceutical care environment by enriching disease pathophysiology knowledge, empowering clinical diagnosis potential, and improving drug-delivery outcomes [1, 2]. As a matter of fact, molecular medicine has emerged in a way to implement clinical practice. Therapeutic issues, such as disease susceptibility behavior and inter-individual pharmacological variability of drug response [inefficiency and/or toxicity causing the emergence of adverse drug reactions (ADR)] have now been better understood [3, 4]. To this direction, the development of specialized nano-vehicles and devices and their application in drug delivery and clinical diagnosis have unanimously also facilitated the integration of genomics knowledge in health care [5, 6]. Gene expression profiling, genotyping and haplotyping analysis, molecular diagnostics use, and biomarkers assessment, just to mention a few, are now evaluated for their clinical exploitation to improve diagnosis and therapy outcomes. As far as the drug-delivery process is

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concerned, this means that the establishment of specific molecular signatures and profiles for genes implicated in drug response, or ADR can be used as specific biomarkers for the prediction of pharmacotherapy outcomes in a given individual, or even for a specific group of patients [7–9]. By extending such a notion to everyday clinical practice and drug prescription, pharmacotyping emerges as a new dimension in pharmacogenomics to describe this transition in pharmaceutical care [1, 6, 10]. Such a case, however, means movement from a drug-selection process mainly based on the physician's own experience, into a more highly integrated, information-based, computer-aided and personalized pharmacotherapy delivery. However, this projection of health environment represents painstaking and time-consuming processes. On the contrary, the recent application of new sophisticated high-throughput tools for extracting, analyzing, and mining genomic data of clinical relevance, along with technological advancements enabling information-based medicine has revived the expectations for a wide application of pharmacogenomics and practical utility of personalized medicine for most people. This notion is further supported by recent achievements in the era of *in silico* modeling for predicting absorption, distribution, metabolism, and excretion (ADME) data, permitting improved development of innovative molecularly targeted drugs with enhanced clinical safety and efficacy [11–16].

7.2 From Genetics and Genomics to the Advent of Pharmacogenomics and Nanomedicine

In the previous years, scientific efforts provided the fertile ground for the transformation of genetics into genomics and the advent of pharmacogenomics from pharmacogenetics (i.e., single gene polymorphisms correlated with pharmacological response) as a new discipline to bridge the gap between pharmacology, genetics, and therapeutics. Undoubtedly, it is now well known that many ADR arise due to inter-individual genetic polymorphisms found in drug-metabolizing enzymes, drug transporters, ion channels, and/or receptors [3, 4, 17]. Indeed, several pharmacogenomics studies have uncovered a number of clinically relevant genetic variations that are well correlated with either altered drug response or the onset of diseases (Table 7.1). Such a direction is facilitated by attempting (a) to understand the clinical relevance that may exist between the genetic make-up of an individual and the various drug effects exerted in the body, and (b) to translate and apply clinically the extracted knowledge toward improving therapeutic outcomes.

Sophisticated target-guided technological nanodevices and information-based advances achieved thus far, pave the way by which genomic knowledge is entering clinical practice and is also being used to analyze complex diseases including cardiovascular diseases, diabetes type-2, asthma, cancer, and degenerative disorders [18–23]. Furthermore, the broad use of automated DNA sequencing techniques and the application of DNA microarray (DNA chip) systems permit the vigorous and

Table 7.1 Selective pharmacogenomic biomarkers for improving drug-delivery and disease prognosis outcomes

Gene/specific allele	Drug	Efficacy/safety outcome
Philadelphia (Ph1) chromosome [t(9;22) translocation][a]	Busulfan	Lower efficacy; treatment is less effective in patients or young children who lack the Philadelphia (Ph**1**) chromosome suffering from either CML[a] or the so-called juvenile type of CML, or even whose CML entered a "blastic" phase
	Dasatinib	Enhanced efficacy; treatment only of adults with Philadelphia chromosome-positive ALL (Ph+ALL) with resistance or intolerance to prior therapy
CYP2C9	Warfarin	Lower safety; ADR; risk of bleeding
CYP2D6	Atomoxetine	Lower safety; ADR; dose reduction for PM patients
	Thioridazine	Lower safety; ADR; QT prolongation, torsade de points
	Codeine	Lower safety; ADR; overdose symptoms for UM patients
CYP2D6	Tamoxifene	Lower efficacy; loss of therapeutic benefit for PMs and/or upon coadministration with CYP2D6 inhibitors
c-kit	Imatinib	Lower efficacy; no response in cancer patients with absence of tumor-activating c-Kit mutations
Del(5q)	Lenalidomide	Enhanced efficacy; Lenalidomide is indicated for the treatment of patients with transfusion-dependent anemia due to low or intermediate-1 risk myelodysplastic syndromes associated with a deletion 5q cytogenetic abnormality with or without additional cytogenetic abnormalities
G6PD deficiency	Chloroquine Primaquine	Lower safety: ADR; hemolytic reactions (moderate to severe) may occur in G6PD-deficient patients
	Rasburicase	Lower safety; ADR; hemolysis in G6PD-deficient patients
EGFR	Erlotinib	Lower efficacy; no response in cancer patients with tumor EGFR-negative expression
	Cetuximab	Lower efficacy; no response in cancer patients with tumor EGFR-negative expression
	Panitumumab	Lower efficacy; no response in cancer patients with tumor EGFR-negative expression
ER	Anastozole Exemestane Letrozole Tamoxifene	Lower efficacy; no response in cancer patients with tumor ER-negative expression
HER2	Trastuzumab	Lower efficacy; no response in cancer patients with tumor HER2-negative expression

(continued)

Table 7.1 continued

HLA-B*1502	Carbamazepine	Lower safety; ADR; severe cutaneous immunoallergic reaction; increased risk for Stevens–Johnson syndrome (Asian)
HLA-B*5701	Abacavir	Lower safety; ADR; increased risk for hypersensitivity reactions
HLA-B*5801	Allopurinol	Lower safety; ADR; increased risk for Stevens–Johnson syndrome
K-RAS	Cetuximab Panitumumab	Lower efficacy; no response in cancer patients with tumor-specific K-RAS mutations
NAT variants	Isoniazid, Rifampin, Pyrazinamide	Lower efficacy in patients defined as slow acetylators; ADR; increased risk for cytolytic hepatitis
	Isosorbide dinitrate Hydralazine hydrochloride	Lower efficacy; ADR; in patients defined as slow acetylators
PML/RARα fusion gene	Arsenic trioxide Tretinoin	Enhanced efficacy; initiation of therapy in APL patients detected with the t(15; 17) genetic marker (PML/RARα fusion gene)
Protein C deficiencies	Warfarin	Lower safety; ADR; increased risk for patients with hereditary or acquired deficiencies of protein C or its cofactor protein S to develop tissue necrosis
SLC01B1*5	Simvastatin	Lower safety; ADR; increased risk for myopathy
TPMT	Azathioprine 6-Mercaptopurine Thioguanine	Lower safety; ADR; neutropenia
UGT1A1*28	Irinotecan	Lower safety; ADR; diarrhea, increased risk for severe neutropenia in high doses of irinotecan
VCORC1	Warfarin	Lower safety; risk of bleeding

Modified from [6]

Abbreviations: *ADR* adverse drug reactions, *ALL* Acute lymphoblastic leukemia, *APL* acute promyelocytic leukemia, *CML* chronic myelogenous leukemia, *ER* estrogen receptor, *Del(5q)* deletion of chromosome 5q, *NSAIDs* nonsteroid anti-inflammatory drugs, *PM* poor metabolizers, *UM* ultra-rapid metabolizers

[a]For more details see URL at FDA: http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm(accessedatDecember1,2010)

systematic assessment of information for a large number of genes implicated in drug response and/or disease pathogenesis [24–26]. Alternatively, and of equal importance, specific gene variants have been found to predispose for the susceptibility of individual persons to disease development and progression (Table 7.1). Importantly, this happens in parallel with efforts focusing on the creation and establishment of unified information-based platforms in genomic medicine to support the clinical application of molecular diagnostics and nanodevices. This is a major advantage, since breakthroughs also in nanomedicine have created specialized technological

platforms for the exploitation of genomic information through guided drug delivery and personalized diagnosis and therapy. Such nanomedicine efforts enable the practical utility of personalized medicine since its major goals are considered to be the anticipation and minimization of individualized risk of disease onset and progression as well as drug therapy toxicity [12, 27–29].

7.3 Pharmacological Response Heterogeneity, Drug Interactions, Metabolizing Enzymes, and Therapy Outcomes

It is now well known that polymorphic gene variants of cytochrome P450 (CYP) drug-metabolizing enzymes contribute to differential pharmacological response of drugs seen among patients suffering from the same disease. Indeed, the knowledge of CYP system allowed significant genomic data to be integrated efficiently in clinical practice. CYP enzymes belonging to families 1, 2, and 3 mediate more than 70% of all phase I-dependent mechanism of marketed drugs. Interestingly, polymorphic enzymes CYP2C9, CYP2C19, and CYP2D6 account for approximately 40% of CYP-mediated drug metabolism. The pharmacogenomics of these enzymes is now considered very important for pharmacotherapy decisions. Ignorance of such information is making the application of a general prescribing scheme for drugs whose disposition is determined by these enzymes problematic [30]. Similarly, assessment of the incidence of ADR has estimated that about 56% of drugs cited in ADR-related studies are metabolized by polymorphic enzymes of phase I, in which approximately 86% account for the CYP-mediated metabolism. On the contrary, only 20% of drugs that are substrates of nonpolymorphic enzymes are involved in the ADR reports [30, 31].

Several examples of drugs whose dosage is related to CYP phenotypes have already been reported that further envisage on the importance of CYP polymorphisms for routine drug prescription and the modulation of pharmacotherapy outcomes. By clinically exploiting this knowledge on drug interactions and more importantly by implementing clinical practice and prescription with CYP system evaluation potential, the empowerment of drug-delivery outcomes in terms of safety and efficacy can be established. Indeed, as has been published earlier [4], by assessing the drug interaction potential of cholinesterase inhibitors, it can be notably evaluated if the individual CYP-isoform implicated in the metabolism of each cholinesterase inhibitor also specifies the type of interacting drugs that could modulate the clinical outcome [32, 33]. To this regard, and in order to further outline such a beneficial notion for pharmacotherapy, two examples of drug classes will be considered: (a) cholinesterase inhibitors, (rivastigmine, tacrine, donepezil, and galantamine), agents used to treat symptoms in patients suffering from AD, and (b) the delivery of the antiestrogen drug tamoxifen [6].

The analysis [4] for cholinesterase inhibitors revealed the following:

1. The metabolism of rivastigmine is mediated predominantly by esterases rather than hepatic CYP enzymes, thus clinically relevant drug interactions implicated to CYP are unlikely to happen. This fact for rivastigmine was indeed verified in a retrospective analysis including four major clinical trials with 2,459 patients, where no increase in adverse events was reported among these elderly populations codelivered with antihypertensives, antihistamines, inflammatory-relief agents, and/or anxiolytic agents.
2. Tacrine is primarily metabolized by the hepatic CYP1A2, (and also to some extent by CYP2D6). As a matter of fact, molecules being either substrates, inhibitors, and/or inducers, mainly of CYP1A2, it is expected to alter the bioavailability of tacrine. The latter, has been confirmed (a) with fluvoxamine (administered in 50 or 100 mg/day), a potent CYP1A2 inhibitor, which caused about 85% reduction in the metabolism of tacrine; (b) with cimetidine, also a CYP1A2 inhibitor, which caused an increase by approximately 30% in the plasma concentrations of tacrine, so that a reduction of its dosage has been advised to these patients; and (c) with estradiol, also metabolized by CYP1A2, which caused an increase of almost 60% in AUC and of about 46% in the mean C_{max} of tacrine. Similarly, tacrine decreased by approximately 50% the clearance of theophylline (also a substrate of CYP1A2), so reduction of theophylline dose has been suggested in this case.
3. The same prediction is indeed verified in certain pharmacotherapy dosage regimens for donepezil that is primarily metabolized by the hepatic CYP3A4 and also CYP2D6: (a) with ketoconazole (200 mg/day), a potent inhibitor of CYP3A4, where a significant increase (23–30% at steady-state) in the plasma concentrations of donepezil was observed; (b) with paroxetine and sertraline, (CYP2D6 inhibitors), where a careful patient monitoring has been recommended due to potential drug interactions.
4. Galantamine is metabolized by CYP3A4 and CYP2D6, so again molecules being potent inhibitors of these isoenzymes it is expected to result in clinically significant drug interactions. Such was indeed the case (a) with paroxetine that caused an increase in the bioavailability of galantamine by 40%, and (b) with ketoconazole that exerted a significant increase in the bioavailability of galantamine by 40%.

Following the previous data for cholinesterase inhibitors, recently, delivery of the anti-estrogen drug tamoxifen to hormone-dependent breast cancer women has also clearly demonstrated the usefulness of CYP2D6 metabolism system, both in terms of drug interactions and pharmacogenomics. Tamoxifen is a pro-drug that needs to be metabolized mainly by CYP2D6 into the pharmacologically active metabolites 4-hydroxytamoxifen and endoxifen that mediate the anti-estrogen action. In particular, it has been postulated that the knowledge of CYP2D6 system could efficiently allow healthcare practitioners to individualize dosage schemes to improve therapy outcomes [34–38]. To this end, CYP2D6 poor-metabolizer (PM) patients (due to polymorphic null-activity CYP2D6 alleles), and also women under tamoxifen

therapy coprescribed with potent CYP2D6 inhibitors (e.g., antidepressant drugs fluoxetine and paroxetine) exhibit an increased risk of breast cancer recurrence and mortality due to decreased levels of active tamoxifen metabolites formed in the body. As a matter of fact, the individualized tamoxifen-dosage scheme can be achieved: (a) for CYP2D6 PM breast cancer patients by avoiding the coprescription of tamoxifen with CYP2D6 inhibitors, adjusting tamoxifen dose, and/or switching hormonal therapy to another drug class (e.g., aromatase inhibitors), and (b) for breast cancer patients exhibiting normal CYP2D6 metabolism by choosing an antidepressant with no CYP2D6 inhibitory effect (e.g., venlafaxine) [6].

The above mentioned drug examples of cholinesterase inhibitors and tamoxifen clearly show the predictive value of CYP-related information for everyday drug prescription in terms of both drug interactions and pharmacogenomics. The avoidance (and/or lowering of the administered dose) of coadministrated drugs that are implicated within the function of the same CYP isoform can ultimately improve pharmacotherapy outcomes. The latter, is more clinically relevant at least: (a) for drugs whose metabolism is the rate-limiting step upon establishment of their therapeutic plasma concentrations, and (b) for patients whose genetic background affects their drug metabolism rate [4, 6].

7.4 Nanotechnology Toward Enabling Personalized Medicine

A person's individuality is reflected also in his pathophysiology. In the same way that genes determine the uniqueness of an organism, genetic variability can characterize a disease phenotype and its response to drugs. Medicine has been deeply affected by this notion during the past decades, switching its interest in the molecular level where nanotechnology applications – as a rapidly emerging field – seem to fit the needs at this size scale properly. Nanomedicine is indeed aiming at providing diagnosis and treatment with speed, accuracy, and effectiveness and thus reinforces also the overall goal of pharmacogenomics and personalized medicine, creating the ground for a fertile interaction between the two scientific fields. Earlier, more sensitive and accurate diagnosis is implemented through the use of nanomaterials in imaging techniques, while on the other hand nanotechnology-based formulated drugs can accomplish a more specific and effective treatment at lower doses. The balance between maximum therapeutic efficacies with lower toxicity is in this way substantially promoted.

New nanotechnology-enabled therapeutics and diagnostics are being developed based on the unique properties that materials exhibit at this size (1–100 nm). The small size, duration of effect, surface properties, and payload density of nanoparticles render them well suited for formulation of either existing or new drugs with a proper pharmacokinetic (PK) behavior and better pharmacodynamic (PD) potential. This can include modulation of an agent's solubility, bioavailability, and half-life (retention time in the body) and, in the case of formulation, sustained and/or environmentally triggered control release of the drug. Most importantly

for personalized medicine, target-specific delivery of drugs can be achieved. For example, conjugation of nanovehicles with an antibody or other ligand is a common approach to guide the accumulation of the carrying drug to a specific diseased tissue (e.g., cancer), where its target antigen is located [12,28]. Such targeted drug delivery ensures a more effective treatment with lower doses administered, thus decreasing the rate of ADR in clinical practice and also increasing patient compliance [27]. It minimizes also the possibility of the influence of interindividual variation as far as PK processes (e.g., ADME) are concerned, where the majority of examples of differential drug behavior apply.

Furthermore, therapeutic advantages of nanovehicles are also urgently used for imaging applications and theranostic approaches, i.e., for systems and strategies in which both disease diagnosis and therapy are combined to benefit personalized medicine [16]. At the same time, specific nanomaterials including nanotubes, dendrimers, liposomes, and quantum dots are being developed as molecular diagnostic probes to target in vivo specific tissues or cells, thus implementing imaging techniques and improving clinical outcomes [39]. The increased surface area per volume of nanoparticles is exploited by coupling them with large payloads of an imaging agent, while additionally, nanoprobes can be specifically directed to tissues and cells providing diagnosis with increased sensitivity and specificity. A really vital application for personalized medicine is the discovery of biomarkers of disease. The identification and quantification of alterations in the molecular level that are related to manifestation, prognosis of a disease, or expected drug response comprise the hallmark of pharmacogenomics and nanodiagnostics that are already contributing to tissue profiling and targeting based on single or multiple markers.

Last, but not least, the development of a variety of nanotechnology-enabled miniature tools and machines is now feasible and cost effective to produce lab-on-chip approaches (including sample mixing, transport, integration, detection, and data processing) that can substitute testing in a clinical laboratory [29]. Also, the capability to generate cheaper high-throughput DNA sequencing and other genomic technologies permits the application of personal genome analysis in clinical practice for each individual to facilitate the movement of pharmacogenomics and personalized medicine toward pharmacotyping in drug prescription.

7.5 Information-Based Infrastructure to Facilitate the Practical Utility of Personalized Medicine

The use of computational and bioinformatics approaches to predict the PK (ADME) and PD properties of a drug is well appreciated throughout the process of drug development and delivery. The application of *in silico* methods and technology to evaluate safety and efficacy issues in pharmacotherapy by predicting mainly the emergence of drug interactions and ADR in clinical practice is now considered a major advancement to improve ultimately the success of new drug discovery and delivery outcomes [40]. Unfortunately, the data and the information generated up

to now through the application of genomic and high-throughput technologies are impressive in scale, but limited in clinical usefulness due to different database system formats and organization. In order for such an effort to finally succeed, information-based platforms in drug development era and health care have to be developed to evaluate and integrate knowledge from different genomic and clinical sources in an "easy for the end-user" manner. To this end, the application of semantic technologies with ontologies able to integrate proper knowledge in a way that will be reusable by several applications and in different scientific areas can be a more beneficial approach toward better and quicker exploitation of genomic information in health care and therapeutics [41].

The way by which pharmacogenomics can enable personalized medicine for most patients is related to the unprecedented load of genomic knowledge to be clinically translated and readily applicable in everyday clinical practice [11, 42]. Unfortunately, such molecular information has had a limited impact for the majority of patients up to now. Part of this delay is attributed to the lack of suitable drug-delivery information-based infrastructure in health care and also limited number of easy-to-use tools to make this knowledge broadly applicable in routine clinical practice. Indeed, this is a real challenge for current drug delivery and biomedicine. The development of suitable computerized and data integration systems in order for them to support the clinical translation of personal genome profiling needs, first, a clinical validation, before making such information applicable in routine patient care. Moving toward practical value of personalized medicine means the existence of tools and molecular diagnostics capable of assessing genome-related clinical information in laboratory medicine to make the extracted information easily applicable by the physician [6, 29]. This is a very difficult task, since before the transfer of molecular techniques to those used for the routine analysis of clinical samples in diagnostic laboratories, such approaches must be extensively clinically assessed and validated [43, 44]. The gradual integration of technologies transferring genomic information into the clinic needs the development of carefully selected and evaluated specific genetic biomarkers for the diagnosis of disease and prediction of drug response, as well as the use and application of genome-wide linkage analysis capable of genotyping, gene array, proteomics, transcriptomics, and metabolomics profiling [4, 45–47] (Fig. 7.1). In order for the individualized drug-delivery profiling based on clinical, genomic, and bioinformatics data to be achieved in pharmacotherapy, e.g., to what is referred as pharmacotyping [1, 4, 10], the developments in laboratory medicine must ensure the level of quality and validity needed in genetic and molecular diagnostics tests.

Another issue is related to the proper education and skills of healthcare practitioners to become familiar with pharmacogenomics knowledge and apply it in their profession [48, 49]. But in order for this attempt to finally succeed, appropriate education must be given to healthcare professionals through the development of new curricula and educational approaches. The training has to be focused on pharmacogenomics, personalized medicine, and pharmacotyping concepts as well as bioinformatics and information-based medical practice. In other words, moving forward for improved prescription of drugs in clinical practice with enhanced

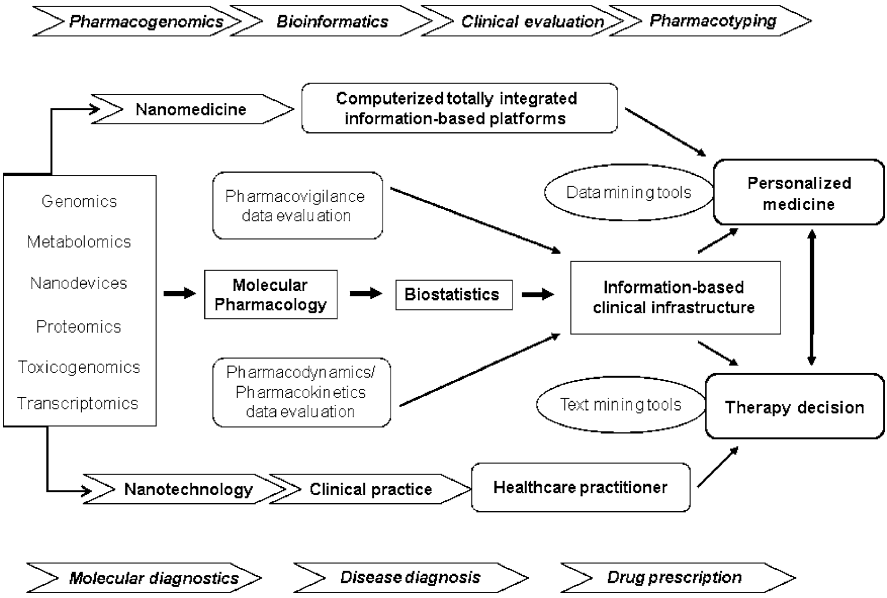

Fig. 7.1 Outline of existing relationship between various disciplines and personalized medicine toward advancing drug prescription profiles (see text for details)

efficacy and safety through the exploitation of genomic knowledge, the drug-delivery era will certainly be advanced and medical practitioners will gain further roles in this challenging health and pharmaceutical care environment. Also, the existing differences and peculiarities in health care and education found between several countries all over the world must be seriously taken into account, when the profound changes in post-genomic drug delivery and clinical practice are organized through the development of information-based platforms to implement and improve patient care. The trends in patient care-related issues or even the changes happening in one part of the world must be carefully examined and then adjusted before their use in other regions and vice versa. And for sure, this will be beneficial for both health and pharmaceutical care, as well as for the society and the public in general. Furthermore, by developing clinically suitable, cost-affordable, and accurate pharmacogenomics approaches, for in vivo data selection and clinical diagnosis, structural and functional genomics analysis, and clinical verification and validation of biological samples, laboratory medicine can be enriched through the integration of compatible information-based platforms to support the utility of genomic knowledge. This means that unified platforms must be developed in order to permit compatibility in handling different data collected from unrelated sources such as those of drug databases, clinical trials, DNA sequencing, and functional genomics analysis, even for the therapy of complex diseases, in a way to support ultimately the broad clinical application of pharmacogenomics, personalized medicine, and pharmacotyping in the years to come [6].

7.6 Toward Advancing Clinical Translation of Genomic Knowledge for Personalized Medicine

The transition into a more genome-enriched clinical pharmacology environment and the feasibility in the application of recent discoveries of molecular pharmacology, biotechnology, bioinformatics, and nanotechnology are crucial factors for implementing clinical pharmacology guidelines with the concepts of pharmacogenomics to better understand drug actions in the body, as well as to improve drug prescription in terms of efficacy and safety [1, 6]. Also, postmarketing drug safety is an essential attainable task toward empowering healthcare infrastructure and improving clinical outcomes [50, 51]. To this respect, the recently released recommendations on enhancing drug safety by the Institute of Medicine clearly address this issue, presenting the ground where improvements can be focused in the years to come [52]. The way by which pharmacogenomics knowledge is affecting drug safety and pharmacovigilance issues in clinical practice can be more easily foreseen by considering the example of cardiac safety upon drug delivery and the attempts of validating predictive molecular markers applicable earlier in the developmental process to assess drug-induced arrhythmias [53]. At first, the clinical observation was that numerous drugs belonging to different pharmacological classes have the propensity of causing prolongation of the QT interval in the electrocardiogram of affected individuals. As a result of this effect, fatal arrhythmias [torsade de pointes (TdP)] were caused that, at least in some cases, were correlated with the property of these drugs to affect also the function of the potassium channel, the major contributor to phase 3 repolarization at the end of QT interval [the potassium channels are known as the rapidly (I_{Kr}) and the slowly (I_{Ks}) activating delayed rectifier] [54–56]. These potassium channels have been found to be encoded by human ether-à-go-go-related gene (*hERG*; alternative nomenclature KCNH2), a fact that later on was successfully intercorrelated with the ability of these drugs to induce QT interval prolongation [57]. Such a status clearly has led to the development of hERG-modulating function assays for the early detection of compounds exhibiting this undesirable side effect. This approach can profoundly enhance cardiac safety in clinical practice by minimizing the number of ADR caused at this level [58]. Furthermore, the pharmacogenomics results on cardiac potassium channels focused on assessing cardiac safety of drugs also led to a more detailed evaluation of ion channel function on the predisposition of some individuals to develop specific cardiovascular disorders. In particular, genetic testing for the identification of specific mutations of cardiac ion channels has led to the clinical evaluation of patients with inherited predisposition to QT interval prolongation and the development of cardiac arrhythmias and sudden death [59]. Such pharmacogenomics data obtained thus far have also influenced regulatory and pharmacovigilance issues applied upon drug discovery and assessment of ADR monitored after the approval of drugs in the market. As far as the cardiac safety is concerned, a more generalized strategy for the implementation of hERG-potassium channel knowledge has been recently adopted

by FDA and EMA upon new drug development and the clinical evaluation of QT interval prolongation and proarrhythmic potential of non-antiarrhythmic drugs through the use of the specific S7A, S7B, and E14 ICH guidance [60–65].

7.7 Toward the Wide-Spread Application of Pharmacogenomics to Enhance the Utility of Personalized Medicine

Recent breakthroughs in science and technology have the potential to transform health practitioners' ability to prevent, diagnose, and treat disease, in a way moving therapeutic strategies toward personalized approaches for individual patients, thus maximizing the benefit of treatments while decreasing their toxicity risks. Toward the direction of enabling nanotechnology and pharmacogenomics knowledge to enter clinical practice and to advance personalized medicine, FDA has created the so-called "regulatory science initiative" to focus on developing new tools, standards, and approaches to assess the safety, efficacy, quality, and performance of innovative drugs, diagnostics, devices, and advanced technologies (see http://www.fda.gov/ScienceResearch/SpecialTopics/RegulatoryScience/ucm228131.htm, accessed at December 8, 2010). Although such an effort is considered crucial to facilitate the clinical translation of pharmacogenomics research data, among the major challenges facing clinicians and researchers engaged in this area has been their successful integration into the clinical pharmacology guidelines for the majority of drugs and for each medical specialty. Moving forward, the successful application of pharmacotyping concepts in pharmacotherapy is eventually based on the practical integration of such guidelines for specific drug dosage scheme recommendations into everyday drug prescription process [1, 6]. This task is expected to be advanced through the development of workflow information-based operating systems in health care to support the utilization, assessment, and outcome of engaged clinical and genomic information. Such a direction would also greatly benefit the revision and adjustment of clinical regulatory issues, the better design of clinical trials, and the registry and evaluation of pharmacovigilance data [1, 6].

For personalized medicine, the development of a multidisciplinary infrastructure and the clinical validation of specific genetic biomarkers for their utility in a large number of diseases and drugs to improve diagnosis and allow the prediction of drug response represent painstaking and time-consuming processes. Especially for the broader application of personalized medicine in the clinics, a highly integrated system also needs to be established to achieve efficient high-throughput clinical data from genotyping, gene array, proteomics, transcriptomics, and metabolomics profiling [66–69]. Also, by moving toward personalized medicine and pharmacotyping, the development of nanotools and molecular diagnostics capable of assessing genome-related clinical information in laboratory medicine to make the extracted information easily applicable by the physician has to be attained. However, this

is considered a very difficult task, since before the transfer of techniques used in genome-related research laboratories to diagnostic laboratories analyzing clinical samples, these methods and techniques must also be assessed for their ethical, social, and cost-benefit consequences [11, 70, 71].

Although there is no single model for pharmacogenomics research to achieve the major benefits in clinical practice and ensure a secure roadmap for pharmacotyping, it is evident that this is fulfilled by building a multidisciplinary structure to integrate expertise from pharmacology, genomics, bioinformatics, nanotechnology and clinical sciences (Fig. 7.1). This direction is obviously supported through the development of specialized pharmacogenomics databases to support the deposition, assessment, organization, and finally dissemination of relevant drug-related data including pharmacological, pharmacogenomics, and clinical ones. Such an example represents the "Pharmacogenetics and Pharmacogenomics KnowledgeBase" (PharmGKB), which is a public database that focuses on genotype and phenotype data relevant to pharmacogenomics [72]. Organization of this knowledge base is being achieved by capturing the relationships between drugs, diseases/phenotypes, and genes involved in PK and PD through literature annotations, primary data sets, PK and PD pathways, and expert-generated summaries of PK/PD relationships between drugs, diseases/phenotypes, and genes. By building such a multidisciplinary infrastructure, the already clinically used approaches of therapeutic drug monitoring and population-based PK/PD models assessing drug levels and behavior in the body could be integrated with pharmacogenomics and genomic medicine concepts. This approach obviously paves the way for pharmacotyping to be greatly facilitated and ensured in routine clinical practice. Also, by establishing such an environment, the education of healthcare practitioners in molecular/clinical pharmacology, drug interactions, pharmacogenomics, and translational medicine should be finally achieved [48, 73].

7.8 Cost Effectiveness of Pharmacogenomics

Although there is a widespread interest for personalized medicine, the broad application of pharmacogenomic testing implies that the validation of clinical improvement outcomes in a cost-effective manner must be clearly demonstrated. However, only a few studies have addressed cost-effectiveness issues of pharmacogenomics application in clinical practice. In one case, for example, thiopurine methyltransferase (TPMT) genotyping for children suffering from acute lymphoblastic leukemia (ALL) and taking thiopurine was assessed in four European countries (Germany, Ireland, the Netherlands, and the UK). Interestingly, the practice of prescribing thiopurine to ALL children based on their TPMT genotype has shown a favorable cost-effectiveness ratio [74]. Further analysis of data collected from the UK and Spain has proposed that key pharmacovigilance data include evidence for azathioprine prescription in favor of routine TPMT testing by reducing the emergence of azathioprine-related ADR [75]. On the contrary, the lack of validated

economic models in a unified basis to examine the costs and benefits of pharmacogenomic testing has been clearly identified in the case of CYP polymorphisms testing upon antipsychotics prescription [76]. Furthermore, the economic evaluation of pharmacogenomic testing is now considered a main barrier hindering the implementation of clinical practice with pharmacogenomics knowledge [77, 78]. Importantly, before moving toward the routine application of pharmacotyping concepts in the healthcare system, the demonstration of economic benefits must accompany the validation of clinical effectiveness of pharmacogenomic testing. Such a direction will allow cost-effectiveness analysis to test relative costs and benefits of pharmacogenomic interventions compared with current practice and create the framework for healthcare providers to make reimbursement decisions [79, 80]. Importantly, this need is further stressed by the fact that in a recent study carried out to assess the clinical effectiveness of pharmacogenomics, only two biomarkers had demonstrated clinical utility, although most of them had demonstrated clinical validity [81]. Development and advancement of a new technology is nothing but expected to be demanding in effort, time, and resources, but its widespread and long-term application will ultimately tend to balance the cost. Especially in cases where the severeness of the disease is high, when suboptimum treatment translates to serious adverse effects or even death, it should comprise the main factor based on which decisions for implementation are made.

However, major changes must be clearly addressed in a cost-affordable way and demonstrated in order to allow (a) the broader clinical utility of genomic data for routine drug prescription, (b) the suitable training of future healthcare professionals, and (c) the integration of genomic medicine into clinical trials [1, 10]. Also, the need for extensive and thoughtful discussions about ethical, societal, and economic impacts arising from the clinical application of pharmacogenomic testing is both a necessity and a major challenge.

7.9 Pharmacotyping Concepts for Individualizing Drug Selection and Dosage Schemes

Until recently, medical practitioners had only their own clinical experience and knowledge upon curing patients and prescribing drugs. In the last few years, substantial experience has been attained on applying computerized systems as an efficient drug prescription tool in clinical practice to improve pharmacotherapy outcomes in terms of efficacy and safety. By integrating in health care genomic drug-related data, pharmacogenomics is moving toward the application of pharmacotyping in drug prescription, i.e., the individualized drug selection and dosage scheme profiling. Such direction will facilitate the physician to prescribe based on patient's genotyping and haplotyping data for genes involved in PD- and PK-related drug actions in the body [1, 6, 10]. This means that drug-delivery environment is changing from a drug-selection process where physicians mainly

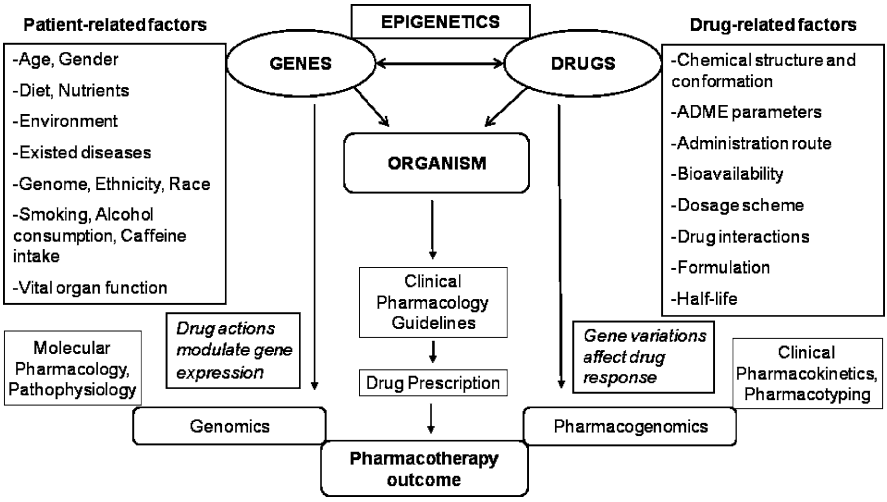

Fig. 7.2 Diagrammatic depiction of genes–drugs molecular interactions, along with the influence of epigenetics and various patient- and/or drug-related factors, to achieve the pharmacotherapy outcome of an organism is indicated (see text for details)

use their own clinical experience into a more highly integrated, information-based and computer-aided process. Indeed, the development of computer-based decision support systems in drug delivery represents an effort for the automation of prescription to achieve quick processing and allow the inter-correlation of multiple patient- and drug-related factors for reducing errors compared to traditional handwritten prescription (Fig. 7.2) [82–84]. However, in order for such a direction to gain full advantage worldwide, it is important for the computer-based decision systems to be designed, managed, and updated in a way to ensure interoperability with other computerized methods for managing healthcare data and common standards between different national systems. Within this frame of development, the creation and establishment of global personalized medicine databases capable of communicating with computerized drug prescribing systems and supporting the integration of pharmacogenomics data can finally assist clinicians to effectively choose and dose medicines. Importantly, these changes ensure personalized medicine for making drug delivery digitized, more efficient, and safer.

In order for the advancement of drug prescription and improvement of clinical outcomes to be broadly achieved through the application of pharmacotyping concepts, a clinically validated information-based system should be organized and established to guide end-user clinicians on how to efficiently integrate successfully (1) the information of drug interactions extracted from clinical and molecular pharmacology sources and (2) the intercorrelation of pharmacogenomic knowledge of genes involved in PK/PD effects of drugs. In such a case, the need for well-educated healthcare practitioners, the development and adjustment of clinical

pharmacology/pharmacogenomic guidelines, and the organization of infrastructure in health care equipped with the proper clinically validated technological methodologies is now, more than ever, stressful and demanding. To this end, last, but not least, it would be of great importance for the conduction of well-designed clinical pharmacogenomic studies to better confirm the utility and also address the question whether personalized medicine and pharmacogenomics are mature enough to be of practical value for the majority of drugs in everyday clinical practice. Such an attempt is considered the "Coriell Personalized Medicine Collaborative," which is a large prospective observational study designed to address the clinical utility of personal genome data in routine patient care [85]. In such well-organized pharmacogenomic models assembling large cohort studies that are assessed by multidisciplinary teams of researchers, the utility of personal genetic information for disease risk assessment and prevention, as well as for pharmacotyping based drug prescription will be better outlined and elucidated. By achieving such a task, major benefits for both the healthcare system and the society are highlighted and more efficiently borne out.

7.10 Future Challenges and Perspectives

By considering the future advancement of diagnosis and therapy outcomes in multifactorial diseases for all patients, for example, degenerative and cardiovascular disorders, type-2 diabetes, and cancer, a more systems pharmacology and personalized medicine approach should be established in both research and the clinics. Undoubtedly, the recent technological advances in nanomedicine and nanotechnology in parallel with the knowledge accumulated thus far from the clinical translation of disease- and drug-related genomic data create the fertile ground for practical utility of personalized medicine. To this end, pharmacogenomics enables drug prescription process toward pharmacotyping, e.g., the individualized specific drug and dosage scheme selection, through coassessment of pharmacological, clinical, and genomics data. On the contrary, the genetic background involved in both the disease pathogenesis and the response of an organism to delivered drugs is complex, and the underlying mechanisms are difficult to be fully elucidated. To this end, toward achieving major benefits for all patients, issues also related to the regulatory environment, healthcare practitioners' education, bioethics, and genomics data dissemination have to be clearly addressed and solved.

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Index

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