

Biosystems & Biorobotics

Hanjoong Jo
Ho-Wook Jun
Jennifer Shin
SangHoon Lee *Editors*

Biomedical Engineering: Frontier Research and Converging Technologies



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Eugenio Guglielmelli, Campus Bio-Medico University of Rome, Rome, Italy
e-mail: e.guglielmelli@unicampus.it

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Aims & Scope

Biosystems & Biorobotics publishes the latest research developments in three main areas: 1) understanding biological systems from a bioengineering point of view, i.e. the study of biosystems by exploiting engineering methods and tools to unveil their functioning principles and unrivalled performance; 2) design and development of biologically inspired machines and systems to be used for different purposes and in a variety of application contexts. The series welcomes contributions on novel design approaches, methods and tools as well as case studies on specific bioinspired systems; 3) design and developments of nano-, micro-, macro- devices and systems for biomedical applications, i.e. technologies that can improve modern healthcare and welfare by enabling novel solutions for prevention, diagnosis, surgery, prosthetics, rehabilitation and independent living.

On one side, the series focuses on recent methods and technologies which allow multiscale, multi-physics, high-resolution analysis and modeling of biological systems. A special emphasis on this side is given to the use of mechatronic and robotic systems as a tool for basic research in biology. On the other side, the series authoritatively reports on current theoretical and experimental challenges and developments related to the “biomechatronic” design of novel biorobotic machines. A special emphasis on this side is given to human-machine interaction and interfacing, and also to the ethical and social implications of this emerging research area, as key challenges for the acceptability and sustainability of biorobotics technology.

The main target of the series are engineers interested in biology and medicine, and specifically bioengineers and bioroboticists. Volume published in the series comprise monographs, edited volumes, lecture notes, as well as selected conference proceedings and PhD theses. The series also publishes books purposely devoted to support education in bioengineering, biomedical engineering, biomechatronics and biorobotics at graduate and post-graduate levels.

About the Cover

The cover of the book series Biosystems & Biorobotics features a robotic hand prosthesis. This looks like a natural hand and is ready to be implanted on a human amputee to help them recover their physical capabilities. This picture was chosen to represent a variety of concepts and disciplines: from the understanding of biological systems to biomechatronics, bioinspiration and biomimetics; and from the concept of human-robot and human-machine interaction to the use of robots and, more generally, of engineering techniques for biological research and in healthcare. The picture also points to the social impact of bioengineering research and to its potential for improving human health and the quality of life of all individuals, including those with special needs. The picture was taken during the LIFEHAND experimental trials run at Università Campus Bio-Medico of Rome (Italy) in 2008. The LIFEHAND project tested the ability of an amputee patient to control the Cyberhand, a robotic prosthesis developed at Scuola Superiore Sant’Anna in Pisa (Italy), using the tf-LIFE electrodes developed at the Fraunhofer Institute for Biomedical Engineering (IBMT, Germany), which were implanted in the patient’s arm. The implanted tf-LIFE electrodes were shown to enable bidirectional communication (from brain to hand and vice versa) between the brain and the Cyberhand. As a result, the patient was able to control complex movements of the prosthesis, while receiving sensory feedback in the form of direct neurostimulation. For more information please visit <http://www.biorobotics.it> or contact the Series Editor.

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Hanjoong Jo · Ho-Wook Jun
Jennifer Shin · SangHoon Lee
Editors

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 Springer

Editors

Hanjoong Jo
Coulter Department of Biomedical
Engineering
Georgia Tech and Emory University
Atlanta, GA
USA

Ho-Wook Jun
Department of Biomedical Engineering
The University of Alabama at Birmingham
Birmingham, AL
USA

Jennifer Shin
Department of Mechanical Engineering Soft
Biomechanics & Biomaterials Lab
Korea Advanced Institute of Science and
Technology
Daejeon (KAIST)
Korea, Republic of (South Korea)

SangHoon Lee
Korea University
Department of Biomedical Engineering,
College of Health Science
Seoul
Korea, Republic of (South Korea)

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Preface

Solving problems in human health, well-being, and disease increasingly requires convergence of scientific and engineering fields with medicine. While some of the last frontiers of biomedicine, such as neuroscience and regenerative medicine, critically demands new ideas and tools from other disciplines, paradigm-shifting technological innovations in information science, nanotechnology, and robotics could open new opportunities in healthcare. At the same time, a new generation of engineers, “fluent” in many different languages of science, are creating entirely new fields to view the old questions with a fresh look. This book aims to provide timely state-of-the-art reviews written by leading biomedical engineers for students, fellows, faculties, and industry investigators in broad biomedical engineering fields.

Acknowledgement

The editors are grateful for overwhelming support by all authors who have contributed their valuable time and effort to share their insights into the biomedical engineering, an exciting field of convergent technology. We also thank Drs. Kookjoon Ahn and Jae H. Kim for their support and encouragement to publish this book. In addition, Ms. Leontina Cecco Di in Springer for her support to get this book published in time.

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About the Editors

Hanjoong Jo



Dr. Hanjoong Jo is John and Jan Portman Professor in the Coulter Department of Biomedical Engineering (BME) at Georgia Tech and Emory University, and Professor of Medicine at Emory University. He is also the Associate Chair of Emory in BME Department. Upon graduation from Korea University, Dr. Jo received PhD under the co-mentorship of Professors John Tarbell (Chemical

Engineering) and Ted Hollis (Physiology) at Pennsylvania State University in 1989. Following postdoctoral training in Jay McDonald Lab at Washington University in St. Louis and University of Alabama at Birmingham, he became Assistant Professor in Pathology and BME. Dr. Jo joined the BME Department at Georgia Tech and Emory University in 2000. He directs the Cardiovascular Mechanobiology and Nanomedicine lab. His lab studies how mechanical force associated with blood flow regulates vascular biology and cardiovascular disease, especially atherosclerosis, aortic valve (AV) calcification, and abdominal aortic aneurysms. He has published more than 140 peer-reviewed papers and edited a book. He developed the mouse model of atherosclerosis, known as partial carotid ligation model, induced by disturbed flow. His work led to the discovery of several genes (mechanosensitive genes and microRNAs) and epigenetic controlling mechanisms that are regulated by bad blood flow and play key roles in atherosclerosis and AAA. By targeting some of these mechanosensitive genes, his lab has been able to treat atherosclerosis and AAA in mice. His lab is now working on nanotechnologies to developing targeted gene and drug therapies in an effort to translate mouse studies toward clinical application. He is an elected fellow of American Institute of Medical and Biological Engineering, Biomedical Engineering Society, American Heart Association and American Physiological Society. He serves as associate editors and editorial board members of several cardiovascular and biomedical engineering journals including Scientific Reports, Circulation Research, Atherosclerosis

Thrombosis Vascular Biology, Am J Physiology, Cell Molecular Bioengineering and Cardiovascular Engineering and Technology. He also has been serving as reviewers and chairs of study sections of the NIH, NSF, Veterans Administration and Am Heart Association. He also organized several international meetings, and in 2012, he served as the Chair of the Annual BME Society Meeting. He is also the founding President of Korean-American Biomedical Engineering Society (KBMES) and Chairs of US-Korea Annual BMES Workshops since 2013. He has been a Distinguished Visiting Professor at Ewha Womans University and Chunbuk National University.

SangHoon Lee



Dr. SangHoon Lee is Professor in the Department of Biomedical Engineering (BME) at Korea University. Dr. Lee received PhD under the Advisor of Professors B.G. Min (Biomedical Engineering) at Seoul National University in 1992. After PhD, he became Assistant Professor in Department of Biomedical Engineering at the medical school of Dankook university in Korea. He also worked as director of BME Department at Dankook university hospital from 1992, and managed the medical device and information system of hospital. At 2006, he moved to medical school of Korea University, and at 2008, he established Department of BME at college of health science. He is now operating 'Intelligent BioMEMS Lab', and his major research area is tissue engineering and regeneration medicine using microtechnology, organ-on-a-chip and electronic skin. He has published more than 150 peer-reviewed papers including "Nature Materials", "Nature Communications", "Advanced Materials", "Biomaterials", "Stem Cells", "Small", "Lab on a Chip" and so forth. He has a great interest in engineering and regeneration of liver, brain, and pancreas. Using diverse primary cell sources, he successfully fabricated complicate liver-ultra structures. He also demonstrated 3D brain tissues networked using nervous system. Another interesting topic is the brain and liver on a chip to simulate liver and CNS disease on the microfluidic platform. Another topic is the electronic skin which is highly stretchable and flexible like skin, and this e-skin is continuously wearable over one week. He is chief editor of 'Biomedical Engineering Letters', and organized several international conferences. He served as committee members of microTAS, ISMM and EMBC. He was dean of 'College of Health Science' at Korea University, and he received several awards due to his academic and industrial achievements.

Ho-Wook Jun

Dr. Ho-Wook Jun is an Associate Professor in the Department of Biomedical Engineering of School of Engineering and School of Medicine at the University of Alabama at Birmingham. Dr. Jun received Ph.D. in Bioengineering from Rice University in 2004. From 2004-2006, he was the Peter and Ruth Nicholas Postdoctoral Fellow at the Richard Smalley Institute for Nanoscale Science and Technology and in the Department of Chemistry at Rice University. In 2006, he joined the faculty at the

University of Alabama at Birmingham. Dr. Jun is also a Co-Founder and Chief Scientific Officer of Endomimetics LLC, a company that develops bionanomatrix coatings for cardiovascular stents and other medical implants.

Dr. Jun group focus on nanostructured biomaterials and stem cell based tissue regeneration to develop an innovative strategy to treat various diseases. His translational research includes 1) a biomimetic hybrid nanosack to increase the efficacy of pancreatic islet transplantation to treat type I diabetes, 2) a native endothelium mimicking nanomatrix coating for stents and cardiovascular devices, 3) a biphasic composite nanomatrix gel for treating severe limb injuries caused by high energy blasts and gunshots in military operations, 4) chondral defect repair using amniotic fluid derived stem cells delivered within a biomimetic nanomatrix gel.

Dr. Jun has published more than 40 peer-reviewed papers and filed 7 patents. Dr. Jun has been recognized by various awards including the Early Career Award from the Wallace H. Coulter Foundation in 2007, the Innovation Award from the American Diabetes Association in 2009, and the Early Career Award from the National Science Foundation in 2010, Dean's Award for Excellence in Mentorship in 2010, Finalist of Alabama Launchpad Governor's Business Plan Competition, and Wallace H. Coulter Fellow in 2011.

Dr. Jun is a Vice President of Korean-American Biomedical Engineering Society (KBMES).

Jennifer H. Shin

Dr. Jennifer H. Shin is an associate professor in Mechanical Engineering Department at Korea Advanced Institute of Science and Technology (KAIST). She obtained B.S., M.S., and Ph.D. in the field of biophysics in Mechanical Engineering Department at MIT in 1998, 2000, and 2004, respectively. After that, she worked as a postdoctoral associate in School of Engineering and Applied Sciences (SEAS) at Harvard University before she started her career at KAIST in 2005. Dr. Shin's research interests center around the emerging

interdisciplinary mechanobiology. At the center of mechanobiology, is the process of mechanotransduction, the way cells sense and respond to various physical stimulations from the microenvironments. Her laboratory focuses on the development of physical stimulation systems that are suitable for biological and biophysical assays to investigate cellular level mechanobiology. Her lab focuses on finding links between cellular biomechanics and physiological changes associated with diseases and differentiation states in light of diagnostics and therapeutics of the diseases, as well as tissue engineering applications. Her lab also studies biological motility at various levels from a single cell, collective cellular clusters, to an organism based on force visualization techniques such as traction force microscopy and monolayer stress microscopy.

She served as a member of the Presidential Advisory Council on Science and Technology (2008-2012) and has been an active board member for 7 academic societies across the fields of core mechanical engineering and biomedical engineering. She has been a leading member to organize several international symposiums and workshops, and also served a committee member of international conferences including Biofabrication, microTAS, and IBEC. She is one of the founding members of Korean-American Biomedical Engineering Society (KBMES) and US-Korea Annual BMES Workshops since 2013. In particular, Dr. Shin displays genuine passion and enthusiasm for teaching students and was recognized with several teaching excellence awards both at the departmental and institutional levels. As a woman faculty in mechanical engineering, she has shown commitments to supporting female students in the field of science and technology through her active contributions at Center for Women in Science, Engineering and Technology (WISSET) and Federation of Women's Science and Technology Association in Korea.

Part I
Nanotechnology and Nanomedicine

Nanoparticles for Imaging and Non-viral Gene Therapy

Yoonjee Park

Abstract Gene therapy has not been investigated as much as pharmacotherapy because of immunogenic issues when a virus was used as a gene delivery vector. Despite the challenges, gene therapy still has attractive aspects. It has less side effects and is more target-specific compared to pharmacotherapy, and it also has potential for generic disease treatment or personalized medicine. Therefore, it would be truly beneficial if safe and reliable vectors are used and targeted for area of interest. Interest in multifunctional nanomedicine for diagnostics and therapeutics has been increasing. For this reason, non-viral gene delivery has been studied, combined with molecular imaging to visualize targeting. In this review, complex nanoparticle systems designed for molecular imaging and gene delivery are discussed. There are design criteria which need to be considered for the nanoparticle complex systems. The criteria are as follows: i) the nanoparticle complex should be stable; ii) it should have efficient targeting capability; iii) controlled release of genes should be available; iv) molecular imaging should be possible; and lastly, v) there should be noticeable therapeutic efficacy. Examples on nanoparticle complex which meet these criteria are described in the review.

Keywords: Molecular imaging · Gene therapy · Nanomedicine · Contrast agents · Non-viral gene delivery · Theranostics

1 Introduction

Gene therapy often requires vectors to deliver genes into cells and the vectors are mainly categorized into two types: viral vectors and non-viral vectors. Although viral vectors, such as a retrovirus and an adenovirus, have high transfection

Y. Park(✉)

Department of Biomedical, Chemical & Environmental Engineering,
University of Cincinnati, Cincinnati, USA
e-mail: yoonjeep@gmail.com

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efficiency, many critical safety issues and concerns still remain [1]. Because of the immunogenic issues, non-viral vectors recently have drawn attention. In addition, in order to visualize the expression of gene transduction in target tissue or specific organs of the body, molecular imaging has been combined with gene therapy.

This chapter highlights the current gene delivery and molecular imaging technologies, involved in novel non-viral nanoparticle complexes as multifunctional “nanomedicine”. Nanoparticles as a gene carrier form a complex where nucleic acid molecules like exogenous DNA are encapsulated or absorbed. The particle size of the nanoparticles is usually 10-100 nm. A high surface-to-volume ratio makes the nanoparticles have outstanding adsorption, which is advantageous for delivering exogenous genes. Non-viral vectors have greater payload capacity than viral vectors [2]. Various types of nanoparticles as a gene carrier will be discussed in Section 2. Molecular imaging techniques will provide a practical and clinically useful way to identify successful gene transduction and expression in patients undergoing gene therapy in a non-invasive manner [3-5].

To achieve molecular imaging, targeted imaging contrast agents are required and the contrast agents are often nanoparticles. Therefore, it is beneficial to develop a nanoparticle complex which has multiple functions for targeted gene therapy and molecular imaging. The design criteria for the nanoparticle complex are as follows: i) the nanoparticle complex should be stable; ii) it should have efficient targeting capability; iii) controlled release of genes should be available; iv) molecular imaging should be possible; and lastly, v) there should be noticeable therapeutic efficacy. Examples on nanoparticle complexes which meet these criteria are described in the chapter.

2 Nanoparticles as a Gene Carrier

2.1 *Liposomes*

Liposomes are spherical vesicles composed of a lamellar phase lipid bilayer and these are formed when energy is supplied, such as heat and stirring, or sonication. Because liposomes are incorporated well within cell membrane and relatively easy to be manipulated, liposomes have been used widely in the analytical sciences as well as for drug and gene delivery. Cationic lipids are usually used for delivering genes which are negatively charged. The positively charged head group allows efficient DNA compaction due to attractive electrostatic forces that occur between the phosphate backbone of DNA and the cationic head groups of the lipids [6]. Loading genes on nanoparticles stably is important; however, at the same time, weak electrostatic interaction will be beneficial to deliver genes [7].

2.2 *Polymers*

With the same reason as described above in Section 2.1., cationic polymer has been shown as a promising carrier among non-viral gene delivery systems.

Because polycation-DNA complexes generally are stable, a number of cationic polymers have been investigated as gene carriers, including polylysine [8], polyethyleneimine [9], polyamidoamine dendrimers [10], poly(α -(4-aminobutyl)-L-glycolic acid) [11] etc.

Block copolymers composed of a cationic segment and a hydrophilic segment spontaneously associate with polyanionic DNA to form block copolymer micelles. The distinct feature of the structure is that the core of the polyion complex between DNA and the polycation is coated by a layer of the hydrophilic polymer. The characteristic core-shell structure endows the complex a high colloidal stability and reduced interaction with blood components [12].

There is no concrete comparison that can easily be made to suggest that one carrier is better than another for all cell types, environments, and applications. While some of the carriers presented above were originally found to yield little to no cytotoxicity for a given cell type, the observation does not necessarily hold true when they are applied to different cell types [13-15]

2.3 Inorganic Nanoparticles

Inorganic nanoparticles, such as gold, silica, iron oxide (ex. magnetofection) and calcium phosphates have been shown to be capable of gene delivery [16]. Some of the benefits of inorganic vectors are in their storage stability, low manufacturing cost and often time, low immunogenicity, and resistance to microbial attack. Nanosized materials less than 100 nm have been shown to efficiently trap the DNA or RNA and allow its escape from the endosome without degradation. Inorganics have also been shown to exhibit improved in vitro transfection for attached cell lines due to their increased density and preferential location on the base of the culture dish. Mirkin and co-workers attached siRNA (small interfering RNA) molecules to the surface of AuNPs via a thiol group [17]. The polyvalent siRNA/nanoparticle conjugates showed a 6 times greater half-life and prolonged gene knockdown compared to free RNA duplexes [18]. Quantum dots have also been used successfully and permit the coupling of gene therapy with a stable fluorescence marker.

2.4 Other Carriers

Chitosan [19], dendrimers [20, 21], and cell-penetrating peptides [22, 23] as a form of nanoparticles have been investigated for potential gene-delivering carriers. Chitosan is a biodegradable polysaccharide [24] extracted from crustacean shells. It has been shown to be non-toxic in a range of toxicity tests, both in experimental animals [25] and humans. Mao et al. [19] demonstrated that the chitosan-DNA nanoparticle system effectively delivered genes to the small intestine of mice. When used to orally deliver pCMVArh2 plasmid, which encoded a dominant anaphylaxis-inducing antigen identified in mice sensitized with peanut, nanoparticle immunization elicited a relatively high IgG2a immune response and

protected the experiment animals from an allergic challenge. A dendrimer is a highly branched macromolecule with a spherical shape. When in the presence of genetic material such as DNA or RNA, a temporary association of the nucleic acid with the cationic dendrimer occurs. Cell-penetrating peptides (CPPs), also known as peptide transduction domains (PTDs), are short peptides (< 40 amino acids) that efficiently pass through cell membranes while being covalently or non-covalently bound to various molecules, thus facilitating these molecules' entry into cells [26]. CPP-decorated LNP (CPP-LNP) for siRNA delivery demonstrated that siRNA in CPP-LNP was efficiently internalized into B16F10 murine melanoma cells in a time-dependent manner, although siRNA in LNP without CPP was hardly internalized into these cells.

2.5 Hybrids

Hybrid systems also have been developed to combine advantages from the materials described above. Elbakry et al. first developed the PEI/siRNA/PEIAuNP system to deliver siRNA into cells and knockdown the expression of target gene based on the self-assembly layer-by-layer technology [27]. PEI (poly ethyleneimine), which has strong escape capacity from the endosome and is usually a gold standard of a polymeric transfection agent, was deposited on the gold nanoparticles to bind siRNA. Guo et al. developed charge-reversal functional gold nanoparticles to reduce high binding affinity between gold nanoparticles and siRNA to release siRNA in cellular cytoplasm easily [28]. When the vector (ternary complexes) is entrapped into the acidic intracellular organelles such as endosomes or lysosomes (pH 5-6), charge conversion facilitates the endosomal escape of the polyplexes through membrane disruption.

3 Nanoparticles as a Molecular Contrast Agent

Over the past decade, there has been an increasing interest and effort in developing molecular imaging. Molecular imaging can be broadly defined as the 'non-invasive and repetitive imaging of targeted macromolecules and biological processes in living organisms.' This field of study has advanced rapidly in recent years, in part due to the application of nanotechnology. The versatility of different imaging modalities has been significantly enhanced by innovative nanoparticle development. These nanoparticles can be used to image specific cells and tissues within a whole organism. An ideal contrast agent should selectively accumulate at the site of interest to be able to interact physically, chemically, biochemically, and functionally with the target and enhance contrast of imaging. Some of the nanoparticles under development may be useful to measure biological processes associated with human disease and help monitor how these change with treatment. In vivo molecular imaging using nanoparticles promises to revolutionize diagnosis and treatment of human diseases, but some practical limitations still need to be resolved.

3.1 *Imaging Contrast Agents Used for Gene Delivery*

Molecular imaging techniques will provide a practical and clinically useful way to identify successful gene transduction and expression in patients undergoing gene therapy in a non-invasive manner. This section will introduce various types of imaging contrast agents which have been combined with gene delivery.

3.1.1 **MRI Contrast Agents**

MRI (magnetic resonance imaging) is based on the response of proton spin in the presence of an external magnetic field when triggered with a radio frequency (RF) pulse. Under the influence of an external magnetic field, protons align in one direction. On application of the RF pulse, aligned protons are perturbed and subsequently relax to their original state. There are two independent relaxation processes: longitudinal (T_1) and transverse (T_2) relaxation, which are typically used to generate the MR images. For T_1 -weighted contrast agents, gadolinium- (Gd^{3+}) or manganese- (Mn^{2+}) chelates are often used. These are paramagnetic, which increases the T_1 relaxation time, resulting in bright contrast T_1 -weighted images. For T_2 -weighted contrast agents, superparamagnetic Fe_3O_4 nanoparticles are used and these reduce T_2 relaxation times, giving rise to dark contrast T_2 -weighted images [29].

Gd^{3+} ion on/in the nanoparticles reduced toxicity of Gd^{3+} , and enhanced T_1 -weighted MR signal [30]. It can also increase the cellular uptake of Gd^{3+} ions through size and shape tuning of the vehicle nanoparticles. Furthermore, biological functional groups can be conjugated on the nanoparticles surfaces for studying dynamic biomolecular phenomena through suitable biochemical reactions and signaling. Superparamagnetic iron oxide nanoparticles (SPIONs) provide the most change in signal per unit of metal, in particular on T_2 -weighted images. SPIONs are composed of biodegradable iron, which is biocompatible and can thus be reused/recycled by cells using normal biochemical pathways for iron metabolism and can be magnetically manipulated [29].

Dixit et al. reported phospholipid micelle encapsulating gadolinium oxide (Gd_2O_3) nanoparticles for molecular imaging and gene delivery [31]. These gadolinium oxide nanoparticles within phospholipid micelles as a novel low cytotoxic T_1 -weighted MRI imaging contrast agent (MGdNPs) can also deliver small molecules such as DNA plasmids. MGdNPs show relatively good MRI relaxivity values, negligible cytotoxicity, excellent cellular uptake and expression of DNA plasmids *in vivo*. Biodistribution studies in mice show that intranasal and intraperitoneal administration of MGdNPs can effectively target specific organs.

As introduced in Section 2.5, cationic polymers have been combined with SPIONs via electrostatic interaction to deliver genes [32]. The transfection efficiency of SPION-gene complexes can be enhanced by magnetic field (magnetofection). Transfection efficiency of SPIONs with PEI on pulsed magnetic fields *in vitro* was 40 times higher than in the absence of a magnetic field [33]. Lee et al. conjugated siRNA onto SPION surfaces using disulfide bonds via a thiol-functionalized PEG (Figure 1) [34]. Targeting and stability of the SPIONs *in vivo* were enhanced.

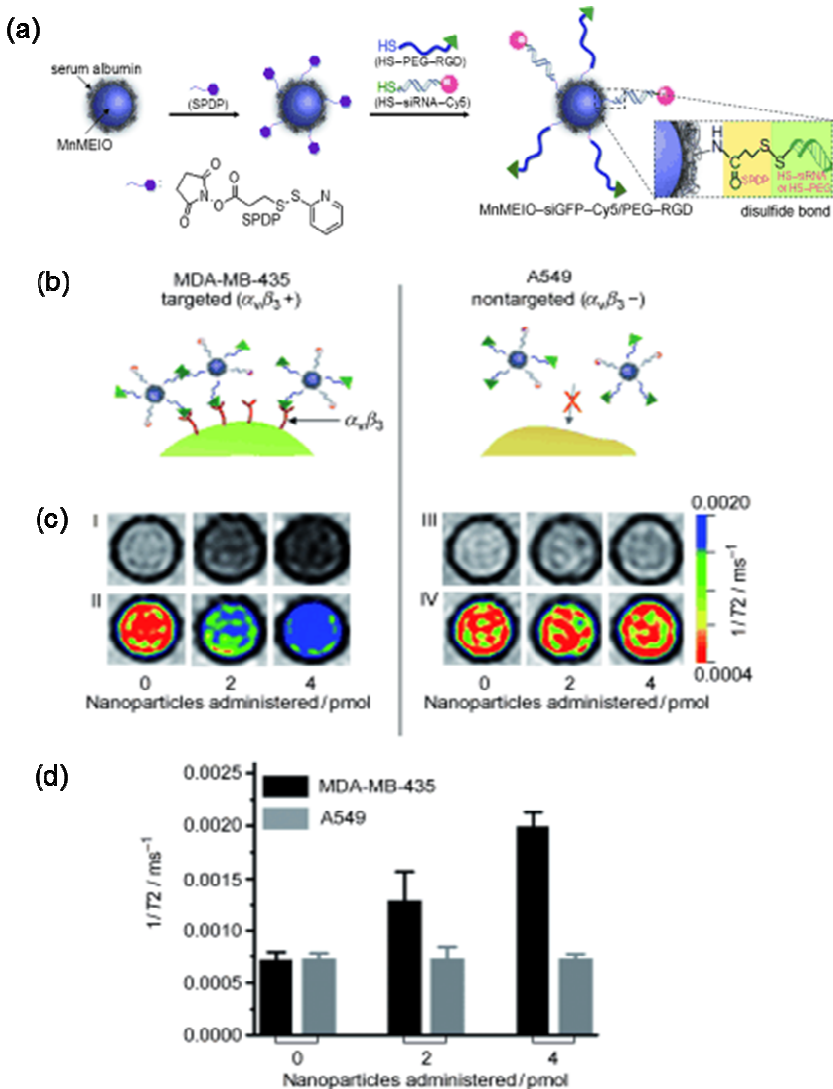


Fig. 1 (a) Fabrication of multimodal MnMEIO-siGFP-Cy5/PEG-RGD. Schematic of MnMEIO-siGFP-Cy5/PEG-RGD. SPDP, which forms disulfide bonds, was used as a linker. (b) Schematic of the target-specific binding of the nanoparticle to $\alpha_v\beta_3$ integrin positive cells. (c) T2*-weighted MR images and their color maps of I,II)MDA-MB-435 and III,IV)A549 cells treated with MnMEIO-siGFP-Cy5/PEG-RGD. Red corresponds to untreated cells and nanoparticle-treated A549 cells, and green and green-blue correspond to treated MDA-MB-435 cells. (d) Values 1/T2 for cells treated with an increasing amount of nanoparticles. (e) Confocal microscopy images showing the distribution of MnMEIO-siGFP-Cy5/PEG-RGD nanoparticles in cells. Nanoparticles appear red, endosomes stained with lysotracker appear blue, merged red and blue regions appear pink. The cell morphology is outlined on the images [34].

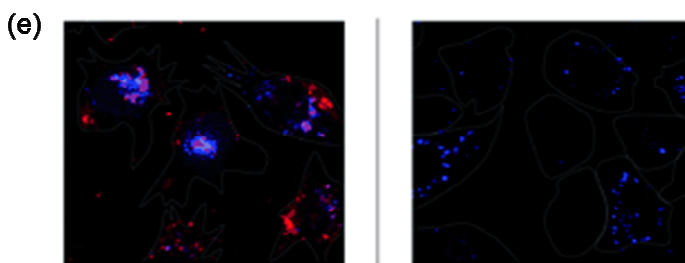


Fig. 1 (continued)

3.1.2 CT Contrast Agents

Computed tomography (CT) is an X-ray-based whole-body imaging technique that is widely used in medicine. Clinically approved contrast agents for CT are iodinated small molecules or barium suspensions. The development of nanoparticle-based X-ray contrast agents is providing an increasing contribution to the field of diagnostic and molecular imaging. The utilization of nanoparticles provides several advantages over the widely used iodinated contrast agent solutions, similar to the Gd^{3+} cases described in the previous section 3.1.1. For example, their surface can be modified to enhance their specificity by attaching targeting moieties, increase their circulation half-life by adding appropriate coatings (e.g. polymers, silica), and improve their functionality by adding other components, including fluorescent markers and therapeutic agents. Gold nanoparticles are a good candidate for CT contrast agents because they induce X-ray attenuation [35]. X-ray attenuation coefficient is determined by the atomic number and electron density of the tissue. The atomic number and electron density of gold (79 and 19.32 g/cm³, respectively) are much higher than those of the currently used iodine (53 and 4.9 g/cm³). In addition, gold nanoparticles provide a high degree of flexibility in terms of ease of producing, modifying the surface for conjugating functional groups, and have also proved to be nontoxic and biocompatible *in vivo*.

3.1.3 Optical Contrast Agents

Fluorescent proteins (FP) have been used as a fluorescent marker for gene activity, protein expression, subcellular components, cell status/fate, among others [36]. Even though there are many advantages of FP-based systems, such as relatively easy and non-cytotoxic expression, this technique does not give sufficient signal for *in vivo* imaging. Low signal to noise (S/N) ratios due to poor tissue penetration of the excitation light and, more importantly, tissue autofluorescence has restricted the utility of most FP-based systems to imaging of cultured cells or superficial tissues [37]

Bioluminescence imaging of luciferase reporter genes is much more sensitive for *in vivo* imaging applications due to the lack of background bioluminescence signal [38]. Luciferases are photoenzymes isolated from a wide variety of insects,

marine organisms, and bacteria, which emit light upon interaction with a specific substrate. Zhou et al. tracked expression of triple fusion (TF) gene which was loaded in liposome nanoparticles by using Renilla luciferase bioluminescence imaging (Figure 2) [39]. Triple fusion (TF) plasmid, pcDNA3.1-RFP-RLuc-HSV-ttk, consisting of red fluorescence protein (RFP), renilla luciferase (RLuc), and herpes simplex virus truncated thymidine kinase (HSV-ttk) reporter genes²² were used for imaging and gene therapy. As is shown in Figures 2 (c), the TF gene entrapped in liposome nanoparticles was found to be expressed at a considerably higher level, in which no significantly prolonged duration was observed.

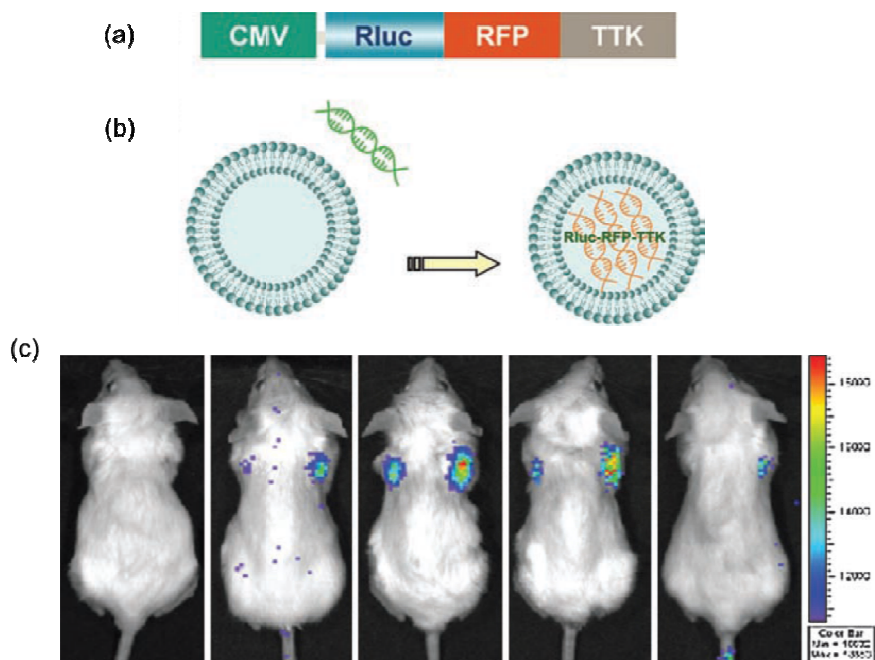


Fig. 2 Schematic of liposome loaded with a tri-fusion reporter gene (TF). (A) Schematic representation of the tri-fusion reporter gene (TF) containing Renilla luciferase (Rluc), red fluorescent protein (RFP) and HSV-ttk driven by a CMV promoter. (B) Schematic representation of liposome nanoparticle and liposome-DNA coupling reaction. Therapeutic liposomes were loaded with Rluc-RFP-ttk gene. (C) The expression of TF transgene was tracked and analyzed by imaging and immunofluorescence. Imaging of Renilla luciferase intensity from one representative BALB/c mouse [39].

3.1.4 Plasmonic Nanoparticles

Metallic nanoparticles, usually made of gold or silver, scatter light with remarkable efficiency by surface plasmonic resonance effect. Typical scattering cross sections of metal nanoparticles greatly exceed the absorption cross-section of fluorescent dyes [40] and of fluorescent proteins [41]. The photostability, water

solubility and nontoxicity of gold nanoparticles make these probes advantageous for biological imaging. Conjugation strategies to attach targeting or delivery moieties are well developed [42]. Gold nanoparticles can provide optical contrast via either absorption, scattering or luminescence. Surface plasmon resonance peak of gold nanoparticles can be tuned to the NIR spectral region (700–850 nm), where tissue is the most transparent, by modifying particle size, shape, material composition or interparticle spacing. [43]. It has been demonstrated that targeted gold nanoparticles can be used to provide high contrast images of cancer cells using a variety of optical modalities to measure light scattered by the nanoparticles, including reflectance confocal microscopy [44], dark-field microscopy[45], phase-sensitive optical coherence tomography [46] and photoacoustic imaging [47]. Aaron et al. demonstrated that scattering spectrum of EGFR-targeted nanoparticles shifts from green to yellow to red as the receptor tracks [44] (Figure 3).

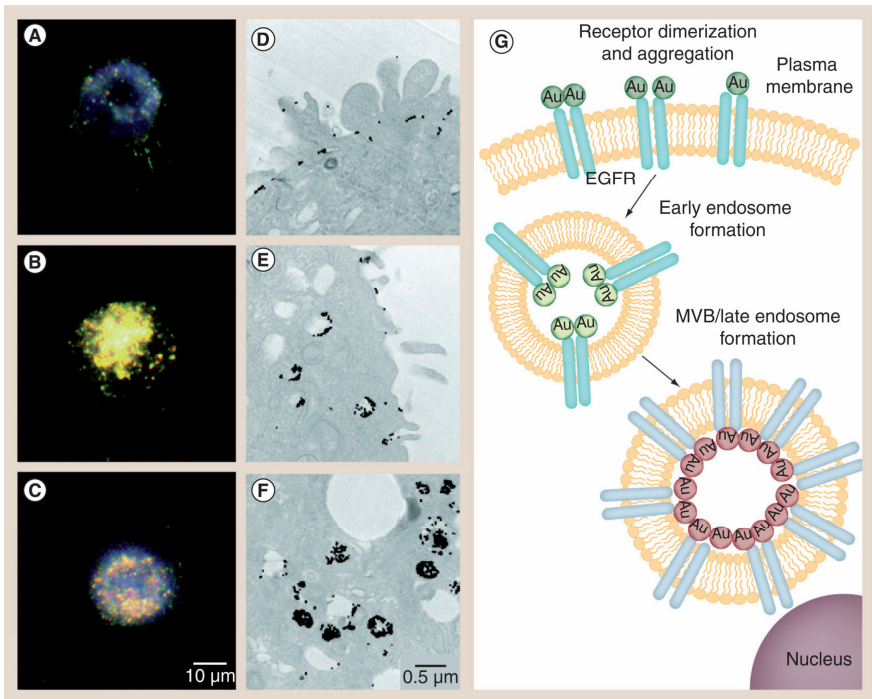


Fig. 3 Gold nanoparticles for dynamic imaging of EGF receptor trafficking in live cells. Dark-field and transmission electron microscopy images of cells labeled with 25-nm anti-EGF receptor (EGFR)-targeted gold nanoparticle conjugates at (A & D) 4°C, (B & E) 25°C and (C & F) 37°C. Labeling at these temperatures arrests the EGFR regulatory process at critical points, with receptors located on the cell membrane at 4°C, endosomal internalization at 25°C and MVB sorting at 37°C. (G) The relationship between EGFR regulation state and the optical signature of the gold nanoparticles in that arrangement. MVB: Multivesicular body [44].

Gold nanorods exhibit intense two-photon luminescence. Two-photon imaging of tissue phantoms treated with 50×15 nm gold nanorods functionalized with EGFR antibodies increased intensity by three orders of magnitude compared with cellular autofluorescence with 760 nm excitation [48].

4 Targeting and Controlled Release of Genes

4.1 Targeting

Targeting is achieved by using a targeting moiety on the surface of a delivery vehicle. Targeting is critical not only for payload delivery to the target to minimize side effects due to non-specific delivery but also for molecular imaging or image-guided delivery using imaging contrast agents. The targeting moiety is an important determinant of the specificity and sensitivity of the contrast agent. The targeted biomarker must be adequately abundant for detection and sufficiently specific to the particular disease or stage of the disease under examination to yield adequate image contrast. Once the target biomarker has been identified, a targeting moiety must be selected. Antibodies, antibody fragments, and peptides identified from phage-display peptide libraries are some of the most commonly used targeting moieties; each class of targeting molecule is associated with different binding kinetics and delivery challenges. A targeting moiety, often via an amine, carboxyl or thiol functional group present in the targeting protein is conjugated to the surface of nanoparticles. Stability, the potential to image multiple targets, and toxicity are important considerations when selecting the targeting moiety. Perhaps the most significant challenge with this approach is the difficulty of achieving high target-to-background ratios. The imaging time window must be optimized, as the target-to-background ratio may remain high if unbound antibodies are still circulating after intravenous (iv.) injection. In addition, antibodies are large molecules (~150 kDa), and this presents barriers to tissue penetration during iv. administration as well as topical application. Engineered antibody fragments are smaller in size and have the potential to improve delivery while maintaining specificity and lowering production costs [49]. Targeting peptides present an attractive alternative to the use of antibodies [50]. Their small size (<10 kDa) reduces barriers to topical delivery and tumor penetration. Also they are less likely to elicit an immune response [51].

4.2 Controlled Release of Genes

As mentioned throughout Section 2, release of genes at the target of interest is as important as carrying genes safely. Even though a technique of electrostatic

binding of genes has been studied extensively, a main issue of releasing genes still remains. A strong binding between the abundant positive charge on the polymer and the negatively charged phosphonic acid of the nucleic acid hinders the release of genes within the cytoplasm thus lowering the efficacy of the gene expression [52]. New materials have been developed to have capability of release via a trigger, including temperature [53], pH [54], redox potential [55], light [56], electric pulse [57], enzymatic degradation [58], and salt levels [59], in order to increase intracellular delivery efficiency. In addition, ultrasound exposure in the presence of microbubbles (e.g. contrast agents used to enhance ultrasound imaging) increases gene transfection efficiency *in vitro* by several orders of magnitude [60]. Park et al. demonstrated via direct observation of vessels-on-a-chip that cavitating microbubbles enhance targeted drug intracellular delivery efficiency. Ultrasound enhanced gene transfer has also been successfully achieved *in vivo*, with reports of spatially restricted and therapeutically relevant levels of transgene expression [60].

In addition, in order for gene therapy to be applicable in clinical medicine, it is imperative that a suitable method for stable controlled release of the required amount of the vector delivered over the desired period of time be developed. A magnetic field has been used with magnetic particles to facilitate gene delivery for a desired period of time, as shown in Figure 4 [61]. Advantages of the method include the rapid accumulation of the therapeutic agent at the target site, thereby reducing the transfection time and increasing the efficiency of transfection while decreasing cytotoxicity. For *in vivo* magnetofection, the magnetic nanoparticles carrying therapeutic genes are generally injected intravenously. As the particles flow through the bloodstream, they remain at the target site because of the application of a very strong high-gradient external magnetic field. Once they are captured at the site, the magnetic particles carrying the therapeutic gene are taken up by the cells in the area, followed by the release of the gene via enzymatic cleavage of cross-linked molecules or degradation of the polymer matrix. If DNA is embedded inside or within the coating material, the magnetic field must be applied to heat the particles and release the gene from the magnetic carrier [62]. In this therapy MNPs were subjected to an oscillating magnetic field, which leads to the generation of heat via two mechanisms depending on the size of the particles: (a) Brownian mode: for MNPs < 100 nm in diameter, heat production is due to the friction between the oscillating particles, and (b) Neel mode: for larger MNPs the heat is produced via the rotation of the magnetic moment with individual field oscillation [63]. Moreover, it would be desirable to be able to stop delivery and expression of the gene as soon as it is decided to stop the treatment and to minimize the significant side effects of this type of therapy.

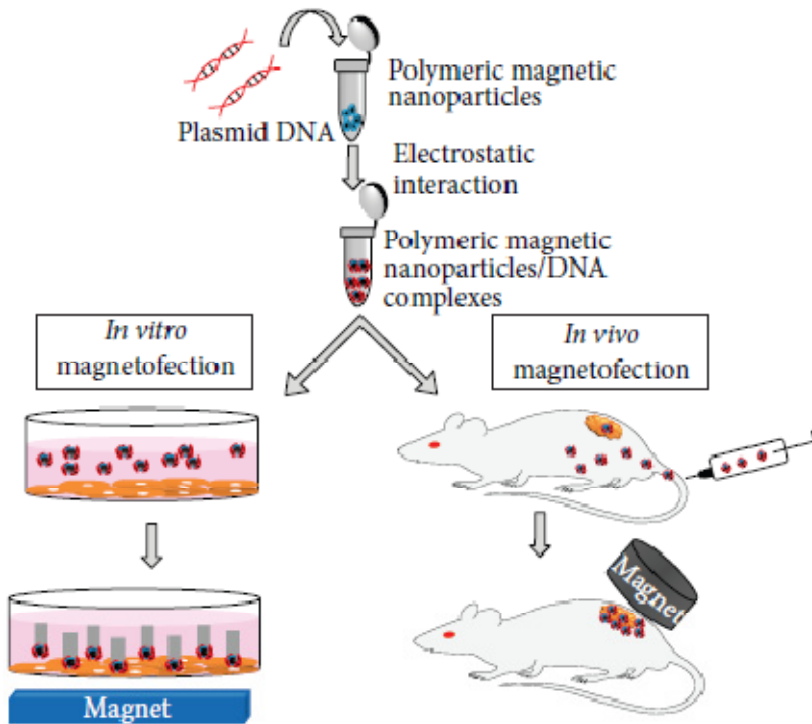


Fig. 4 Schematic representation of in vitro and in vivo gene delivery using magnetofection (grey color pattern on the left corner of the image represents the direction of movement of MNPs under the influence of magnet) [61]

5 Therapeutic Efficacy and Safety

5.1 Current Status of Non-viral Gene Delivery with Molecular Imaging

Up to date, approximately 70% of gene therapy clinical trials have used modified viruses such as retroviruses, lentiviruses, adenoviruses and adeno-associated viruses (AAVs) to deliver genes. However, developments in material sciences, such as new polymers and lipids, as well as in nanotechnology, nanoparticles for gene delivery have shown a rapid progress. siRNA, miRNA, and DNA delivery have been tried clinically using non-viral vectors. Table 1 shows non-viral gene delivery systems which completed clinical trials. Mostly the systems consist of liposomes or polymers. One study which used PEI as a plasmid DNA delivery vehicle for intermediate-risk superficial bladder cancer showed serious adverse events for 3 patients out of 47 (6.38%) (Clinicaltrials.gov identifier: NCT00595088). Therefore, development of new materials which can deliver genes

into cytosol without cytotoxicity is critical. Truong et al. developed degradable poly(2-dimethylaminoethyl acrylate) (PDMAEA), which enables the release of siRNA after endocytosis. The polymer changes to a negatively charged and non-toxic polymer after the release of small interfering RNA, presenting potential for multiple repeat doses and long-term treatment of diseases [64].

None of clinical trials which involve non-viral gene therapy with contrast agents has been found. This might be in part due to lack of signal intensity for combined systems. Development of new imaging contrast agents which can guide gene delivery and enable us to track gene expression over time without toxicity is necessary. Overall, ideal materials for nanoparticle complexes should be biocompatible, nonimmunogenic, and multifunctional for carrying and releasing genes and for molecular imaging.

Table 1 Non-viral gene delivery systems which completed clinical trials

Delivery system	Gene type	Condition	phase	Clinicaltrials.gov identifier
Cationic Liposome	cystic fibrosis gene (pGT-1)	Cystic fibrosis	I	NCT00004471
Cholesterol-fus1 Liposome Complex (DOTAP:Chol-fus1)	fus1 plasmid DNA	Non-small cell lung cancer	I	NCT00059605
DOTMA/Cholesterol [Ratio 1:0.5(-/+)] Liposomes	interleukin-2 gene	Head and neck cancer cells	II	NCT00006033
LErafAON (liposomal c-raf antisense oligonucleotide)	c-raf antisense oligonucleotide	Neoplasms	I	NCT00024661
PEG-PEI-Cholesterol Lipopolymer	IL-12 Plasmid EGEN-001	Peritoneal Carcinoma/Recurrent Ovarian Carcinoma	II	NCT01118052
ALN-PCS02 Liposomes	siRNA KIF11 and VEGF	Solid tumours	I	NCT01158079
ALN-PCS02 Liposomes	siRNA PCSK9	Hypercholesterolemia	I	NCT01437059

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