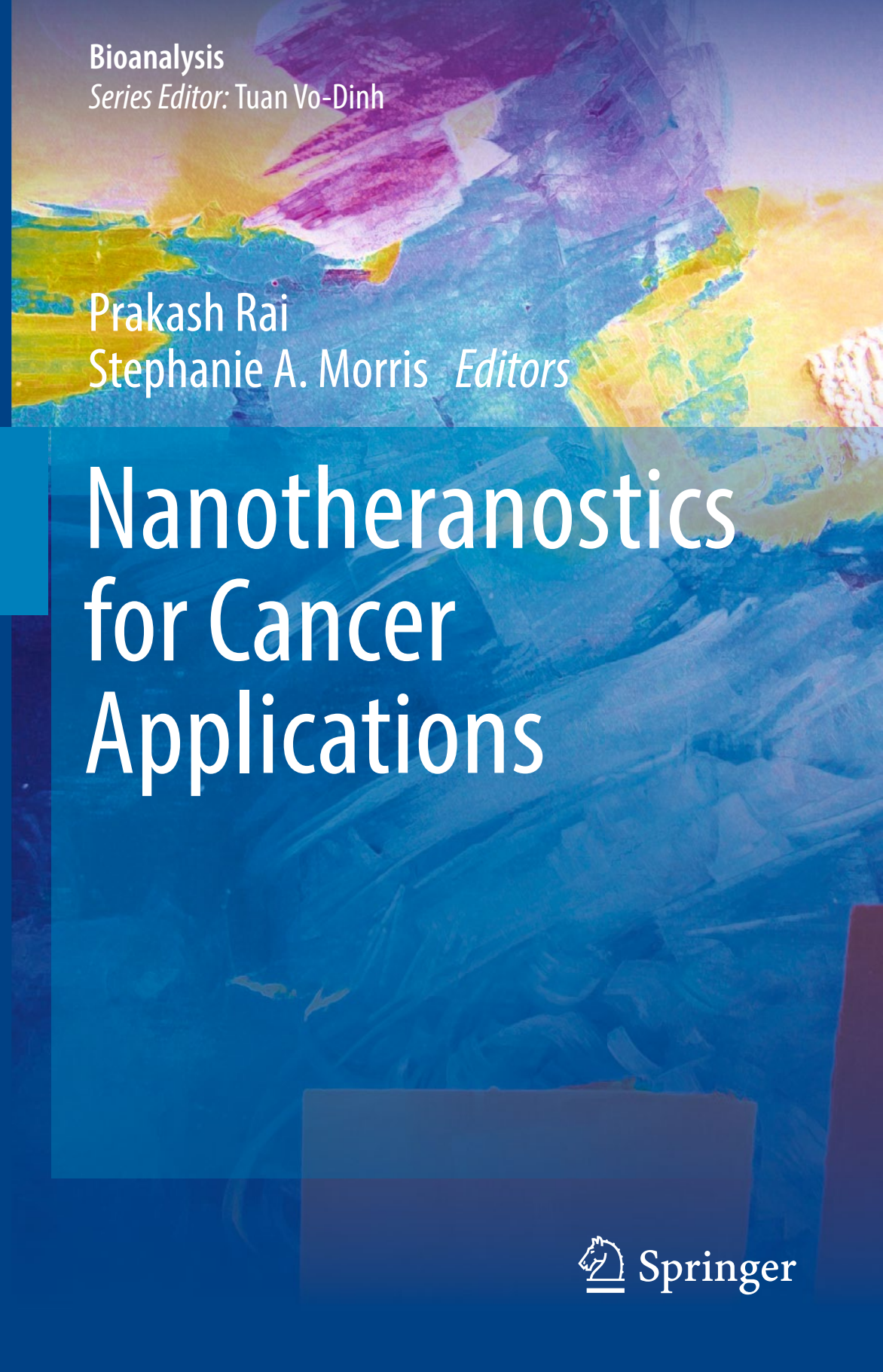
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Tuan Vo-Dinh
Fitzpatrick Institute for Photonics
Duke University
Durham, NC, USA

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Prakash Rai • Stephanie A. Morris
Editors

Nanotheranostics for Cancer Applications

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Editors
Prakash Rai
Department of Chemical Engineering
University of Massachusetts Lowell
Lowell, MA, USA

Stephanie A. Morris
National Cancer Institute, NIH
Nanodelivery Systems and Devices Branch
Cancer Imaging Program, Division of
Cancer Treatment and Diagnosis
Rockville, MD, USA

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Contributors

Hunter N. Bomba Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, Raleigh, NC, USA

Erica N. Bozeman Department of Surgery, Emory University School of Medicine, Atlanta, GA, USA

Xiaoyuan Chen Laboratory of Molecular Imaging and Nanomedicine, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD, USA

Bomy Lee Chung David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Department of Chemical Engineering, Cambridge, MA, USA

Alexander J. Donovan Department of Chemical Engineering, University of Illinois at Chicago, Chicago, IL, USA

Keyvan Farahani National Cancer Institute, National Institutes of Health, Rockville, MD, USA

Rachael A. Floreani Bioengineering Program, College of Engineering and Mathematical Sciences, College of Medicine, University of Vermont, Burlington, VT, USA

Mechanical Engineering Program, College of Engineering and Mathematical Sciences, University of Vermont, Burlington, VT, USA

Venkateswara Gogineni Departments of Biomedical Engineering & Radiology, Medical College of Wisconsin, Milwaukee, WI, USA

James Grant School of Dentistry, University of California, Los Angeles, Los Angeles, CA, USA

Jordan J. Green Department of Biomedical Engineering, Translational Tissue Engineering Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Zhen Gu Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, Raleigh, NC, USA

Division of Pharmacoengineering and Molecular Pharmaceutics and Center for Nanotechnology in Drug Delivery, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Christopher M. Hartshorn National Cancer Institute, NIH, Nanodelivery Systems and Devices Branch, Cancer Imaging Program, Division of Cancer Treatment and Diagnosis, Rockville, MD, USA

Anthony J. Hickey RTI International, Research Triangle Park, NC, USA

Dean Ho SINAPSE, Departments of Biomedical Engineering and Pharmacology, and Biomedical Institute for Global Health Research and Technology (BIGHEART), National University of Singapore, Singapore, Singapore

Department of Bioengineering, University of California, Los Angeles, Henry Samueli School of Engineering and Applied Science, Los Angeles, CA, USA

Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA, USA

California NanoSystems Institute, Los Angeles, CA, USA

Kathryn Hughes Center for Drug Evaluation and Research, US Food and Drug Administration, Silver Spring, MD, USA

Rahul Jadia Department of Biomedical Engineering and Biotechnology, University of Massachusetts Lowell, Lowell, MA, USA

Jaidip M. Jagtap Departments of Biomedical Engineering & Radiology, Medical College of Wisconsin, Milwaukee, WI, USA

Yalia Jayalakshmi C2CBio Consulting, Sunnyvale, CA, USA

Amit Joshi Departments of Biomedical Engineering & Radiology, Medical College of Wisconsin, Milwaukee, WI, USA

Nazila Kamaly Technical University of Denmark, Department of Micro and Nanotechnology, DTU Nanotech, Lyngby, Denmark

Joseph Kaplinsky Technical University of Denmark, Department of Micro and Nanotechnology, DTU Nanotech, Lyngby, Denmark

Mamta Kapoor Center for Drug Evaluation and Research, US Food and Drug Administration, Silver Spring, MD, USA

C. Katherine Wang Formerly of BRIM Biotechnology, Taipei, Taiwan

Theodore Kee SINAPSE, Departments of Biomedical Engineering and Pharmacology, and Biomedical Institute for Global Health Research and Technology (BIGHEART), National University of Singapore, Singapore, Singapore

Manish Kohli Department of Oncology, Mayo Clinic, Rochester, MN, USA

Janel L. Kydd Department of Biomedical Engineering and Biotechnology, University of Massachusetts Lowell, Lowell, MA, USA

Robert Langer David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Department of Chemical Engineering, Cambridge, MA, USA

Phuong Le Micro and Nanotechnology Laboratory, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Jung-Rok Lee Division of Mechanical and Biomedical Engineering, Ewha Womans University, Seoul, South Korea

Youngshin Lee School of Dentistry, University of California, Los Angeles, Los Angeles, CA, USA

Christina H. Liu National Cancer Institute, National Institutes of Health, Rockville, MD, USA

Gang Liu State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics & Center for Molecular Imaging and Translational Medicine, School of Public Health, Xiamen University, Xiamen, China

Ying Liu Department of Chemical Engineering, University of Illinois at Chicago, Chicago, IL, USA

Department of Biopharmaceutical Sciences, University of Illinois at Chicago, Chicago, IL, USA

Liang Ma Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Micro and Nanotechnology Laboratory, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Yusra Betul Medik Laboratory of Nano- and Translational Medicine, Carolina Center for Cancer Nanotechnology Excellence, Carolina Institute of Nanomedicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Department of Radiation Oncology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Tianxin Miao Bioengineering Program, College of Engineering and Mathematical Sciences, College of Medicine, University of Vermont, Burlington, VT, USA

Darron Miya School of Dentistry, University of California, Los Angeles, Los Angeles, CA, USA

Stephanie A. Morris National Cancer Institute, NIH, Nanodelivery Systems and Devices Branch, Cancer Imaging Program, Division of Cancer Treatment and Diagnosis, Rockville, MD, USA

Mana Naeim School of Dentistry, University of California, Los Angeles, Los Angeles, CA, USA

Chin Chun Ooi Department of Chemical Engineering, Stanford University, Stanford, CA, USA

Abdul K. Parchur Departments of Biomedical Engineering & Radiology, Medical College of Wisconsin, Milwaukee, WI, USA

Uma Prabhakar Formerly of Office of Cancer Nanotechnology Research, NCI, Bethesda, MD, USA

Prakash Rai Department of Biomedical Engineering and Biotechnology, University of Massachusetts Lowell, Lowell, MA, USA

Department of Chemical Engineering, University of Massachusetts Lowell, Lowell, MA, USA

Kyle C. Roche Laboratory of Nano- and Translational Medicine, Carolina Center for Cancer Nanotechnology Excellence, Carolina Institute of Nanomedicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Department of Radiation Oncology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Zach Rodgers Department of Chemistry, School of Physical and Mathematical Sciences, Westminster College, New Wilmington, PA, USA

Luisa M. Russell National Cancer Institute, National Institutes of Health, Rockville, MD, USA

Christie M. Sayes Department of Environmental Science, Baylor University, Waco, TX, USA

Gayatri Sharma Departments of Biomedical Engineering & Radiology, Medical College of Wisconsin, Milwaukee, WI, USA

Andrew M. Smith Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Micro and Nanotechnology Laboratory, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Wujin Sun Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, Raleigh, NC, USA

Division of Pharmacoengineering and Molecular Pharmaceutics and Center for Nanotechnology in Drug Delivery, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Pushpa Tandon National Cancer Institute, National Institutes of Health, Rockville, MD, USA

Katherine M. Tyner Center for Drug Evaluation and Research, US Food and Drug Administration, Silver Spring, MD, USA

Praveena Velpurisiva Department of Biomedical Engineering and Biotechnology, University of Massachusetts Lowell, Lowell, MA, USA

Andrew Z. Wang Laboratory of Nano- and Translational Medicine, Carolina Center for Cancer Nanotechnology Excellence, Carolina Institute of Nanomedicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Department of Radiation Oncology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Jinqiang Wang Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, Raleigh, NC, USA

Division of Pharmacoengineering and Molecular Pharmaceutics and Center for Nanotechnology in Drug Delivery, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Shan X. Wang Department of Materials Science and Engineering and Electrical Engineering, Stanford University, Stanford, CA, USA

Sam Warner Laboratory of Nano- and Translational Medicine, Carolina Center for Cancer Nanotechnology Excellence, Carolina Institute of Nanomedicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Department of Radiation Oncology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Sarah B. White Departments of Biomedical Engineering & Radiology, Medical College of Wisconsin, Milwaukee, WI, USA

Lily Yang Department of Surgery, Emory University School of Medicine, Atlanta, GA, USA

Yanqi Ye Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, Raleigh, NC, USA

Division of Pharmacoengineering and Molecular Pharmaceutics and Center for Nanotechnology in Drug Delivery, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Camila Gadens Zamboni Department of Biomedical Engineering, Translational Tissue Engineering Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA

About the Editors

Prakash Rai, Ph.D. joined the Department of Chemical Engineering at the University of Massachusetts Lowell as an Assistant Professor in September 2012. Prior to this, he was an Instructor at the Center for Engineering in Medicine at Massachusetts General Hospital (MGH) and Harvard Medical School. He received his Bachelor's degree in Chemical Engineering from the University of Mumbai, India, in 2003 and his Ph.D. in Chemical and Biological Engineering from Rensselaer Polytechnic Institute in 2007. He is currently working on developing translational nanotechnology-based platforms for the imaging, prevention, and treatment of cancer. His research involves designing, synthesizing, characterizing, and evaluating the efficacy of these multifunctional nanoparticles in cell culture as well as animal models of the disease. He has authored over 30 publications in peer-reviewed journals with several first author publications in journals including *Nature Biotechnology*, *Proceedings of the National Academy of Sciences*, *Angewandte Chemie*, and *Cancer Research*. In 2010, he was chosen to receive the "Scholar in Training" award by the American Association of Cancer Research. He has also received a cancer nanotechnology "Pathway to Independence" grant award from the National Cancer Institute at the National Institutes of Health to carry out research in the area of cancer theranostics.

Stephanie Morris, Ph.D. joined the National Institutes of Health (NIH) as a Program Director at the National Cancer Institute in 2012 where she managed a portfolio of nanotechnology research projects and centers in the Alliance for Nanotechnology in Cancer program. She also developed new research initiatives such as the Innovative Research in Cancer Nanotechnology program and participated on several NIH and interagency committees and working groups, especially those focused on nanotechnology data sharing. She received her Bachelor's degree in Biology and Neuroscience and Behavior (dual majors) from Wesleyan University in Middletown, CT, and her Ph.D. in Biochemistry and Biophysics from the University of North Carolina at Chapel Hill where she was a Ford Foundation Fellow. She is also the recipient of a UNCF-Merck Postdoctoral Research Fellowship, which she received while in the National Cancer Institute's Laboratory

of Receptor Biology and Gene Expression. Her research background includes the study of transcription and chromatin-modifying enzymes. Dr. Morris recently moved to the Office of Strategic Coordination in the National Institutes of Health Office of the Director where she is involved in strategic planning for the NIH Common Fund and serves as a Program Officer for the Molecular Transducers of Physical Activity and the Somatic Cell Genome Editing programs.

Part I
Introduction to Cancer and Nanotechnology

Chapter 1
Introduction

Praveena Velpurisiva, Janel L. Kydd, Rahul Jadia, Stephanie A. Morris, and Prakash Rai

Traditional medicine has been in use since ancient times to treat various forms of illness. The modern practice of medicine has evolved into advanced surgery, endoscopy, laser treatments, radiation therapies, and chemotherapies. Newer modalities of treatment include immunotherapies and genetic therapies that aim to be molecularly targeted to a patient's disease. These advancements have enhanced healthcare leading to improved treatment outcomes. However, these forms of treatments tend to be invasive and pose side effects due to their non-specificity for the diseased tissue. Since existing options do not provide a go-to solution for every disease, some of the emerging pharmacological technologies focus on providing personalized and targeted treatments that minimize toxic effects while effectively treating the illness.

Success stories in drug discovery have led us to identify a vast library of therapeutics that can be very effective in in vitro and in vivo models of diseases but often fail to produce the desired response in humans [1]. Maladies that continue to plague mankind are considered caused by "drug delivery problems" as opposed to "drug discovery problems," which have been conventionally thought of as root sources of

P. Velpurisiva · J. L. Kydd · R. Jadia
Department of Biomedical Engineering and Biotechnology, University of Massachusetts Lowell, Lowell, MA, USA

S. A. Morris (✉)
National Cancer Institute, NIH, Nanodelivery Systems and Devices Branch, Cancer Imaging Program, Division of Cancer Treatment and Diagnosis, Rockville, MD, USA
e-mail: morriss2@mail.nih.gov

P. Rai (✉)
Department of Biomedical Engineering and Biotechnology, University of Massachusetts Lowell, Lowell, MA, USA

Department of Chemical Engineering, University of Massachusetts Lowell, Lowell, MA, USA
e-mail: prakash_rai@uml.edu

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failure in successful disease-ameliorative measures. Delivering the "right drug" to the "right place" at the "right time" in the "right dose" could help improve treatment outcomes across all disease types. As Sir Richard Feynman had envisioned the field of nanotechnology in the mid-twentieth century [2], key nanoscale properties like high surface area to volume ratio and smaller size have revolutionized various fields like drug delivery, diagnostics, 3D printing, cosmetics development, food processing, nano-fertilization for agriculture, miniaturization of chips and semiconductors in electronic devices, composite coatings for automobiles, and decontamination of water and soil [3]. Nanoscale materials vary from bulk or macroscale materials in their mechanical, electrical, optical, and magnetic properties [4]. Exploiting these properties in nanomaterials for applications in medicine, referred to as nanomedicine, can address concerns about the location, time, dose, and ability to deliver the most appropriate drug and will be the focus of this book.

1.1 Nanotechnology in Medicine

Traditional drug treatments in medicine are riddled with acute toxic side effects, long-term potential disruption of vital organ function, drug resistance, and overall unsatisfactory response rates and likelihood of recurrence [5, 6]. Therapeutic approaches using nanomedicines are designed to allow for lower doses of drug(s) being required to elicit a favorable treatment response compared to the naïve formulation of the drug [7–9]. Nanomedicines are used in treatments for various diseases like malaria, multiple sclerosis, and autoimmune diseases and have been shown to provide new avenues for patient care that aim to ameliorate disease and reduce unnecessary harm to healthy tissue by targeting specific areas of diffuse disease [6, 10–14].

Nanomedicines have several virtues that enable them to improve the future of medicine and healthcare. They can be targeted by surface decoration via bioconjugation with ligands such as antibodies, peptides, or aptamers. Such targeted nanomedicines offer less non-specific cytotoxicity, which is common to chemotherapies, and can be designed to have multiple functionalities [15, 16]. The simplicity in the formation of nanoparticles holds a potent punch vis-a-vis the possibilities of diagnosis and prognosis and in the treatment of several ailments ranging from cardiovascular to infectious diseases [9]. The goal of such delivery systems is to provide a means by which sufficient quantities of relevant cargo (usually toxic) are brought to specific areas of the body while being protected from the surrounding environment within the body cavity. Effective treatment regimens necessitate a means by which a disease process can be addressed safely and reliably. This needs to be achieved while preserving the integrity of healthy tissue and treating the site of interest in an efficient manner that will induce less stress to patients and enhance positive outcomes such as greater overall survival, lower morbidity, and an improved quality of life. Nanomedicine certainly can help usher in such effective diagnostic and

treatment regimens for better disease management while reducing the overall cost of healthcare.

1.2 Nanodiagnostics and Prognostics

Diagnostic and prognostic nanotechnologies have been implemented in areas of disease research such as brain trauma, Alzheimer's disease, kidney and cardiovascular disease, as well as others. Iron oxide nanoparticles, for example, have been used to track neural stem cells in patients with brain trauma by the use of magnetic resonance imaging (MRI) [17, 18]. In addition, early-stage Alzheimer's disease can be detected through MRI using a probe that is specific to Aß oligomers, amyloid fibril precursor peptides that cause plaque formation in the brain and subsequent neurodegenerative disease [17]. Gold nanoparticles have been researched for some time. Nanoparticles, such as gold, which are inert and nontoxic and possess X-ray diffraction properties, or nanoparticles that have magnetic properties, for example, iron oxide nanoparticles, are common modalities in nanotheranostics. Nanotheranostics is an area of nanomedicine that combines therapy and diagnostics through imaging techniques, including MRI, positron-emission topography, and computerized tomography [19]. Common medical procedures such as endoscopy and colonoscopy may be improved with the help of light scattering gold nanoparticles, an example of how versatile such nanoplatforms can be in routine medical procedures and preventative care [20]. Nuclear magnetic resonance-based MRI scans are improved by the use of diamond chips that are composed of nitrogen-vacant centers where magnetic field sensitivity and subsequently MRI resolution are enhanced, another example of ongoing advancements in the application of nanotechnology in medicine [21–23]. Multifunctional nanoparticles, such as those used in dual imaging and drug delivery, provide a means by which treatment of disease occurs simultaneous to image capture and monitoring of the disease process.

Another area, point of care technology, is integral in nanoparticle research and disease process monitoring and diagnosis [24, 25]. This is especially relevant in low- and middle-income countries for use in infectious disease and endemic metabolic disease detection, both universal health concerns [24]. Devices that are easy to use, patient friendly, accurate, inexpensive, portable, chip-based, and self-contained are desirable in these technologies. Nanodevices are being designed to address these needs such as those that are paper-based with microfluidic or screen printing modes of use [26]. A nano-calorimeter type of biosensor has been developed to screen a pinprick droplet of blood for metabolic diseases such as phenylketonuria, for example, while *Streptococcus pyogenes*, a pathogenic cause of rheumatic heart disease, can be detected within minutes using a DNA probe that is immobilized on gold nanoparticle multi-walled carbon nanotube hybrids [25–28]. In addition, a low-cost method to detect glucose oxidase activity in diabetes has been tested using nano-optical sensors [29]. The possible applications cut across several diseases for which medical intervention and detection can be utilized at the point of care for patients.

1.3 Nanotherapeutics

Several exciting applications of nanotechnology in the treatment of diseases ranging from genetic disorders to infectious diseases and cardiovascular diseases to cancer are currently being developed. Increasingly the use of nanoparticles for treating diseases at the genetic level is being explored by turning specific genes either off or on. Andrew Fire and Craig Mello discovered the role of RNA interference (RNAi), a novel mechanism of harnessing the function of small interfering RNA strands (siRNA) to direct gene silencing, for which they were awarded the 2006 Nobel Prize in Medicine or Physiology. The power of RNAi has been adopted by Alnylam Therapeutics for developing a new class of therapeutics that target genes involved in disease initiation and progression [30]. Integrating nanotechnology to deliver genes to target sites further enhanced innovative research in this field of gene therapy. The first FDA-approved gene therapy, Luxturna™ granted to Spark Therapeutics, employs an adeno-associated viral vector (a nano-sized particle) to deliver gene therapy to patients with a specific type of retinal dystrophy [31]. Other forms of gene therapy use the recently discovered CRISPR/Cas9 gene-editing technology, which has the capability of altering, deleting, or repairing genes. Several companies are investigating the use of this technology for targeted treatment of diseases. For example, CRISPR Therapeutics in collaboration with other companies uses this technology to find treatments for sickle cell disease, β-thalassemia, hemophilia, Duchenne muscular dystrophy, and so on. While CRISPR technology has shown great potential, some of the concerns related to its delivery using viral vectors are addressed by pioneers in the field of drug delivery, where they used lipid nanoparticles as carrier modalities. Some other current studies that have focused on exploring the benefits of nanotechnology are listed in Table 1.1.

As an exciting example of nanomedicines for managing infectious diseases, recent advances show that protein nanoparticles encapsulating a core of influenza matrix protein 2 ectodomain (M2e) with a shell of head-removed (hr) HA domains can provide a onetime vaccination, shielding us from various strains of influenza [44]. The shape of nanoparticles can play a crucial role in designing a drug delivery system. A star-shaped polymeric oral delivery capsule containing drugs to treat HIV proved to offer sustained release of the encapsulated drug over a week [45]. Some researchers have also designed a mechanism to fight off antibiotic-resistant bacteria causing peripheral wounds or skin infections. Synthesized quantum dots made of cadmium telluride, which can be excited by a green LED light, generate reactive oxygen species (ROS). This made the bacteria 1000 times more susceptible to antibiotic [46].

Some exciting and ingenious ideas have recently helped transform the fields of nanomedicine as well as drug delivery. For example, dissolvable microneedles were synthesized for efficient delivery of siRNA to target melanoma [47]. A step forward in enabling minimally invasive drug delivery to the brain was achieved by implanting tiny microfluidic probes in mice that release dopamine with a remote trigger. These implants may later be used to deliver disease-specific drugs in the brain. This

Table 1.1 List of nanomaterials engineered to treat various disease forms

Disease	Nanomaterial	Drug	Surface modification	Size (nm)	PDI	Zeta potential	Route	Ref.
Alzheimer's disease	PEGylated P(HDCA-*co*-MePEGCA)	NA	Anti-Aβ $_{(1–42)}$	125 nm	0.1–0.2	(−20 to −30 mV)	Intravenous	[32]
Parkinson's disease	Chitosan/ Mangafodipir coacervate	Anti-*eGFP* siRNA	NA	122 nm	NA	NA	Intranasal	[33]
Cystic fibrosis	Poly(lactic/ glycolic acid) (PLGA)	Ciprofloxacin	NA	190.4 nm	<0.090	−22.5 ± 5.4 mV	Pulmonary	[34]
Tuberculosis	Mannosylated nanostructured lipid carrier (NLC)	Rifampicin	NA	315 nm	<0.2	NA	Pulmonary	[35]
Duchenne muscular dystrophy	CRISPR-Gold	Cas9, gRNA	DNA-SH, donor DNA, poly (N-(N-(2-aminoethyl)-2-aminoethyl) aspartamide), silicate	500 nm	NA	18 mV	Muscle injection	[36]
Inflammatory bowel diseases	Ginger-derived nanoparticles	NA	NA	231.6 nm	0.176	(−12.9 mV)	Oral	[37]
Obstructive nephropathy	Chitosan/siCOX-2 nanoparticles	COX-2 siRNA	N/A	226 nm	NA	28 mV	Intraperitoneal	[38]
Corneal allograft rejection	Poly(d,l-lactic-co-glycolic acid)	Dexamethasone sodium phosphate	NA	200 ± 8 nm	0.12	(−8 mV)	Subconjuctivital injection	[39]
Atherosclerosis	Hyaluronic acid nanoparticles	NA	NA	237 ± 10 nm	NA	NA	Tail vein injection	[40]

(continued)

Table 1.1 (continued)

Disease	Nanomaterial	Drug	Surface modification	Size (nm)	PDI	Zeta potential	Route	Ref.
Human immunodeficiency virus (HIV) infection	Polyanionic carbosilane dendrimers (G3-S16 and G2-NF16)	NA	NA	~2 nm	NA	NA	NA	[41]
Hearing impairment (outer hair cell pathology)	Poly(lactic/ glycolic acid) (PLGA)	NA	P407/ chitosan / methoxy poly(ethylene glycol) (mPEG)	100–200 nm	NA	NA	Intratympanic	[42]
Visceral leishmaniasis	Liposomes[a]	Amphotericin B	NA	NA	NA	NA	Parenteral	[43]

[a]FDA approved

technique can also be applied to other diseases in controlling the release of drugs for localized delivery [48].

In this book, we describe the innovative approaches that are being developed using multifunctional nanotechnology in cancer research, which can offer novel ways to diagnose disease, initiate treatment, and monitor treatment response. Cancer continues to remain a major global healthcare problem [49]. The following chapters will initially divulge at length the biology of cancer with a focus on the biological barriers to drug delivery followed by an introduction to nanotechnology. The chapters that follow will elaborate on the applications of nanotechnology in medicine especially in vitro and in vivo cancer diagnostics and therapeutics administered through various modes and their challenges, such as regulatory approval. Regulatory approval is also discussed in this book as a separate chapter about the role of the Food and Drug Administration (FDA) in approving drug products containing nanomaterials. The later chapters deal with the multifunctional aspects of nanoparticles for image-guided drug delivery and their role as theranostics for real-time monitoring of treatment outcomes in cancer patients (Fig. 1.1). Nanotheranostics research lies at the intersection of nanotechnology, diagnostics, and therapeutics. Thus, the last portion of this book focuses on nanotheranostic applications to cancer following a thorough introduction to these topics. Not unlike other approaches to disease diagnosis and treatment, nanotechnology requires thorough research and development that may take decades. This book provides a review of various nanotechnology applications to cancer and discusses the relatively new development of nanotheranostics as the next stage of this technology (Fig. 1.2).

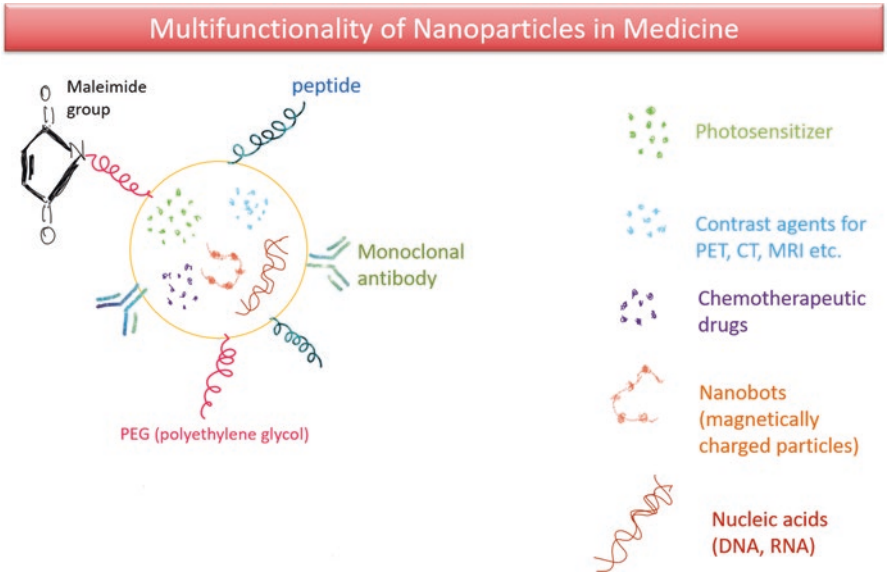

Fig. 1.1 Role of nanoparticles in medicine

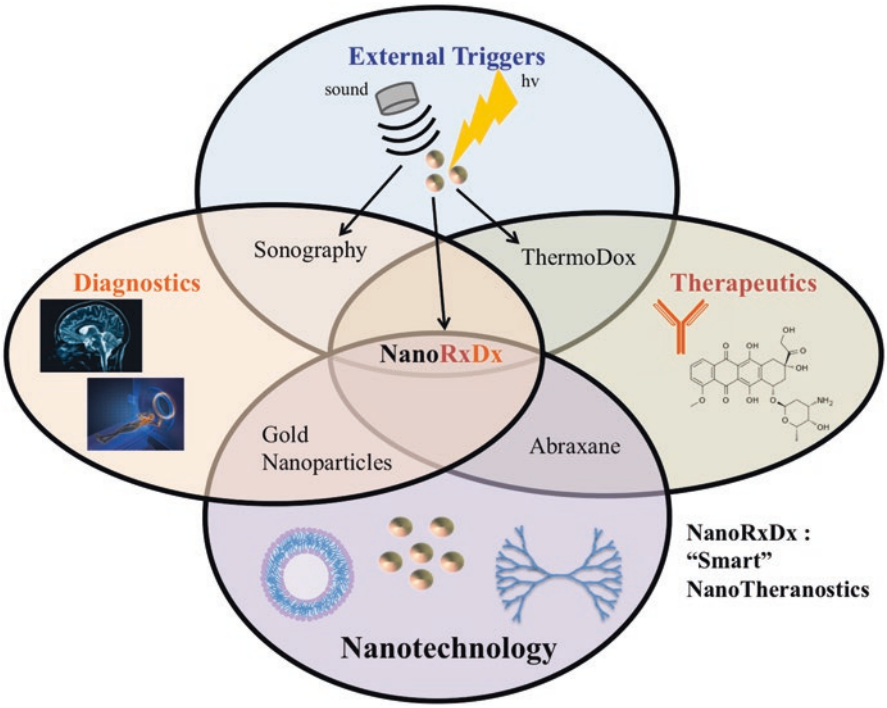

Fig. 1.2 "Smart" nanotheranostics (NanoRxDx) lie at the intersection of nanotechnology, therapeutics, as well as diagnostics and require external triggers to cause therapeutic effects in the diseased tissue

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Chapter 2
Biological Events and Barriers to Effective Delivery of Cancer Therapeutics

Erica N. Bozeman and Lily Yang

Cancer is one of the leading causes of death worldwide with an estimated 70% increase in new cases over the next two decades [1]. While the mortality rates associated with many other diseases have declined in recent decades, cancer-related deaths have remained relatively constant. A variety of strategies and therapies have been developed to combat this deadly disease ranging from localized radiation therapy and systemic chemotherapy to more targeted approaches, including antibody-based therapies, molecular-targeted small molecules, and immunotherapy.

Potentially cancerous cells originate frequently in human bodies due to the accumulation of mutations and alterations in signal pathways that enable them to acquire characteristics to undergo deregulated proliferation, develop resistance to cell death, and evade immune detection [2]. The majority of these premalignant and malignant cells are eliminated by activation of apoptotic cell death and the host's immune system, which is constantly surveying the body for the presence of abnormal cells, a process that F. M. Burnet called "immune surveillance" [3, 4]. At a point, these "precancerous" cells can form an "equilibrium" and coexist within the host without leading to an invasive tumor phenotype for many years even decades. However, over time, due to immune-selective pressure, a small percentage of these cells can acquire sufficient mutations that are necessary to "escape" immune detection and to gain unbalanced cell proliferation and death, leading to a cancerous phenotype. Specific characteristics or "hallmarks of cancer" as reported collectively by Hanahan and Weinberg include (1) resisting cell death, (2) inducing angiogenesis, (3) enabling replicative immortality, (4) evading growth suppressors, (5) sustaining proliferative signaling, and (6) activating invasion and metastasis [5]. With this knowledge, a

E. N. Bozeman · L. Yang (✉)
Department of Surgery, Emory University School of Medicine, Atlanta, GA, USA
e-mail: lyang02@emory.edu

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number of therapies have been developed that target one or multiple hallmarks. During the last decade, additional acquired capabilities of tumor cells that are needed for tumor growth and progression have been identified. Those include avoiding immune destruction, deregulating cellular energetics, genome instability, and tumor-promoting inflammation [6]. All of which are targets under active investigation toward the development of more effective cancer therapeutics.

Although the principles guiding cancer treatment for different types of human cancers share similarities, specific treatment strategies vary depending on the tumor type, disease stage, pathological characteristics, and the presence of known genetic mutations. Human cancer types consist of solid tumors in organs or tissues and hematological malignancies derived from the blood cell lineage, such as leukemia. Solid tumors typically consist of carcinomas, sarcomas, and lymphomas. The development of human cancers is a multistage process that involves a series of alterations in genetic and signal pathways, interactions of tumor cells with their microenvironment, and angiogenesis (the formation of new blood vessels). Transformation from precancerous to malignant cells leads to the development of an early-stage cancer lesion at the original cell or tissue site (in situ tumor) [7]. In response to the malignant transformation in epithelial cells, surrounding stromal fibroblasts and macrophages are activated. Proliferation of those stromal cells and infiltration of myeloid-derived cells and other immune cells, as well as the enrichment of extracellular matrix, form a dense tumor stromal layer that limits migration and invasion of tumor cells. Depending on tumor biology and aggressiveness of the tumor cells, these early-stage tumors can remain in the in situ stage for an extended period of time (i.e., years), but some of them progress into invasive cancers in a relatively short time. Invasive carcinomas are initially retained within the organ or tissue as primary tumors. However, some tumor cells migrate via the lymphatic system or blood vessels to the draining lymph nodes or distant organs to develop metastatic tumors. Many types of invasive tumors, such as pancreatic cancer and triple negative breast cancer, retain stroma-rich histological characteristics. Intensive stromal components create physical and biological barriers for delivery of imaging and therapeutic agents (Fig. 2.1).

The detection of the early-stage tumor followed by surgery treatment is the most effective way for cancer treatment. However, a high percentage of cancer patients are diagnosed at the stage when the tumors have already invaded locally to normal organs and/or metastasized to distant organs. Surgery in combination with systemic chemotherapy or radiation therapy has been used to treat some of these patients with locally advanced tumors that are resectable. For patients with unresectable or metastatic tumors, systemic chemotherapy, small molecule targeted therapy, local radiation therapy, and immunotherapy are commonly used therapeutic approaches. However, systemic chemotherapy can lead to detrimental adverse effects on healthy cells placing patients at risk for additional health complications.

Despite our growing knowledge of how cancer develops and the correlations of genomic and phenotypic characteristics with its progression, current diagnostics and therapies are markedly limited in their clinical efficacy with the majority of

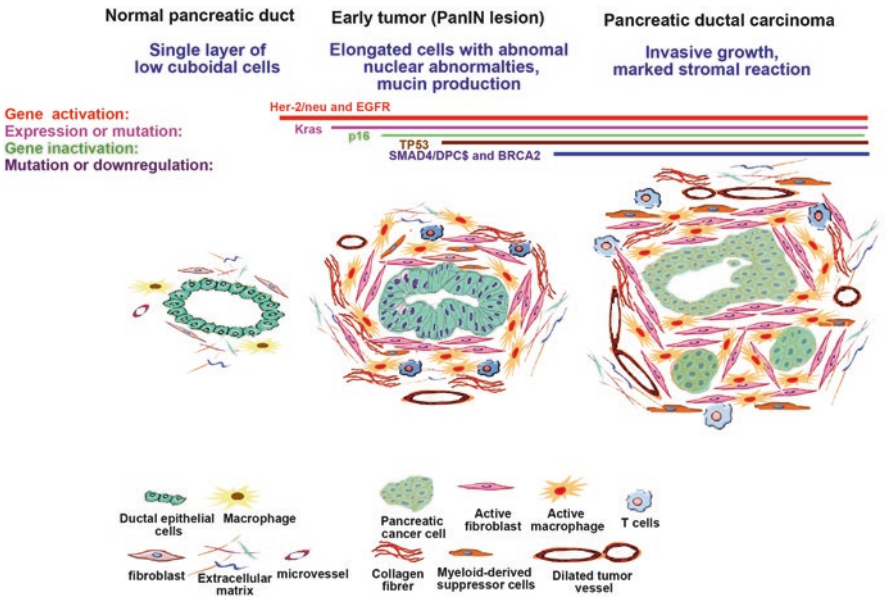

Fig. 2.1 The development of tumor stromal barriers for drug delivery during tumorigenesis of pancreatic cancer. Genetic mutations and alterations lead to the transformation of pancreatic ductal epithelial cells into early stage pancreatic cancer (PanIN). Extensive tumor stromal barriers develop at the early stage of pancreatic cancer development mediated by proliferation and infiltration of active stromal fibroblasts and macrophages as well as an increase in production of extracellular matrix proteins. Further genetic mutations and abnormalities in signal transduction pathways promote the development of invasive pancreatic ductal carcinoma with dysfunctional tumor vessels. About 50–85% of the pancreatic tumor consists of stromal components. Therapeutic agents must overcome the following three major stromal barriers before reaching tumor cells: (1) dysfunctional blood vessels and lack of tumor vessels in hypoxic areas, (2) extensive tumor stromal fibroblasts and macrophages, and (3) dense extracellular matrix

patients being diagnosed at late stage of disease, developing metastatic disease, experiencing tumor recurrence, or succumbing to the disease. Chemotherapy, radiation therapy, surgery, and biological-based therapies (including antibody-based therapies or small molecule inhibitors) comprise the cocktail of strategies to combat tumor progression. Each of which is presented with a number of hurdles and at the center being inefficient delivery of therapeutics and diagnostics into the tumor [8]. The biological barriers present within the tumor microenvironment that impede targeted and sustained drug delivery will be discussed in this chapter including the roles played by the tumor vasculature as well as the heterogeneous cell populations present in the tumor, including carcinoma, stromal, immune, and cancer stem cells, and their relative contributions to the development of drug resistance. Emerging strategies that seek to exploit or circumvent these barriers to enhance delivery efficacy will also be highlighted.

2.1 Barriers to Early Cancer Detection and Diagnosis

Detection of premalignant and malignant diseases at the early stage remains a huge clinical focus; however it has been challenging for several reasons. Appropriate screening technologies must be available that are both highly specific and sensitive to cancer cells, which require that biomarkers and relevant molecular targets of early-stage disease be identified and validated. The most impactful approach for the early detection of cancers in the patient population without symptoms or specific symptoms would be cancer biomarker detection in the blood and other body fluids. However, most serum biomarkers, such as alpha-fetoprotein, CA 19-9, CA-125, carcinoembryonic antigen (CEA), and Her-2/Neu, are used to monitor tumor growth, therapeutic response, and recurrence. They typically lack specificity and sensitivity for diagnosis of tumors at the early stage. Prostate-specific antigen (PSA) is one of the few biomarkers that has been used as a biomarker for both early detection and monitoring [9]. Furthermore, several new types of blood-based biomarkers have shown promise toward early detection including the presence of mutant gene DNA fragments, microRNA markers (noncoding, single-stranded RNAs), tumor-associated exosomes, and autoantibodies directed toward tumor proteins [10].

Current advances in clinical imaging devices, imaging methods, and contrast agents offer an opportunity to detect tumors noninvasively based on blood flow, soft tissue contrast, and tumor metabolic activities. However, those imaging modalities, such as MRI, CT, SPECT, and PET, in general, fail to detect small, early tumors. Although fluorine-18-fluoro-2-deoxy-*D*-glucose (FDG) contrast-mediated positron-emission tomography (PET)/CT is the most sensitive in the detection of metabolically active tumor cells among clinical imaging modalities, it is not tumor specific and has a low resolution to precisely locate small tumors in organs [11]. Recently, various biomarker-targeted imaging based on optical, nuclear, or nanoparticle imaging probes have been developed for molecular cancer imaging and have shown improved sensitivity and specificity in cancer detection. However, the efficiency of imaging using tumor cell-targeted techniques requires specific binding of the imaging probes to the surface of tumor cells. Extensive tumor stroma also creates a barrier that prevents the interaction of the targeted imaging probes with tumor cells and traps the imaging probes non-specifically, resulting in reduced sensitivity and specificity in imaging early tumor lesions. Since more than 50% of a pancreatic tumor mass consists of tumor stromal components, obtaining the necessary contrast between the tumor and surrounding stromal tissues is challenging for diagnostic screening by CT and MRI of pancreatic cancer [12].

2.2 Basic Mechanisms of Delivery of Therapeutic Agents

In order to treat a potentially systemic disease such as cancer, chemotherapy is the most widely used therapy that seeks to non-specifically kill highly proliferative tumor cells via the administration of cytotoxic drugs. It is administered singularly

or in combination with several drugs that act on different cytotoxic mechanisms to enhance the therapeutic effect on tumor cells. Chemotherapy can also be used as an adjuvant therapy before or after surgery or in combination with radiation therapy or immunotherapy. Depending on their chemical and biological properties, chemotherapy drugs can disrupt proliferation/cell cycle in a number of different ways, including disrupting DNA synthesis and inducing DNA damage (e.g., alkylating drugs, heavy metal, antimetabolites, antitumor antibiotics, and topoisomerase inhibitors) or interfering with cell division/cell cycle and mitosis (e.g., plant alkaloids) [13, 14]. While generally effective at killing tumor cells, the main issues with the delivery of conventional chemotherapy drugs are the lack of specificity, insufficient level of drug delivery into the tumor, and drug resistance. A number of normal cells with a high proliferative capacity are found throughout the body, such as hair follicles, bone marrow cells, and gastrointestinal cells, which are also affected by chemotherapy drugs [15, 16]. As a result, one of the major issues for chemotherapy is severe systemic toxicity that limits the dose of drugs that can be administrated into patients and produces detrimental side effects to patients' health and well-being. Furthermore, small molecule chemotherapy drugs are cleared from the blood circulation quickly due to a short blood half-life ($t_{1/2}$) from 15 to 30 min. Only a small percentage of the injected drug molecules (i.e., 0.001–0.05% of total injected dose (ID)/gram) can be delivered to the tumor site [17–19].

To address the issues concerning the lack of specificity, antibody-based delivery of therapeutics seeks to deliver drugs more specifically to the tumor. By targeting proteins that are typically overexpressed by tumors, these antibody-drug or antibody-toxin conjugates have shown proven efficacy in both solid and hematological cancers [20]. Due to the large size of antibodies (MW 150 kDa, ~10 nm in size), antibody-based therapeutics have a longer blood half-life and increased delivery efficiency (8–20% ID%/gram) into tumors compared to chemotherapy drugs [21]. However, one issue of antibody-mediated therapy is that diffusion deep into the tumor tissues is significantly limited due to its large size. Additionally, the high affinity binding nature of most antibodies can further inhibit their tissue penetration by binding to their antigens of interest on the tumor periphery and not being able to reach additional tumor antigens found at the center of the tumor [22].

Lastly, therapeutic agents can be delivered via nanoparticle-based delivery. Nanoparticle drug carriers, with particle sizes <100–200 nm, can be delivered to tumor tissues and accumulate in the perivascular area (the area around blood vessels) via the enhanced permeability and retention (EPR) effect when passing through the leaky tumor vasculatures as shown in Fig. 2.2 [23, 24]. While this effect (discussed in more detail in later section) relies on passive diffusion into the tumor site, many classes of nanoparticles can be conjugated with targeting proteins/ligands that mediate more "active targeting" to the tumor environment. The existence of the EPR effect had been conclusively demonstrated primarily in animal models of human cancer; however, recently this phenomenon has been observed in patients [25]. Tumor vasculatures in human tumors are highly heterogeneous in their distribution and angiogenesis. Unlike tumors developed in experimental animal tumor models, many human solid tumors have relatively slow growth rates and low angiogenesis.

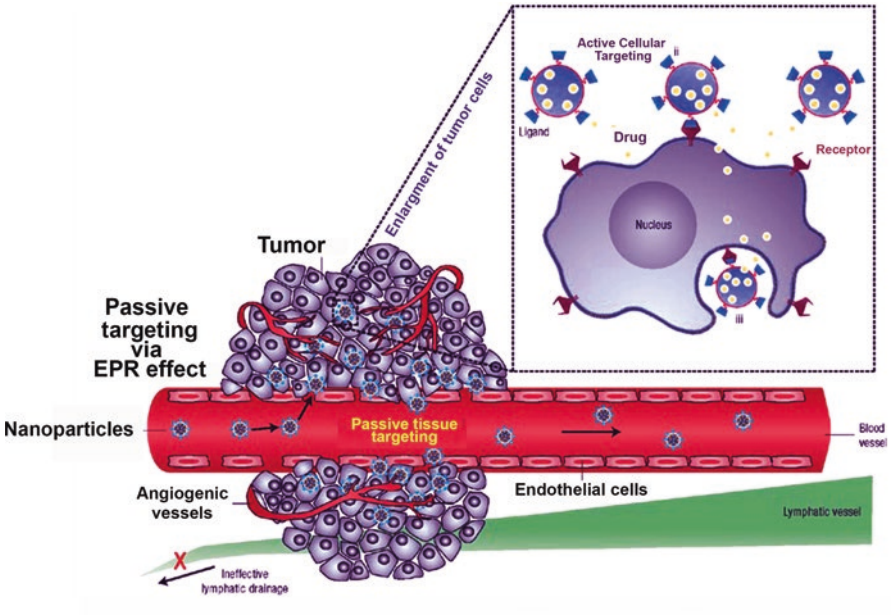

Fig. 2.2 Mechanism of delivery of nanotherapeutics into tumors. Due to their small size (<100 nm), most nanoparticles can effectively navigate the "leaky" vasculatures found within tumors allowing them to enter and be retained within the tumor microenvironment at a higher concentration than other drug formulations. This phenomenon is referred to as the enhanced permeability and retention (EPR) effect. Additionally, nanoparticles can be modified with a variety of ligands that can mediate more active, cell-specific drug targeting based on receptor expression on the tumor cells. (Adapted by permission from Macmillan Publishers Ltd. [23])

Therefore, it is likely that active targeting of drug delivery into tumor cells in human solid tumors is critical due to a low level of tumor areas with leaking vasculatures for the EFR effect-mediated nanoparticle delivery.

2.3 Routes of Administration for Therapeutics

The routes that cancer therapeutics can be administered include intravenous (i.v.), oral, intratumoral, intraperitoneal (i.p.), and inhalation (Fig. 2.3). Physical and biological barriers for drug and nanoparticle delivery via various administration routes are also shown in Fig. 2.3. The vast majority of delivery mechanisms are based on i.v. administration as it allows for direct entry into the systemic blood circulation to reach the tumor site. For small molecular drugs and therapeutic antibodies, systemic administration leads to a high level of distribution into almost all normal organs and tissues. However, i.v. delivery of therapeutic agents with a size over 10 nm, such as engineered viral vectors and nanoparticles, results in non-specific accumulation of

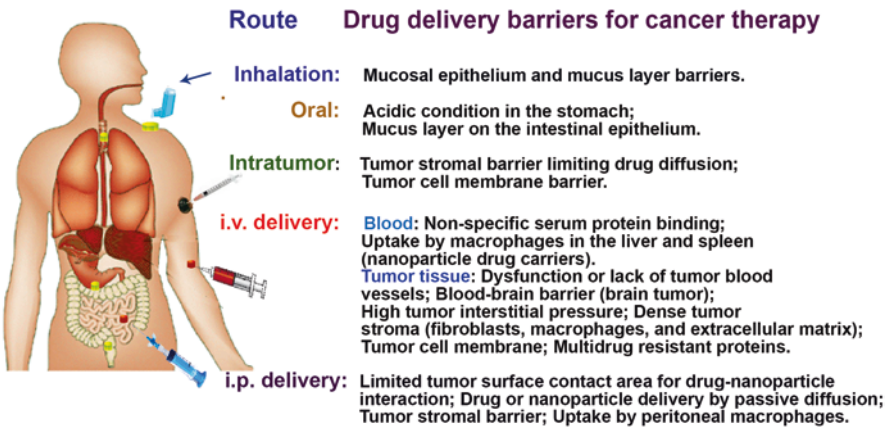

Fig. 2.3 Physical barriers for drug delivery by different routes of administration. Cancer therapeutics can be administered via a number of different routes including inhalation, oral, intratumor, intravenous (i.v.), or intraperitoneal (i.p.). However, each route poses unique barriers for effective drug delivery. (1) Inhalation: Drug or nanoparticle drug carriers are delivered via mucosal epithelium in the pharynx and the lung. The mucus layer creates a drug and nanoparticle delivery barrier. (2) Oral: Acidic condition in the stomach may inactivate drugs or destroy nanoparticle drug carrier. Specific nanoformulation can protect drugs and conditionally release drugs in the intestines. The mucus layer on the intestinal epithelium reduces absorption. This route is not appropriate for delivery of targeted nanoparticles. (3) Intratumor: Local therapy with limited amount of drug delivery and poor intratumoral drug and nanoparticle distribution due to tumor stromal barrier, no mechanism for drug delivery into tumor cells. (4). i.v. delivery: In the blood circulation, nonspecific serum protein binding and uptake by macrophages in the liver and spleen reduce efficiency of nanoparticle drug delivery into tumors. Delivery barriers in tumors include dysfunctional or lack of tumor blood vessels, blood–brain barrier (brain tumor), high tumor interstitial pressure, dense tumor stroma (fibroblasts, macrophages, and extracellular matrix), tumor cell membrane, and multidrug resistance proteins. (5) i.p. delivery: Treatment of tumors in the peritoneal cavity needs to overcome the tumor stromal barrier in the peripheral and inside tumors. Since passive diffusion of the drug and nanoparticles only reach 2–3 mm deep inside the tumor mass, active targeting using penetrating nanoparticles has the potential to increase intratumoral drug delivery and distribution [26]. Additionally, nonspecific uptake of nanoparticle drug carriers by peritoneal macrophages decreases the drug delivery into tumors. (Human body image is adapted from: http://www.nature.com/nrd/journal/v1/n7/fig_tab/nrd836_F1.html)

the therapeutic agents in the reticuloendothelial system (RES) of the spleen and liver, reducing the amount of drug molecules that are available to be delivered to the tumor site [27, 28]. Additionally, interactions of therapeutic agents with serum proteins affect biodistribution, pharmacokinetics (how the body affects the drug), bioactivity of the drug, and targeting ability of targeted agents and nanoparticle drug carriers [27, 29]. Therapeutic agents that are smaller than 5 nm in size, such as chemotherapy or small molecule drugs, can be cleared out from the blood circulation in relatively short time through the kidneys and liver. Large macromolecules (some proteins and antibodies), viral vectors, and nanoparticles cannot be eliminated by the kidneys and, therefore, have longer blood circulation time, allowing increased delivery into the tumor [30]. However, as the size of the therapeutic agent

increases to >200–400 nm, they can be cleared from the blood circulation in relatively short time through enhanced macrophage uptake. The effect of the therapeutics can be significantly impacted by the route of administration. For instance, therapeutics administered orally can be inactivated in an acidic environment or prematurely absorbed and metabolized in the liver and gastrointestinal tract (first-pass metabolism), thus significantly reducing systemic bioavailability. Nanoparticle-formulated drugs for oral administrations have been developed and demonstrated protective and conditional release effect in animal tumor models [31]. Although the feasibility of intratumoral delivery of nanoparticles for cancer therapy has been demonstrated, such an approach can only be applied to specific tumor types. Additionally, limited intratumoral distribution of the nanoparticles following direct injections will be an issue for effective cancer therapy.

2.4 Physical Barriers Impeding Delivery of Therapeutics into Tumor Cells

The environment surrounding cancerous cells, termed the tumor microenvironment, is an active, vital part of the development and progression of the tumor. Tumor stromal cells expand and infiltrate in response to the malignant transformation of cancer cells to form a protective barrier that limits tumor cells in their initiation site [32] (Fig. 2.1). However, interactions of tumor cells with surrounding stromal cells lead to the activation of stromal cells, which, in turn, produce cytokines, cellular factors, and proteases (cleavage enzymes) to promote aggressive tumor biology, resistance to cell death, as well as invasion and metastasis of tumor cells [32, 33]. For example, extensive proliferation of stromal fibroblasts and infiltration of active macrophages are detected in early pancreatic cancer lesions (PanIN). Extensive tumor stromal components further create a physical barrier that prevents infiltration of immune cells, especially cytotoxic T cells, into the tumor cell nest. This stromal barrier also blocks efficient delivery of therapeutic agents, including small molecules, chemotherapeutics, antibodies, viral vectors, cellular therapeutics, and nanoparticles into the tumor microenvironment [34].

Following systemic delivery, therapeutic agents have to overcome the tumor blood vessel barrier to extravasate into the tumor interstitial space (Figs. 2.1, 2.2, and 2.3). The blood vessels in normal organs and tissues are typically well-formed and organized mediating smooth, unilateral entry and exit. However, to support the fast-growing tumor cells, tumors not only “hijack” normal blood vessels (angiogenesis) to obtain nutrients but also generate new blood vessels, a process known as neovascularization (Fig. 2.1). This process is regulated based on the balance between pro- and antiangiogenic factors that are secreted by cancer cells and tumor-associated stromal cells within the tumor microenvironment. Vascular endothelial growth factor (VEGF) is the primary angiogenic factor to promote the “angiogenic switch” leading to the formation of additional vasculature. These blood vessels are comprised of two main cell types: endothelial cells that line the walls of vessel tubes and pericytes, which provide support and help maintain over-

all structure. The endothelial cells are also irregular in size and shape with highly variable and abnormal pericyte coverage of tumor vessels leading to a defective vascular monolayer. Large gaps or spaces are typically present between the endothelial cells (up to 2 μm), which permit fluid accumulation and the extravasation of larger molecules and nanoparticles (<200–400 nm) more readily than normal vessels [35] (Fig. 2.2). Therapies inhibiting VEGF and other proangiogenic factors have shown clinical success for many cancers; however, this response is often transient in nature due to the development of resistance. Tumors acquire adaptive mechanisms to evade and/or counterbalance the effects of angiogenic inhibitors leading to the reinitiation of neovascularization and the continuation of angiogenesis including the following: (1) induction of alternate proangiogenic signaling pathways, (2) recruitment of bone marrow-derived proangiogenic cells, (3) enhanced coverage by pericytes, and (4) activation of metastatic pathways to promote access to normal tissue vasculature [36].

Tumor blood vessels, mostly found in the stromal areas, differ significantly based on their structure and organization and intratumoral distribution. Due to a disorganized layout of blood vessels, there is a high degree of heterogeneity of blood flow throughout the different regions of the tumor leading to areas that are highly perfused (usually the tumor periphery), while other areas have limited blood flow making these tumor sites oxygen deprived or hypoxic. These hypoxic areas are heterogeneously dispersed throughout the solid tumor mass. Hypoxic and necrotic tumor sections can be further exacerbated by compressed blood vessels caused by increased interstitial pressure from fluid accumulation and stress of expanding tumor cells [37]. When the vascular permeability increases it allows for elevated amounts of fluid and plasma proteins to enter the tumor coupled with poor lymphatic drainage at the tumor site causing the fluid pressure within the tumor to become elevated. Whereas, solid stress is primarily "growth-induced" based on the rapid proliferation of tumor cells and accompanying extracellular matrix (ECM) components consisting of stromal cells, fibroblasts, and immune cells [38]. As diffusion is the main transport method for therapeutic agents in the tumor microenvironment, the abnormal tumor vasculatures and lack of adequate blood vessels are significant barriers to efficient delivery. Another pathway for transport of some of macromolecules and specific peptide-conjugated nanoparticles across endothelial cell layer is mediated by a transcytosis mechanism [39]. Currently, the effect of antiangiogenic therapy, which seeks to destroy or normalize the tumor vasculature, on the efficiency of intratumoral delivery of therapeutic agents, especially nanoparticle drug carriers, has yet to be determined [40]. It is likely that reduction in blood vessel density and permeability by antiangiogenic agents may decrease nanoparticle delivery into tumors.

The bioavailability of nanoparticle-based therapeutics is also impacted by macrophages, which are one of the many types of immune cells that reside in the tumor microenvironment. The tumor microenvironment consists of resident and infiltrating immune cells, as well as fibroblasts. Macrophages are a class of immune cells that possess a high capacity to non-specifically engulf foreign particles via a process known as phagocytosis. As a result, prior to therapeutic or diagnostic nanoparticles

reaching their intended targets within the tumor, they can be cleared quickly by macrophages [41]. Many human tumors have a high level of tumor-associated macrophages, such as in pancreatic and triple-negative breast cancers [42, 43]. At present, strategies to avoid clearance by macrophages in the RES in the liver and spleen include making smaller-sized nanoparticles (<10 nm) coated with the polymer polyethylene glycol (PEG) or antifouling polymers [44, 45]. This enables the delivery system to minimize non-specific uptake by macrophages, resulting in increased blood circulation time and improved efficiency of intratumoral drug delivery. However, it is still to be determined whether such nanoparticle modifications reduce non-specific uptake of intratumorally delivered nanoparticles. On the other hand, several groups are exploring the feasibility of using macrophages as drug carriers to bring therapeutics into tumors, especially in hypoxic and necrotic tumor areas that lack blood vessels [46, 47].

Activated tumor-associated fibroblasts are the primary type of tumor stromal cells that form a dense fibrotic barrier in the perivascular areas to prevent therapeutic agents to diffuse into tumor cells. These fibroblasts also produce extracellular matrix, such as collagen, to further trap the therapeutics, especially macromolecules and nanotherapeutics in the tumor stroma [48].

Additionally, the results of recent studies have shown that tumor stromal fibroblasts and macrophages and extracellular matrix not only create a physical barrier for infiltrating immune cells, such as T cells, and therapeutic antibodies into the tumor cell nest, but they also express high levels of the inhibitory molecule PD-L1 (programmed death-ligand 1) [49]. This immune checkpoint regulator interacts with PD-1 (programmed cell death protein 1) on activated T cells, inhibiting the function of cytotoxic T cells [50]. Systemic delivery of anti-PD-L1 antibodies that blocks binding of PD-L1 on tumor and stromal cells with PD-1 on T cells has demonstrated therapeutic effects on the activation of tumor-specific T cells and tumor growth inhibition in animal tumor models and in cancer patients [51, 52].

2.5 The Effect of Intra- and Intertumoral Heterogeneity on Drug Delivery

The concept of heterogeneity is critically important to the discussion of tumor-derived barriers and for effective delivery of therapeutics and diagnostics. Human cancer cells and tumor-associated stromal cells are heterogeneous in their histological characteristics, cell populations and localizations, and genetic and phenotypic features [53]. Tumor response to a given therapeutic agent is largely affected by intratumoral drug delivery and distribution, which are determined by heterogeneous tumor microenvironments, as well as intrinsic drug sensitivity of heterogeneous tumor cells. Each patient's tumor is highly unique based on cellular and microenvironment compositions. The biodistribution and efficacy of drug delivery is further complicated by additional layers of complexity, the variability exhibited within a

single patient's primary and/or metastatic tumors (intratumoral heterogeneity) and between patients with the same tumor type (intertumoral heterogeneity) [54]. Both levels of heterogeneity highlight potential reasons that patients respond differently to the same treatment, have partial responses within a given tumor, and develop resistance to therapeutic agents. Therefore, effective cancer therapy requires personalized therapeutic approaches that are designed based on genetic and pathological characteristics of both cancer cells and tumor stroma of individual patients at different stages of cancer development. Although genomic characterization of human cancers will play a major role in the selection of appropriate therapeutics for cancer patients, it may not be able to predict the effects of heterogeneous distribution of tumor blood vessels and tumor stroma on drug delivery efficiency in the primary and metastatic tumors in individual patients. It is likely that real-time, image-guided drug delivery and therapeutic effect monitoring will be an important component in designing a personalized therapy.

2.6 Drug Resistance

As previously discussed, heterogeneous tumor vessels and stromal structures within a tumor can contribute to low drug delivery and poor therapeutic response to chemotherapy in cancer patients. For the small percentage of drug molecules that are able to reach tumor cells, the cell membrane presents another barrier. The uptake and accumulation of chemotherapeutic drugs inside the cells are mediated by different mechanisms, including passive or facilitated diffusion, active drug transporters, or pinocytosis. Human tumor cells have a wide range of efficiency in taking up different drugs. Therefore, one of the clinical challenges in cancer therapy is that the limited amounts of drug molecules to be delivered into tumor cells are not sufficient to overcome drug-resistant mechanisms in tumors to achieve a good therapeutic response. Insensitivity of tumor cells to drug treatment can be due to either intrinsic or acquired resistance (Fig. 2.4). Intrinsic resistance occurs when a tumor initially fails to respond to a drug treatment due to apoptotic resistance developed during the carcinogenesis process. Prolonged exposure to a drug can lead to acquired resistance, when the cancer cells lose drug responsiveness over time. Acquired drug resistance can be mediated by drug-selective pressure that allows for the growth of a subpopulation of tumor cells that are insensitive to the drug or, as the result of new genetic mutations and cellular abnormalities, make the cells more resistant to cell death induced by therapeutics [55].

The uptake and accumulation of several chemotherapeutic drugs are mediated by drug transporters. One of the well-known multidrug-resistant (MDR) mechanisms is the upregulation of ATP-binding cassette (ABC) transporters on the cell membrane [56]. P-glycoprotein (P-gp) is a member of the ABC transport superfamily which is a well-characterized and highly efficient drug efflux pump. Goldstein and his colleagues reported over two decades ago that this energy-dependent, multidrug efflux pump was expressed in human cancers and was capable of conferring a drug-resistant

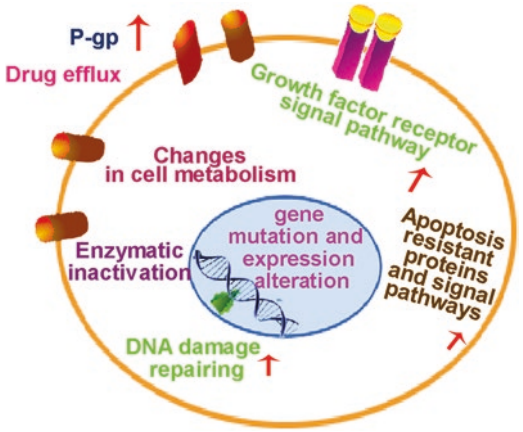

Fig. 2.4 Drug-resistance mechanisms in human cancer cells. Tumors have developed a variety of mechanisms to promote cell proliferation, survival, and resistance to therapeutic agents. The efficacy of therapeutics is hindered due to the overexpression of the drug-efflux pumps, such as P-gp, that efficiently pump drugs out of the tumor cells. Additional mechanisms include changes in the cellular metabolism and enzymatic inactivation which further reduces the intracellular concentration of the drugs in the tumor microenvironment, as well as an increase in DNA damage repair and upregulation of anti-cell death (anti-apoptosis) mechanisms

phenotype in many human cancers [57]. P-gp promotes multidrug resistance through its recognition of hundreds of compounds ranging in size from 330 to 4000 Da and acts primarily by pulling substrates from the lipid bilayer of cancer cells and actively pumping them out of the cells [58]. These molecular efflux pumps are not only expressed by most tumor cells but also highly upregulated in tumors with cells possessing cancer stem cell (CSC)-like properties. About 1–5% of tumor cells in the overall tumor mass are considered to be the initiators of tumor progression. CSCs are similar to normal stem cells in that they possess self-renewal, multipotency, and proliferative capabilities. CSCs can be found throughout the tumor tissue; however, they are often localized in the hypoxic tumor areas where most therapeutics are unable to reach. As a result, these cells often persist after the completion of chemotherapy cycles. Additionally, CSCs are capable of existing in a dormant or quiescence state, protecting them from the cytotoxic action of most chemotherapeutics. The persistence of these cells often results in recurrence or relapse of the cancer or the development of distant metastasis in a high percentage of cancer patients following surgery and chemotherapy [59].

Drug inactivation is another strategy utilized by tumor cells and CSCs to mediate multidrug resistance by reducing the intracellular concentration of drugs. For example, increase in cytochrome P450 (CYP) enzyme activity promotes the metabolism of approximately 50% of current drugs, and elevated expression of glutathione-S-transferase (GST) enzymes enhances the detoxification of anticancer drugs [60, 61].

Many chemotherapy drugs require metabolic activation to exert their effector function and acquire clinical efficacy. A high level of the aldehyde dehydrogenase isoform 1 (ALDH1) enzyme is frequently associated with drug-resistant CSCs [62]. Additionally, thymidylate synthase (TS) is an enzyme that catalyzes deoxyuridylate to deoxythymidylate, an essential process for DNA synthesis. 5-Fluorouracil (5FU) treatment-induced thymidylate synthase upregulation has been known to be a mechanism for 5FU resistance [63].

2.7 Interaction of Targeted Therapeutics with Tumor Cells

Most antibody-based and vesicle-based therapies, such as engineered viral vectors and nanoparticles, rely on a process called receptor-mediated endocytosis for cell internalization [64]. In this scenario, the therapeutic, which has been modified to contain a specific ligand or recognition motif, will bind to a protein or cell surface receptor highly expressed on the surface of a tumor cell that has the ability to mediate uptake into that cell. There are several factors that will impact the efficiency of this interaction including affinity, charge, and size of the therapeutic agents [65]. As discussed previously, binding affinity of the therapeutic can impact its ability to penetrate deeply into the tumor mass; however, it must be capable of binding tightly to its receptor in the appropriate conformation to initiate cellular uptake. Another important factor is the charge of the particle. While having a neutral or slightly negative surface charge is preferred for longer circulation half-life, more positively charged particles mediate enhanced endosomal release once inside the tumor cell [66]. Many drug delivery systems utilize the natural pH gradients within the tumor microenvironment (pH 6.5–7.2) and in the endosomal/lysosomal compartments (pH 4.0–6.5) to release therapeutic agents. This pH-sensitive drug release or activation leads to a high drug concentration within the appropriate cellular compartment while reducing systemic toxicity [67].

However, not all therapies have to be internalized to be effective. Some antibody-based therapies and small molecule inhibitors can inhibit cell growth by merely binding to their respective ligand/receptor on the tumor cell surface. One mechanism where the monoclonal antibody therapy trastuzumab (Herceptin) kills tumor cells is by binding to HER-2 expressed by breast and ovarian cancer cells preventing the binding of growth factors as well as preventing dimerization with other HER family members, which inhibits downstream kinase activity responsible for cell growth [68]. Induction of antibody-dependent cell-mediated cytotoxicity (ADCC) is also one of the mechanisms for tumor growth inhibition. For immunotherapy, antibodies to PD-1 or PD-L1 bind to T cells or PD-L1 expressing immune suppressor cells in the tumor microenvironment, respectively, which mediates tumor cell killing by tumor-specific cytotoxic T cells [50].

2.8 Biology of the Immune System

As discussed in previous sections, a variety of cells are found within the tumor microenvironment, many of which being resident and infiltrating immune cells. The cellular composition of the tumor often serves as a biological barrier to efficient delivery of therapeutics and contribute to the tumor's responsiveness to treatment. Tumors with a high infiltration of T cells, a critical immune cell that mediates tumor cytotoxicity, are more susceptible to therapeutic intervention such as PD-L1 blockade despite acquired resistance becoming more prevalent [69–71]. Based on the cytokines and other factors that are secreted by the tumors as well as other immune cells, such as dendritic cells, these T cells can be reprogrammed to differentiate into immune-suppressive cells known as regulatory T cells (Tregs). When found within the tumor, Tregs along with myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages promote tumor development. Targeting these suppressive cell populations for depletion is a promising immunotherapeutic approach toward enhancing responsiveness to treatment.

Alongside depletion of immune regulatory cells, identifying and effectively targeting key biomarkers with the tumor microenvironment are critical for a successful therapeutic response. While expression of inhibitory/checkpoint proteins such as PD-1/PD-L1, cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte activation gene 3 (LAG3), and indoleamine (2,3)-dioxygenase (IDO) have been effectively targeted leading to several FDA-approved immunotherapies, resistance remains a major clinical hurdle [52]. Targeting signaling pathways involved in cancer progression such as the RAS/RAF/MEK pathway is becoming a viable approach seeking to improve clinical patient outcomes [72]. Additionally, using tumor overexpression of proteins such as HER-2, epidermal growth factor receptor (EGFR), insulin growth factor 1-receptor (IGF-1R), and prostate-specific membrane antigen (PSMA) to more efficiently deliver therapeutics to the tumor microenvironment has proven highly effective. As resistance and efficient delivery of therapeutics remain critical barriers for effective cancer treatment, a combinatorial approach targeting multiple proteins and pathways will likely prove most advantageous for cancer patients.

2.9 Concluding Thoughts

The design of future therapeutics and diagnostics must reflect a heightened understanding of our growing knowledge of cancer biology, the tumor microenvironment, and mechanisms of drug resistance. Advances in identification of biomarkers and the development of new methods for serological detection and noninvasive imaging have the potential for early cancer detection such that patients can be treated by curative surgery. For patients with advanced disease, personalized and targeted therapy will be designed based on the characteristics of tumors in individual patients. An ideal therapeutic agent should have selective activity to the dominant cellular

pathways found within a cancer patient's tumor, which play critical roles in tumor proliferation and invasion. It is also important to combine therapeutic agents acting upon different key signaling molecules to generate a synergistic, therapeutic effect. Furthermore, success in combating highly heterogeneous, human tumors requires integrated diagnostic and therapeutic strategies, including early detection, molecular imaging, genetic and pathological characterization, targeted cancer therapy, image-guided drug delivery, and therapeutic response monitoring. Tumor metastasis is the major cause of cancer mortality. Even among patients without detectable metastatic tumors by conventional diagnostic and imaging approaches, many of them have tumor cells that have already invaded into distant organs and tissues. Therefore, systemic targeted therapy to treat potentially small metastatic tumor lesions before surgery may be important for those patients with lesions currently defined as localized tumors. Additionally, image-guided surgery assisted by molecular imaging probes should significantly reduce both local tumor recurrence and distant metastasis. This strategy is likely to mediate more complete removal of tumor cells in the surgical cavity and invaded tumor cells in the surrounding normal tissues and draining lymph nodes that are not visible by conventional surgery and pathological analysis, thus obtaining truly negative tumor margins and improve survival of cancer patients.

Considerations for the development of better targeted therapeutics include the choice of therapeutic, cellular target (receptor), type and affinity of targeting ligands, and chemical composition, size, and charge of the drug carrier. Biological and pathological characteristics of tumors of individual patients should also be taken into considerations. It is expected that future precision oncology will be built upon our profound knowledge in the roles of various genetic mutations and cell signal alterations in the initiation and development of specific tumors. Genomic and pathological analyses of tumor biopsy samples will provide critical information about the road map of the tumor and key molecular targets for designing targeted therapy for individual patients. There will be a series of molecular-targeted small molecules and biomarker-targeted drug carriers loaded with different therapeutics for the selection of the best combination for a specific cancer patient. Novel approaches for image-guided drug delivery will be applied in clinic to assess drug delivery efficiency in cancer patients. Multifunctional theranostic nanoparticles will be used to efficiently deliver drugs into tumor site and to overcome barriers in tumor microenvironment, while drug delivery and therapeutic response could be detected using noninvasive imaging.

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Chapter 3
Cancer Diagnostics and Therapeutics

Uma Prabhakar, Yalia Jayalakshmi, and C. Katherine Wang

3.1 Introduction

Cancer, which is the second most common cause of death in the USA, represents uncontrolled growth and spread of abnormal cells [1]. The disease is dynamic and involves a network of time-dependent and constantly altering molecular and cellular interactions in multiple cellular pathways that can escape routine monitoring or manipulation. The complexity of cancer is further confounded by the fact that the associated networks also undergo constant reshaping that in turn conform to pliable signaling processes/responses. Therefore, it is not enough to disrupt a single node or specific nodes in any network. Being patient dependent, the challenge then is to understand and implement selective, targeted, and personalized-perspective approaches to combat these diseases without drastically altering the physiological milieu and associated pathways thereby minimizing bystander effects.

According to the American Cancer Society, and based on 1998–2012 incidence data reported by the North American Association of Central Cancer Registries (NAACCR), in 2016 it is estimated that in the USA alone 1,685,210 new cases of cancer will be diagnosed, and a projected 595,690 deaths will occur [1]. Generally, treatments have included surgery, radiation, chemotherapy, hormone therapy, immune therapy, and targeted therapy. The significant progress made in the treatment and diagnosis of both common and rare cancers has not only offered visible and significant improvement in patient performance with regard to overall and

U. Prabhakar (✉)
Formerly of Office of Cancer Nanotechnology Research, NCI, Bethesda, MD, USA

Y. Jayalakshmi
C2CBio Consulting, Sunnyvale, CA, USA

C. Katherine Wang
Formerly of BRIM Biotechnology, Taipei, Taiwan

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progression-free survival and quality of life (QoL) but has also impacted patterns of patient care [2]. The long-lasting remission, improved predictability of the drug effects, etc. are exemplified by the increased use of combination therapies; increase in the approvals of new targeted therapies over new chemotherapies, harnessing the immune system to specifically target tumor cells to induce tumor-specific immunological memory; and development of diagnostic tools to help the oncologist select the right choice of treatment for the individual patient.

However, despite these promising efforts in overall cancer diagnosis and treatment, the 5-year survival outlook, particularly for cancers of the pancreas, liver, and lung, continues to be low and still in the single or low double-digit percentages as shown in Table 3.1 [1].

These troubling statistics have resulted in an increase in concerted efforts to identify and advance new targeted drugs that can be used as single agents, or in multiple and complex drug combinations, and across the cancer care spectrum from prevention, screening, chemotherapy, surgery, and radiation to molecularly targeted therapies and immune therapies. Additionally, regulatory health agencies have introduced novel approaches and mechanisms such as "Fast Track, Breakthrough Therapy, Accelerated Approval, Priority Review" to accelerate the review and approval of drugs that provide improvements over existing therapies, as well as innovative products that serve previously unmet medical needs or otherwise improve patient care and public health [3].

In oncology, diagnostic tests have also contributed to effective and early diagnosis of disease and clinical decision-making. In recent years, the diagnostic process and predictive biomarker use is more strongly driven by the need to preselect patients based on drug labels (exemplified by HER2 (ERBB2) expression for trastuzumab treatment of breast cancer), as more effective patient selection clearly contributes to improved success rates of new therapies. Furthermore, biomarkers provide an ideal and holistic approach to defining the most successful strategy for guiding therapeutic interventions by assessing drug safety, evaluating target engagement and the immediate consequence on biological processes (pharmacodynamic biomarkers, such as the well-known proliferation markers Ki67 or BCL-2), predicting outcome to therapy (surrogate biomarkers such as prostate-specific antigen (PSA), for prostate cancer), and monitoring disease progression or therapeutic efficacy [4].

A companion diagnostic is a specific test developed during or after a drug is made available on the market to determine if that therapy is suitable for a patient to

Table 3.1 Cancer 5-year survival data (USA only) during the time intervals as noted

	1975–1977	1987–1989	2005–2011
All cancers	49%	55%	69%
Liver	3%	5%	18%
Pancreas	3%	4%	8%
Lung	12%	13%	18%

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ensure that the most optimal treatment decision is made for the patient [5]. Currently, the companion diagnostics market for targeted drugs (e.g., KIT D816V mutation detection for Gleevec® eligibility in aggressive systemic mastocytosis (ASM)) is worth $42 billion across indications and predicted to reach $60 billion by 2019 [6]. Note that 42% of all drugs across therapeutic areas and 73% of oncology drugs in development are targeted drugs [7]. The launch of the Obama Precision Medicine Initiative [8], and the "Cancer Moonshot" to "accelerate our understanding of cancer and its prevention, early detection, treatment, and cure" [9], marks a hallmark of the future of healthcare and end of the traditional "one-size-fits-all" drug model. The challenge in the new model of healthcare will be convincing medical insurance companies of the importance of prevention and early detection and assessing the value of companion diagnostics. Highlights of the development of innovative diagnostics and companion diagnostics using novel platforms are described next in this chapter. Also addressed in this chapter are the challenges that need to be overcome for early, correct diagnosis and effective treatment of cancer.

3.2 Cancer Diagnostics

Because carcinogenesis is a multifocal and multistep process, cancers take several years to progress from the start of acquiring genetic instability and mutation until the actual malignancy and metastasis occur [10]. For example, pancreatic cancer can take 15–20 years from disease initiation to cancer death, with breast and prostate cancers perhaps taking even longer [10]. Most often, cancer is detected at a stage when it has already compromised several vital functions/organs and is widespread within the body. Early detection and diagnosis are critical to improving the chances of treatment being effective, which are difficult challenges in and of themselves. However, overdiagnosis and overtreatment also need to be managed, as they too are prevalent and can cause unnecessary burden on the patient and the overall healthcare system. Therefore, more sensitive, specific, and dependable diagnostic methods need to be developed to enable earlier and more accurate cancer detection.

Current methods of cancer diagnosis, in addition to physical exam, complete medical history, and biopsies of suspected cancerous tissue, involve evaluating for the presence of biomarkers from appropriate available clinical samples or using imaging modalities. To minimize invasive procedures and unnecessary exposure to radiation, initial cancer diagnosis is best confirmed through sensitive biomarker evaluations in easily obtained samples, such as sputum, nipple discharge, urine, stool, whole blood, serum, or plasma. These biomarker assessments could estimate risk of disease, screen for primary cancers, distinguish benign growths from malignant ones, provide prognosis and prediction for patients diagnosed with cancer, or monitor the status of the disease (potential recurrence or response to therapy). In order for the biomarker to be recognized and utilized for specific endpoints, they have to be validated appropriately in three major steps: analytical validity, clinical

validity, and clinical utility [11–13]. Analytical validity can be defined as being able to accurately and consistently measure the biomarker in the sample of interest in the assay. To determine analytical validity, the biomarker is tested for sensitivity (proportion of individuals confirmed with disease who test positive for the biomarker), specificity (proportion of individuals who test negative for the biomarker), robustness, accuracy, and reproducibility. Clinical validity has to be demonstrated by dividing a population into two distinct groups based on the biomarker and determining if that specific population validates the original hypothesis of the biomarker. Finally, clinical utility is based on whether or not the biomarker will add useful information for clinical decision-making, such as assessing the effectiveness of the biomarker and the benefit-to-harm ratio. Currently, more than 20 cancer biomarkers have been approved for clinical use in the prediction of cancer and cancer progression (Table 3.2).

Aside from diagnosing cancers through physical samples taken from patients, medical imaging can also be used as a diagnostic tool. Imaging modalities include computer tomography (CT), positron emission tomography (PET), and magnetic resonance imaging (MRI). X-ray, ultrasound, and endoscopy are also used to determine the presence of abnormal growths (Table 3.3). However, these traditional imaging methods may only detect cancers larger than 1 cm, which may preclude them from being used to detect early-stage cancers [14]. While increase in sensitivity may be achieved through combining imaging modalities with small molecule contrast agents such as 2-deoxy-2-(^{18}F)fluoro-D-glucose (FDG) or chelated gadolinium, these small molecules have poor stability, fast clearance, and low signal intensity that often limit their use for early detection [14], resulting in the need for better imaging agents for more specific diagnosis at earlier stages. Improvement in earlier detection may be achieved with molecular imaging, which combines these imaging modalities with agents that more sensitively target cancerous cells, such as the use of targeted contrast imaging agents, theranostics, or targeted metallic nanoparticles.

The initial detection of the disease is often followed by a biopsy of tissue used for additional testing to further understand the tumor, including looking at histology, immunohistochemistry (IHC), and genetic mutations. Genetic mutations or overexpressed proteins are often correlated with the resulting effect from blocking the associated signaling pathways, which in turn has paved the path for a new set of diagnostics called companion diagnostics. These companion diagnostics not only can pinpoint a specific event resulting in the tumor's continual proliferation, but they are also able to direct treatment options that can successfully block the specific event and help kill the tumor. Several companion diagnostic test kits have been developed using various labeling techniques or gene expression/genome analyses. Table 3.4 summarizes a list of FDA-approved companion diagnostics using these technologies. The therapeutic/drug prescribed for the companion diagnostic is also shown in the table. For example, the ALK gene mutant can be detected by immunohistochemistry (IHC) from a formalin-fixed paraffin-embedded (FFPE) tissue using Ventana ALK D5F3 companion diagnostic or by conducting fluorescence in situ hybridization (FISH) on FFPE tissue using Abbott's VYSIS ALK Break Apart FISH

Table 3.2 FDA-approved biomarkers used in clinical practice

Biomarker	Clinical use	Cancer type	Specimen
Pro2PSA (truncated form of prostate-specific antigen, PSA)	Discriminating cancer from benign disease	Prostate	Serum
ROMA (HE4 + CA-125) (Risk of Ovarian Malignancy Algorithm)	Prediction of malignancy	Ovarian	Serum
OVA1 (panel of multiple proteins)	Prediction of malignancy	Ovarian	Serum
HE4 (human epididymis protein)	Monitoring recurrence or progression of disease	Ovarian	Serum
Fibrin/fibrinogen degradation product (DR-70)	Monitoring progression of disease	Colorectal	Serum
AFP-L3% (alpha-fetoprotein protein isoform)	Risk assessment for development of disease	Hepatocellular	Serum
Circulating tumor cells (EpCAM (epithelial cell adhesion molecule), CD45, cytokeratins 8, 8+, 19+)	Prediction of cancer progression and survival	Breast	Whole Blood
p63 protein	Aid in differential diagnosis	Prostate	FFPE tissue
c-Kit	Detection of tumors, aid in selection of patients	Gastrointestinal stromal tumors	FFPE tissue
CA19–9 (cancer antigen 19–9)	Monitoring disease status	Pancreatic	Serum, plasma
Estrogen receptor (ER)	Prognosis, response to therapy	Breast	FFPE tissue
Progesterone receptor (PR)	Prognosis, response to therapy	Breast	FFPE tissue
Her-2/neu	Assessment for therapy	Breast	FFPE tissue
CA-125	Monitoring disease progression, response to therapy	Ovarian	Serum, plasma
CA15–3	Monitoring disease response to therapy	Breast	Serum, plasma
CA27.29	Monitoring disease response to therapy	Breast	Serum
Free PSA	Discriminating cancer from benign tissue	Prostate	Serum
Thyroglobulin	Aid in monitoring	Thyroid	Serum, plasma
Nuclear mitotic apparatus protein (NuMA, NMP22)	Diagnosis and monitoring of disease (professional and home use)	Bladder	Urine
Alpha-fetoprotein (AFP)	Management of cancer	Testicular	Serum, plasma, amniotic fluid
Total PSA	Diagnosis and monitoring of disease	Prostate	Serum
Carcinoembryonic antigen	Aid in management and prognosis	Not specified	Serum, plasma
Human hemoglobin (fecal occult blood)	Detection of fecal occult blood (home use)	Colorectal	Feces

Adapted with permission [15]

Table 3.3 Characteristics and cost of different quantitative imaging modalities in clinical use for cancer detection/diagnosis

Technique	Resolution	Depth	Time	Imaging Agents	Target	Cancer technique clinically used for	Average cost of one procedure without insurance
X-Ray	50–100 μm	No limit	Minutes	None	Anatomical, physiological	Bone, breast, lung	$
MRI	10–100 μm	No limit	Minutes to hours	Paramagnetic chelates, magnetic particles	Anatomical, physiological, molecular	Brain, breast, liver, ovarian, prostate	$$
Endoscopy	10–71 μm	Surface	Live	None	Anatomical, physiological	GI, Stomach	$
CT	50 μm	No limit	Minutes	Iodinated molecules	Anatomical, physiological	Brain, colorectal, low-dose for lung in smokers, liver, lymphoma, ovarian	$
Ultrasound	50 μm	cm	Seconds to minutes	Microbubbles	Anatomical, physiological	Gastric, liver, ovarian, pancreas	$
PET	1–2 mm	No limit	Minutes to hours	^{18}F-, ^{64}Cu- or ^{11}C-labelled compounds	Physiological, molecular	Brain, breast, colorectal, esophageal, head & neck, lymphoma, melanoma	$$$
SPECT	1–2 mm	No limit	Minutes to hours	^{99m}Tc- or ^{111}In-labelled compounds	Physiological, molecular	NA	$$

Adapted with permission [16]

Abbreviations: *CT* computed tomography, *GI* gastrointestinal, *MRI* magnetic resonance imaging, *PET* positron emission tomography, *SPECT* single photon emission computed tomography, *$* <US$1500, *$$* $1500–$3000, *$$$* > $3000

Table 3.4 Approved Cancer Companion Diagnostic Kits

Gene mutant	Test method	Diagnostic	Company Name	Drug	Indication
ALK	FFPE IHC tissue stain (qualitative)	Ventana ALK D5F3 CDx Assay (conducted in combination with Benchmark automated staining instrument)	Ventana	Xalkori (crizotinib)	NSCLC
ALK	FISH from FFPE tissue (qualitative)	VYSIS ALK Break Apart FISH Probe Kit	Abbott Molecular Systems	Xalkori (crizotinib)	NSCLC
BRAF V600E	RT-PCR from FFPE melanoma tissue (qualitative)	Cobas 4800 BRAF V600 Mutation test	Roche Molecular Systems	Zelboraf (vemurafinib)	Melanoma
BRAF V600E, V600K	RT-PCR from FFPE melanoma tissue (qualitative)	THxID™ BRAF Kit	bioMérieux Inc.	Mekinist (tramatenib); Tafinlar (dabrafenib)	Melanoma
BRCA1/ BRCA2	NGS from FFPE ovarian tumor tissue (quantitative)	FoundationFocus™ CDx_{BRCA}	Foundation Medicine	Rubraca (rucaparib)	Ovarian cancer
BRCA1/ BRCA2	SNPs by PCR using whole blood and looking at germline DNA (qualitative)	BRACAnalysis CDx™ (performed at Myriad Labs)	Myriad Genetic Laboratories	Lynparza (olaparib)	Ovarian cancer
C-KIT protein	IHC from FFPE of normal and neoplastic tissue (qualitative)	DAKO C-KIT PharmDx (Kit)	Dako North America	Gleevec/Glivec (imatinib mesylate)	GIST
EGFR	IHC on routinely fixed normal and neoplastic tissue (qualitative)	DAKO EGFR PharmDx Kit	Dako	Erbitux (cetuximab), Vectibix (panitumumab)	Colorectal cancer (CRC)
EGFR	RT-PCR from FFPE NSCLC tumor tissue (qualitative)	Cobas EGFR Mutation Test v2	Roche Molecular Systems	Tagrisso (osimertinib)	NSCLC

(continued)

Table 3.4 (continued)

Gene mutant	Test method	Diagnostic	Company Name	Drug	Indication
EGFR L858R mutant	RT-PCR from FFPE tissue (qualitative)	Therascreen EGFR RGQ PCR Kit	Qiagen Manchester Ltd	Gilotrif (Afatinib); Iressa (gefitinib)	NSCLC
EGFR L858R	RT-PCR from FFPE NSCLC tumor tissue (qualitative)	Cobas EGFR Mutation Test	Roche Molecular Systems	Tarceva (erlotinib)	NSCLC
HER2 overexpression	Immunocytochemical assay (semi-quantitative) in FFPE breast cancer tissue and metastatic gastric/ gastroesophageal junction adenocarcinoma	HercepTest	Dako Denmark	Herceptin(trastuzumab);Perjeta (pertuzumab);Kadcyla(ado-trastuzumab emtansine)	Breast cancer, metastatic gastric cancer/ gastroesophageal junction adenocarcinoma
HER2	FISH in FFPE breast cancer tissue and metastatic gastric/ gastroesophageal junction adenocarcinoma (quantitative)	HER2 FISH PharmDx Kit	Dako Denmark	Herceptin(trastuzumab);Perjeta (pertuzumab);Kadcyla(ado-trastuzumab emtansine)	Breast cancer, metastatic gastric cancer/ gastroesophageal junction adenocarcinoma
Her2/Neu	FISH (gene amplification) from FFPE breast tissue (qualitative)	INFORM HER-2/NEU	Ventana Medical Systems	Herceptin (trastuzumab)	Breast cancer
Her2/Neu	FISH (gene amplification) from FFPE breast tissue	PathVysion HER-2 DNA probe kit	Abbott molecular Inc	Herceptin (trastuzumab)	Breast cancer
Her2/Neu	IHC in FFPE breast tissue (semi-quantitative)	PATHWAY ANTI-HER2/ NEU rabbit monoclonal primary antibody (lab test)	Ventana Medical Systems	Herceptin (trastuzumab)	Breast cancer

Gene mutant	Test method	Diagnostic	Company Name	Drug	Indication
Her2/Neu	IHC in FFPE breast tissue (semiquantitative)	INSITE HER-2/NEU Kit	Biogenex Laboratories Inc.	Herceptin (trastuzumab)	Breast cancer
Her2/Neu	CISH (chromogenic in situ hybridization) in FFPE breast tissue (quantitative)	SPOT-Light HER2 CISH Kit	Life Technologies	Herceptin (trastuzumab)	Breast cancer
Her2/Neu	IHC from FFPE breast tissue (semiquantitative)	Bond Oracle Her2 IHC System	Leica Biosystems	Herceptin (trastuzumab)	Breast cancer
Her2/Neu	CISH in FFPE breast tissue (quantitative)	HER2 CISH PharmDx kit	Dako Denmark	Herceptin (trastuzumab)	Breast cancer
Her2/Neu	ISH (in situ hybridization) in FFPE cancerous breast tissue	INFORM HER2 Dual ISH DNA probe cocktail	Ventana Medical Systems	Herceptin (trastuzumab)	Breast cancer
KIT	PCR from fresh bone marrow samples	KIT D816V Assay	Arup Laboratories	Gleevec (imatinib mesylate)	Aggressive systemic mastocytosis (ASM)
KRAS	RT-PCR from FFPE tissue (qualitative)	*Therascreen* KRAS RGQ PCR Kit	Qiagen Manchester Ltd	Erbitux (cetuximab), Vectibix (panitumumab)	Colorectal cancer (CRC)
KRAS	RT-PCR from FFPE tissue	Cobas® KRAS mutation test (Kit)	Roche molecular systems	Erbitux (cetuximab), Vectibix (panitumumab)	Colorectal cancer (CRC)
LSI TP53	FISH from peripheral blood specimens	Vysis CLL FISH Probe Kit	Abbott	Venclexta (venetoclax)	B-cell chronic lymphocytic leukemia (CLL)

(continued)

Table 3.4 (continued)

Gene mutant	Test method	Diagnostic	Company Name	Drug	Indication
PDGFRB	FISH from fresh bone marrow samples	PDGFRB FISH for Gleevec Eligibility in Myelodysplastic Syndrome/Myelo-proliferative Disease (MDS/MPD)	Arup Laboratories	Gleevec (imatinib mesylate)	Myelodysplastic syndrome/ myeloproliferative disease (MDS/MPD)
PD-L1	IHC from FFPE tissue (qualitative)	PD-L1 IHC 22C3 pharmDx	Dako, North America	Keytruda (pembrolizumab)	NSCLC
PD-L1	IHC from FFPE tissue (qualitative)	PD-L1 SP142 CDX Assay	Ventana Medical Systems	Tecentriq (atezolizumab)	Metastatic urothelial cancer

FDA website, [17]

Abbreviations: *CISH* chromogenic in situ hybridization, *CRC* colorectal cancer *FFPE* formalin-fixed paraffin-embedded, *FISH* fluorescence in situ hybridization, *GIST* gastrointestinal stromal tumor, *IHC* immunohistochemistry, *NGS* next-generation sequencing, *NSCLC* non-small cell lung cancer, *RT-PCR* real-time polymerase chain reaction, *SNP* single nucleotide polymorphism

Probe Kit. Both kits are used to determine whether the patient should take Xalkori® for non-small cell lung cancer (NSCLC); however, the results of the two tests differ in that one is using IHC and the other is using FISH. It is up to the discretion of the physician as to which diagnostic kit to use when there are several for the same gene mutant and there are no guidelines approved by the National Comprehensive Cancer Network (NCCN). The reader is directed to the FDA companion diagnostic website for the most updated information [17].

Diagnostic kits to determine the genetic signature of the biopsy are also currently used to determine what types of treatments the tumors may be likely to respond to in addition to providing information on whether the tumor may recur. For example, MammaPrint®, developed by Agendia, measures the activity of 70 genes from FFPE breast cancer samples. The gene expression of these 70 genes is then used by the diagnostic to predict the patient's likelihood of having their breast cancer recur. Oncotype® and Prosigna® are two other breast cancer diagnostics measuring gene activity that use the genetic information to determine how patients may respond to treatments or which treatments patients should use. Of these kits, only Oncotype has been included for use in breast cancer in the NCCN [18] and American Society of Clinical Oncology (ASCO) guidelines [19]. Exact Science's diagnostic, Cologuard®, is approved for the detection of colorectal cancer and colorectal neoplasia. Their diagnostic is able to predict the presence of colorectal cancer based on the elevated levels of 11 DNA biomarkers in the patient's stool sample that are associated with colorectal cancer.

3.3 Novel Diagnostic Approaches

The methodologies and efficiency of cancer diagnosis continue to improve through the use of companion diagnostics and more targeted diagnostics. Diagnostic methods and kits mentioned thus far generally detect single or dual biomarker proteins or genetic mutations from a single sample. To expand the versatility and accuracy of cancer detection, simultaneous detection of multiple markers and the use of blood-based or "liquid biopsies," such as circulating tumor cells and circulating DNA, are new directions that are being pursued. The simultaneous detection of multiple markers, also known as multiplexing, is being developed using various encoding methods, including bead-based technologies, planar arrays, and distinct barcodes for multiple samples that can be produced from particles or fluorescent reporter probes (e.g., NanoString technologies) [20] using a variety of sophisticated detection methods [20–27].

The use of tissue biopsy specimens, both for the detection and subsequent decisions about treatment regimens, is often limited by the invasive nature of the procedures, the heterogeneity of the disease, and that it represents a snapshot of the disease in time. In recent years, liquid biopsies from a simple blood draw are being explored for downstream analysis. These biopsies include rare circulating tumor

cells (CTCs) and cell-free circulating nucleic acids, such as microRNAs [28–31], noncoding RNAs [32, 33], and cell-free circulating tumor DNA (ctDNA) [34].

CTCs have the potential to provide valuable insights on the molecular changes happening in the tumor in real time and are amenable to longitudinal monitoring which can help the physician gain a broader understanding of the disease and its response to therapy. The only US FDA-approved CTC diagnostic (CellSearch) uses ferromagnetic beads coated with EpCAM antibodies for the capture and detection of CTCs for metastatic breast, prostate, and colorectal cancer [35]. It must be recognized that the majority of cells, cell-free nucleic acids, microRNAs, and exosomes in a liquid biopsy will have originated from normal cells with numbers fluctuating as a consequence of biological variations, and laboratories undertaking these approaches must be scrupulous in their methodologies to avoid erroneous results [36]. While these various molecules are promising in their correlation to many different cancers (lymphoma [37], leukemia [38], glioblastoma [39], gastric [40], colorectal [41], lung [42], liver [43], breast [44], prostate [45], pancreatic cancer [46]), the challenge of validating these as biomarkers still looms and will need to be overcome before they can be used in cancer diagnosis. As of June 2016, US FDA approved the first liquid biopsy test to identify metastatic NSCLC patients with EGFR mutation who are eligible for the EGFR-targeted therapeutic erlotinib (Tarceva®) [47], and likely more will follow in the future.

Although sensitivity and specificity of cancer diagnosis and diagnostic markers are continuing to improve, current approved methods and technologies still have limitations. There are recent promising technologies and approaches that are gaining momentum to better define cancer diagnostic strategies. The first is "next-generation" functional diagnostic technology that integrates functional testing with next-generation sequencing and immunoprofiling to precisely match combination therapies to individual cancer patients [48]. Remarkable advances in next-generation sequencing (NGS) technology have also enabled the identification of large numbers of mutations in tumors of cancer patients that, in turn, allow for identification of "long tail" mutations that occur only in a minor subset of patients. The power of functional testing lies in the fact that it complements genetic sequencing by providing response to live patient cells without prior knowledge of the mechanism of drug activity using target and pathway-based methods, direct cytotoxicity, and ex vivo and in vivo methods [48]. Using a novel approach known as conditional reprogramming (CR), Crystal et al. [49] developed a large number of ex vivo-derived models of tumors from patients with non-small cell lung cancer (NSCLC) who showed resistance to targeted therapies. Using these models, they demonstrated that a novel combination of targeted therapies against the MAPK/ERK kinase (MEK) and anaplastic lymphoma kinase (ALK) could combat resistance to a single agent ALK inhibition in ALK-mutant NSCLC. Thus, multiple diagnostic technologies on a single patient biopsy could enable the best "personalized" choice from an armamentarium of several single and combination regimens.

Building on the intersection of information technology and healthcare, another important and emerging trend is the application of machine learning (ML) to clinical and genomic big and mixed data in cancer diagnosis, prognosis, and prediction.

ML, a branch of artificial intelligence, uses new deep learning algorithms and databases of cancer diagnosis images to detect new cancers and predict prognosis, recurrence, survival, etc. It is still an evolving but promising area with the potential of influencing clinical decision-making [50]. ML tools, including artificial neural networks (ANNs), Bayesian networks (BNs), support vector machines (SVMs), and decision trees (DTs), have been widely applied in cancer research for the development of predictive models, resulting in effective and accurate decision-making. However, for the successful adoption of any new applications in everyday clinical practice, an appropriate level of validation is required, and it will be no different for the use of ML in the cancer diagnostic arena.

While technologies such as NGS and ML are able to use computational power to enhance data interpretation, producing accurate diagnostic data at earlier stages of disease is still an area that needs to be pursued. Nanotechnology platform-based diagnostics may be able to address these limitations and also hold considerable promise for early detection of different types of cancers due to their inherent size and versatility of use. By virtue of the fact that nanoparticles (NP) possess long-term stability, they are amenable to the design of sensitive and multiplexed bioassays for the simultaneous measurement of multiple markers of a disease [51] and have been used as ultrasensitive probes for the ex vivo detection of cancer biomarkers. Nanomaterials such as nanoparticles (NPs), nanowires, nanocantilevers, nanotubes, and nanodevices have all been used in this context. High-resolution in vivo tumor imaging was demonstrated recently by combining existing optical imaging technologies with sophisticated NP-based optical contrast agents. Further, various novel assemblies of NP systems have been reported as nanoprobes for the early detection of cancer. Nanomaterials such as NPs, nanowires, nanotubes, and nanodevices have been explored as ultrasensitive probes to detect cancer biomarkers. Through these different avenues, nanotechnology may be able to further push the sensitivity and precision of cancer diagnosis and be integrated with other burgeoning technologies to move this field forward.

3.4 Challenges Associated with Diagnostics

New diagnostics development collectively faces continuous challenges scientifically, technically, commercially, and from the regulatory perspective even beyond approval and product launch. Scientifically, understanding of the biology of the disease or clinical condition, quality of the related diagnostic test, and the extent to which the test result can be tied to definitive decisions around therapeutic selection are key considerations. Technologically, the platform per se must help drive adoption and commercial success and not pose a risk to the drug if it is a companion diagnostic. The detection and diagnosis of cancer at its earliest stage using traditional biomedical imaging tools of magnetic resonance imaging (MRI), ultrasound, and positron emitting tomography (PET) is hampered by inadequate imaging period, risk of renal toxicity, and limitations in detecting small tumors [52], thus

encouraging the use of nanoparticles as diagnostic tools. Gold nanorods decorated with antibodies that can bind cancer-specific biomarkers in the blood have been demonstrated for their ability to be an inexpensive tool and a sensitive method of detecting early-stage cancer.

From the business perspective, with increased use of diagnostics to personalize treatments for patients, medical insurance companies have begun to demand proof of clinical validity that the tests would positively shape clinical decision-making and patient outcomes. The demand for evidence required prior to providing coverage for many of the approved tests is often unrealistic [53]. Educating physicians, in addition to insurance companies and regulators so they stay abreast of scientific advances, is yet another huge challenge.

Therefore, when considering development of a companion diagnostic for a specific therapeutic, the following points must be addressed: (i) proven clinical utility of the biomarker and companion diagnostic, (ii) having robust analytics and standardized methodologies, and (iii) having reliable and easy-to-use systems [54]. The companion diagnostic developer and regulatory agency must determine early on in development if the therapy and diagnostic will be co-developed and approved together and determine the appropriateness of cross-labeling the products. The agency encourages sponsors to seek early inputs while engaging in such efforts so as to influence and shape the sponsor's strategic thinking and prevent future delays in reviews of future formal regulatory submissions. In general, to help overcome these challenges in diagnostics development and commercialization, it is important for developers to continue to maintain an open dialogue with both end users (e.g., physicians, insurance companies) and the regulatory agency to ensure that the products developed fulfill unmet medical needs and can reach the market in an efficient manner.

3.5 Cancer Therapeutics

The principal goal of cancer treatment is to cure the disease, i.e., to achieve disease-free long-term survival. If a cure is not possible, cancer treatments aim at palliative care to control symptoms and improve quality of life while prolonging the patient's life. When cancer is locally advanced, a cure is possible for several cancer types; however, once metastasized, for most of the cancers, the treatment options are only palliative. Localized cancers are treated effectively with surgery and radiation. Systemic chemotherapy is used once the cancer has spread to multiple organs. As shown in Fig. 3.1, while surgery, radiation, and chemotherapy were used in the 1900s as principal treatment options, advancement in the understanding of the molecular origins of cancer and the decoding of the human genome in 2003 has led to novel treatment modalities such as molecularly targeted therapies and immunotherapies, including therapeutic vaccines [55].

The treatment options for cancer in general largely depend on the type and stage of cancer, possible side effects, and the patient's preferences and overall health. In

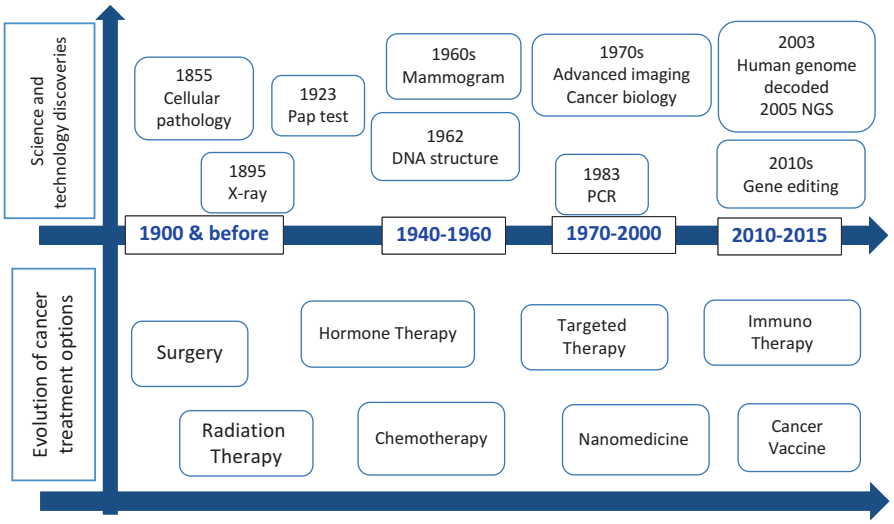

Fig. 3.1 Illustration of the time evolution of cancer treatment options. Highlighted are the timeline of key science and technology discoveries that have fueled the growth of modern cancer treatment options. [55, 60, 61]

cancer care, physicians with different subspecialties often work together to create a patient's overall treatment plan that combines different types of treatments. In the following section, a high level overview of the principles of cancer treatment, major treatment options, their brief history and evolution, major accomplishments, and continuing challenges will be provided. The readers can find multiple excellent reviews and books on each of these topics for an in-depth understanding, some of which are referenced here [56, 57]. The readers are also directed to a few web links, which provide the most updated information on cancer therapy options [1, 55, 58–61].

3.6 Current Treatment Approaches

3.6.1 Surgery

Most solid tumor therapies involve surgery and are curative when cancer is local. Surgery to remove tumor masses was already in practice in the 1700s and advanced considerably in the next two centuries with the discovery of anesthesia in 1846 and cellular pathology in the 1900s that enabled diagnosis of resected tissues for cancerous cells [55]. Modern advancement in surgical tools and imaging techniques has led to less invasive and more precise surgeries.

The type of surgery depends on the treatment goals, such as disease prevention, diagnosis, staging, cure or debulking, palliative, supportive, or restorative. When necessary, surgery is combined with radiation therapy and/or chemotherapy preoperatively (neoadjuvant) or postoperatively (adjuvant) to maximize removal of all cancerous cells and improve overall efficacy and safety outcome [55, 58].

3.6.2 Radiation Therapy

Radiation was first used in 1903 to successfully treat skin cancer 5 years after Marie Curie discovered radium [60]. It is currently successfully used in treating sarcomas, gliomas, lymphomas, thyroid, head and neck, and anal cancers, while pancreatic and melanoma do not respond as well.

The forms of radiation used in cancer include ionizing radiation (X-rays and gamma rays) and nonionizing particle radiation (electrons and protons), which can be delivered via external or internal sources. From these radiation sources, high energy is delivered to cells, which damages and kills cellular DNA over time. This therapy results in tumor shrinkage, reduced spread of remaining cancer cells after surgery, or palliative effects for advanced cancers and can be used alone or in combination with surgery, chemotherapy, and other pharmacological therapies. Radiation-induced damage more quickly affects actively dividing cells, such as cancer cells and fast-dividing normal cells (e.g., skin, bone marrow, intestinal lining), but side effects are also observed later on in slow-growing tissues such as the nerve, bone, breast, and brain [58, 59].

To maximize and concentrate dose onto the tumor while minimizing exposure to the normal tissues, radiation therapy planning is guided by simulations and imaging techniques. Treatment regimens can last several weeks, and doses are fractionated to reduce side effects.

3.6.3 Chemotherapy

One of the major milestones in the treatment of cancer was the introduction of chemotherapy. The original discovery dates back to World War II, with the accidental observation of the curative effect of nitrogen mustard on lymphoma. Later in the 1950s, Sydney Farber and colleagues at Harvard Medical School systematically studied aminopterin, a vitamin folic acid derivative, and showed remission in pediatric acute lymphoblastic leukemia (ALL) patients [57]. Since then, more than 100 chemotherapeutic agents have been developed and continue to be used in effectively treating multiple types of cancer.

Table 3.5 Common classes of chemotherapy agents, their mechanism of cytotoxicity. [1, 55, 61]

Mechanism of toxicity	DNA level effects	Cell cycle	Examples
Alkylating agent	Crosslinks with DNA, interferes with replication and transcription	All phases	Temozolomide, cyclophosphamide, carmustine, platinum (cisplatin, etc.)
Antimetabolites	Substitutes for DNA/RNA building blocks causing false messages	S phase	5-FU, capecitabine, gemcitabine, cytarabine, antifolates (pemetrexed, methotrexate)
Antitumor Antibiotics	Intercalates within DNA base pairs	All phases	Anthracyclines (doxorubicin, etc.), Antibiotics (mitomycin-C, etc.)
Mitotic inhibitors	Interferes with micro / tubulin function during mitosis	M phase	Taxanes: (paclitaxel, docetaxel) Vinca alkaloids (vinorelbine, vincristine)
Topoisomerase inhibitors	Inhibits enzymes involved in DNA cleavage and rejoining	M phase, All phases	Topo I: camptothecins (topotecan, irinotecan, etc.) Topo II: etoposide

Chemotherapy agents generally share a common mechanism of action, such as targeting DNA, causing direct toxicity, or interfering with DNA synthesis, replication, and cell division, resulting in cell death. Table 3.5 summarizes common chemotherapy compound classes, their mechanism of action on cellular DNA processes, and example drugs. Their use results in side effects as a consequence of unilaterally targeting all fast-dividing cells in addition to cancer cells and includes alopecia (hair loss), anemia, bone marrow depression, immune suppression, nausea, and emesis [59]. As such, there have been numerous strategies to maximize treatment benefit over toxicity and/or overcoming multidrug resistance (MDR) by using adjuvant/neoadjuvant therapies, combining with other drugs and treatments, and more recently, using specific and selective targeting and drug delivery approaches [62, 63].

3.6.4 Hormone Therapy

Hormonal therapies leverage the underlying biochemical pathways in which estrogen and androgen function and are used to treat and prevent breast, uterine, and prostate cancers whose growth is fueled by these hormones. Estrogen blockers (tamoxifen, raloxifene) and aromatase inhibitors (anastrozole, exemestane) are used to treat breast cancer, and androgen inhibitors (bicalutamide, flutamide, and nilutamide) are used to treat prostate cancer [58].

3.6.5 Prodrugs

Prodrug strategy for cancer therapy intends to address the selectivity challenges of chemotherapeutic agents by chemically modifying the bioactive molecule and enabling site-specific drug delivery [64]. Prodrugs of active moieties can be designed to improve the physicochemical properties and pharmacokinetics of the drug and enhance drug targeting and release to the tumor within the tumor microenvironment. Prodrug designs include attaching ligands that specifically target antigens or enzymes overexpressed in a tumor, polymers that extend circulation half-life and enable enhanced permeation and retention in the tumor, or attaching a cleavable linker that is activated by the tumor microenvironment (e.g., acidic pH or hypoxia). Etirinotecan pegol (NKTR-102) is a PEGylated conjugate of irinotecan, which is in phase 3 for metastatic breast cancer [65]. Polymer-drug prodrug conjugates that self-assemble into micellar nanoparticles are also in early-stage clinical studies in the USA and Asia [66].

3.6.6 Targeted Therapies (Small Molecule Inhibitors and Antibodies)

Mutant or overexpressed genes confer abnormal growth and survival signals in cancer cells. As a result, in many types of cancers, cancer cells and the surrounding tissues express specific protein targets and/or exhibit altered signaling pathways [67]. Targeted therapies refer to a class of drugs that inhibit these target proteins/signaling pathways and can either be small molecule chemicals [68] or biologics (e.g., monoclonal antibodies or mAbs) [69–72]. Typically, biologic drugs can only access the cell surface targets due to their large size, whereas small molecules are directed toward the intracellular domains of these surface proteins as well as cytoplasmic targets. Notable examples of small molecule-based targeted therapies are tyrosine kinase enzyme inhibitors that compete with ATP and block kinase activity.

In some advanced non-small cell lung cancers (NSCLC) and other cancers, tumor cell growth and survival depends on specific gene mutations in the epidermal growth factor receptor (EGFR) tyrosine kinases that result in the constitutive activation of certain tyrosine kinases (i.e., without the on/off regulation). Using tyrosine kinase inhibitors (TKIs) to treat these patients have resulted in improved efficacies with reduced toxicities compared to just using standard chemotherapies [68]. Several kinase inhibitors have been approved and are being developed along with the diagnostic markers for the prospective identification of patients with or without these specific gene mutations. Some representative examples are shown in Table 3.6.

Monoclonal antibodies (mAbs) are recombinant (man-made) proteins that bind to specific antigens found on cancer cells or neighboring cells. This class of therapeutic is exemplified by rituximab (Rituxan®, FDA approved in 1997) and trastu-

Table 3.6 A representative example of approved targeted therapy drugs (small molecules and antibodies) and their molecular targets. [68, 72]

Type of inhibitors	Cancer target	Example drugs, their brand names, and cancer indications
Signal transduction inhibitors	ErbB1 (EGFR) ErbB2 (HER2) TKs (EGFR) TKs (BCR-ABL, KIT, PDGFR) Kinases (B-raf, VEGFR2, EGFR, PDGFR) BTK PI3K	Cetuximab (Erbitux®): CRC, HNSCC Trastuzumab (Herceptin®): breast and stomach cancer Gefitinib (Iressa®): NSCLC Erlotinib (Tarceva®): NSCLC, renal, pancreatic cancer Imatinib mesylate (Gleevec®): GIST, CML Sorafenib (Nexavar®): kidney and liver cancer Sunitinib (Sutent®): GIST and kidney cancer Ibrutinib (Imbruvica®): CLL Idelalisib (Zydelig®): CLL
Apoptosis inhibitors	20s proteasome	Bortezomib (Velcade®) and Carfilzomib (Kyprolis®): multiple myeloma
Angiogenesis inhibitors	VEGF	Bevacizumab (Avastin®): CRC, GBM, ovarian, cervical and kidney cancer
Immune cell targeted	CD20 CD19, CD3	Rituximab (Rituxan®): B-cell lymphoma Blinatumomab (Blincyto®): ALL
Immune checkpoint inhibitors	PD1, PD-L1, CTLA4	Nivolumab (Opdivo®): NSCLC, kidney cancer, Pembrolizumab (Keytruda®): NSCLC, melanoma Atezolizumab (Tecentriq®): NSCLC Ipilimumab (Yervoy®): melanoma

Typically, antibody names end with "mab" whereas small molecule kinase inhibitor names end with "nib"

zumab (Herceptin®, FDA approved in 1998), two molecularly targeted monoclonal antibody therapies for the treatment of non-Hodgkin lymphoma and breast cancer, respectively [72]. Rituximab targets a specific protein called CD20 on B cells of the immune system, and trastuzumab targets HER2/Neu protein, which is overexpressed in 15–20% of patients with breast cancer. Bevacizumab (Avastin®, FDA approved in 2003) is an anti-angiogenic mAb that stops the growth of new blood vessels in the tumor microenvironment by blocking the vascular endothelial growth factor (VEGF) and inhibiting its binding to its receptor on blood vessels, thereby starving tumor cells of nutrients. Today, there are more than 50 therapeutic antibodies approved in Europe/USA for the treatment of several solid tumors and hematological cancers. A complete list of approved small molecule and biologics targeted therapies for the treatment of specific cancer subtypes can be found in reference number [72]. Table 3.6 is a representative example of some of these targeted therapies.

Antibody-drug conjugates (ADCs) leverage the specific antigen targeting property of mAbs to deliver highly toxic drugs to cancer cells by joining the two together through

chemical conjugation [73]. Brentuximab vedotin (Adcetris®) and ado-trastuzumab emtansine (Kadcyla®) are examples of marketed ADCs [74, 75]. Due to their specificity to cancer cells/tissues, targeted therapies in general are less toxic compared to traditional chemotherapies. The type and extent of toxicities depend on the target and whether the target is also present on healthy tissues (e.g., skin rashes with EGFR inhibitors, hypertension with anti-VEGF therapies) [76]. Many of these therapies are given in combination with conventional chemotherapies and/or surgery and radiation. It must be noted that mAb therapies are expensive but rarely curative and also involve complex manufacturing processes [77, 78]. While the majority of small molecule drugs tend to be given orally, antibodies are typically administered intravenously or subcutaneously.

3.6.7 Immunotherapies

The immune system consists of multiple specialized cells and substances that are part of the innate or adaptive immunity. The job of the immune system is to protect from infections and cancer and hence has checks and balances in place to recognize self (host) from foreign (graft). Despite having a healthy immune system, cancer can still grow, either because the immune system is not strong enough to fight cancer or because cancer has developed mechanisms to suppress or evade the immune attack. Immune therapy involves mechanisms to stimulate the immune activation pathways or block the negative signals that suppress immune function, thereby enhancing tumor-specific T-cell responses or externally deliver molecules that supplement one's own immune function [55, 58]. Different kinds of cancer immunotherapies that have been developed in the past two decades are discussed below.

3.6.8 Monoclonal Antibodies (mAbs)

In addition to the direct effect of antibodies blocking a specific receptor or its ligand as targeted therapies, mAbs can in some cases, induce anticancer effects by immune activation through their Fc region by complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC). Rituximab and the new generation of molecules that bind to CD20 antigen on B lymphocytes are example molecules that activate effector immune response and tumor cell killing [79]. Blinatumomab is an example of a bispecific mAb that contains only the two Fab regions, each of which recognize one antigen, CD19 antigen on lymphoma cells, and CD3 antigen on immune T cells, triggering an immune attack of tumor cells [80, 81]. Blinatumomab (Blincyto®) was approved in 2014 for the treatment of patients with Philadelphia chromosome-negative precursor B-cell ALL.

3.6.9 Immune Checkpoint Inhibitors

Recently, revolutionary improvement in cancer treatment was shown by mAbs directed against immune checkpoint molecules CTLA-4, PD-1, and PD-1's ligand PD-L1 on T cells. Cancer cells express tumor-specific antigens, which in a functioning immune system will be recognized by the immune cells and trigger T-cell activation and downstream effector response, leading to tumor eradication. However, immune regulatory pathways are frequently compromised in cancer due to negative signals in the tumor microenvironment. Immune checkpoint inhibition is a mechanism to downregulate these negative signaling pathways and unleash the immune response on tumors. Between 2010 and 2016, the following immune checkpoint inhibitors were approved: pembrolizumab (Keytruda®, FDA approved in 2014) and nivolumab (Opdivo®, FDA approved in 2014) for melanoma and NSCLC, ipilimumab (Yervoy®, FDA approved in 2011) for melanoma, and atezolizumab (Tecentriq®, FDA approved in 2016) for NSCLC and urothelial cancer [82–85]. Long-term sustained treatment effects have been seen in melanoma and NSCLC [86, 87] with combination therapies showing even greater promise [88–90]. The Kaplan-Meier survival curve shown in Fig. 3.2 [87] for the PD-1 inhibitor nivolumab is a representative example highlighting the far superior survival benefits seen with immunotherapy compared to traditional chemotherapy in the treatment of advanced NSCLC patients in the second-line setting. However, the same agent failed to improve survival in the treatment naive (first-line setting) advanced NSCLC [91] patients, suggesting the need for more research in the field.

The early promise of checkpoint inhibitors has led to increased trends in combining these agents with various oncology drugs, be they chemotherapeutics, small molecules, therapeutic vaccines, or more advanced cell therapies. The obvious reasons for combining checkpoint inhibitors with other therapies is based on the fact that two therapies given together can be more powerful than one, and using lower doses of two agents simultaneously may contribute to less toxicity. Pembrolizumab, nivolumab, and atezolizumab are some of the checkpoint inhibitors that have been combined with other therapies in solid tumors, NSCLC, etc. [92]. While the side effects are mostly manageable, in some cases, the side effects of boosting the immune system are severe [93].

3.6.10 Cytokines

Cytokines are the chemical messengers between cells of the immune system. They help communicate and coordinate immune response in a regulated fashion. They are produced by the immune cells as a response to pathogens or tumor antigens and mediate the growth and activity of certain immune cells, thus improving overall immune response. They also increase the recognition of tumor cells by the immune system by stimulating the immune effector cells directly at the tumor site, thereby

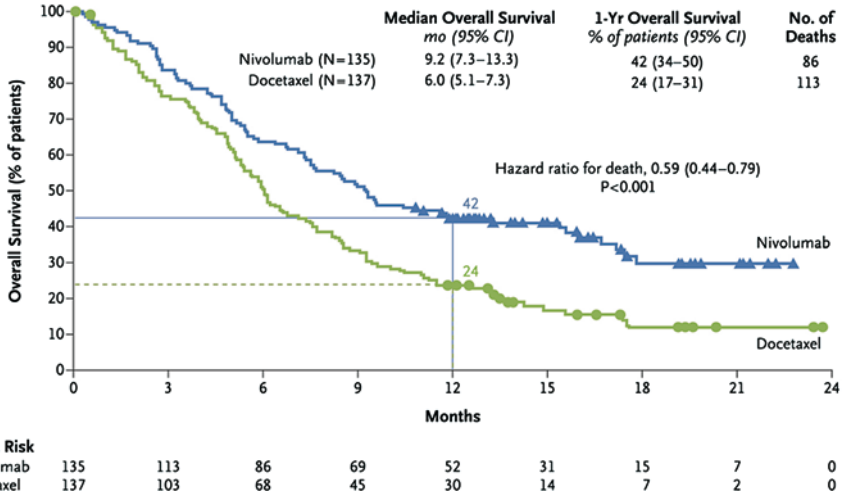

Fig. 3.2 Kaplan-Meier curves showing improved and sustained overall survival with nivolumab (PD-1 inhibitor) vs. docetaxel (chemotherapy)**.** Data are for advanced NSCLC patients who have disease progression during or after chemotherapy. Horizontal lines indicate the rates of overall survival at 1 year. (Reprinted with permission from [87])

mounting a tumor attack. Cytokine therapy in cancer exploits their immense signaling network. There are two FDA-approved cytokine therapies: interleukin-2 (IL-2) for treatment of kidney cancer and advanced melanoma and interferon-alpha (IFN-α) for treatment of some leukemias and lymphomas [94, 95]. Many more are in clinical development. They may be used as single agents, adjuvants with other immune therapies, or in combinations with chemotherapy. Cytokine immune therapy is complex due to their dual role of immune activation and suppression and their redundancy in function. A key challenge is finding the balance between efficacy and toxicity. Attaching a polyethylene glycol (PEG) polymer to interferon-alpha prolongs drug exposure by extending its circulation half-life while reducing its peak concentration. This delivery strategy has resulted in reduced toxicity and improved convenience of dosing frequency and is approved as adjuvant therapy for stage III melanoma. Cytokines also play a key role in cancer vaccine and adoptive cell therapy. In these applications, they help in the ex vivo production of immune cells and serve as adjuvants in vivo in enhancing and maintaining a robust immune response.

3.6.11 Cancer Vaccines

Certain types of cancers are caused by the same viruses that cause infectious diseases, for example, cervical cancer by human papilloma virus (HPV) and liver cancer by long-term infection with hepatitis B virus (HBV). Therefore, the same

traditional vaccines administered to healthy individuals against these viruses also have the potential to prevent such cancers. HPV quadrivalent vaccine (Gardasil®), the first cancer prevention vaccine, was approved in 2006 for the prevention of cervical cancer [96].

However, the majority of cancers are not caused by infections. Therapeutic cancer vaccines are given to patients to treat cancer. In this type of therapy, cancer cells or specific cancer antigens or immune cells are removed from a patient's body, manipulated ex vivo to boost immune response, and reinjected to the same patient (autologous therapy) [97]. The treatment improves the detection of tumors by the immune cells, resulting in an immune attack toward eradicating the tumors. Adjuvants are often included with cancer vaccines to further enhance the potency and durability of the immune attack. With the memory function of the immune system, these vaccines potentially could prevent tumors from coming back. Currently, sipuleucel-T (Provenge®) to treat hormone refractory prostate cancer is the only FDA-approved therapeutic cancer vaccine. With this therapy, white blood cells from a patient, primarily dendritic cells, are taught to recognize a specific prostate cancer antigen [98]. These are complex and expensive therapies. Many more cancer vaccines are in development, including allogeneic therapies, where well-established human cancer cell lines are used. Allogeneic therapy overcomes many of the challenges of working with autologous therapy, such as an unlimited source for tumor antigens, easier ex vivo manipulation, and standardized, scalable, and cost-effective manufacturing and therapy to multiple patients. In some cases, cancer antigens are delivered using special vehicles that can enhance their efficacy. These include modified viruses, bacteria, or other germ vectors that have been altered to suppress their reproductive potential [97].

Oncolytic viruses are yet another type of therapeutic vaccine where certain live viruses are genetically modified to selectively replicate within the tumor cell (but not in the normal cell) and induce direct tumor cell lysis, which may in turn stimulate systemic immune response against the tumor. While the concept and development of oncolytic anticancer therapy was already attempted in the 1990s [99], talimogene laherparepvec (Imlygic®), which is injected intratumorally for the treatment of melanoma, is the only FDA-approved oncolytic viral therapy [100, 101]. Imlygic® is a herpes simplex virus type 1-derived oncolytic virus that produces the immune modulatory protein granulocyte macrophage colony-stimulating factor (GM-CSF). Several other candidates are in early and mid-stage clinical trials [98]. Since immune cell infiltrated tumors are more responsive than checkpoint inhibitors, oncolytic viral therapy is also being investigated in combination with immune checkpoint inhibitors [102, 103]. Optimal delivery methods, overcoming inactivation of the virus by the host antibodies, and manufacturing issues are some of the challenges of oncolytic viral therapy [99].

3.6.12 CAR (Chimeric Antigen Receptor)-T-Cell Therapy

Chimeric antigen receptor-T-cell therapy (CAR-T) is one of the newly emerging immune therapies that has shown great promise in early clinical trials targeting CD19 antigen in B-cell malignancies. Complete remissions are seen in relapsed and refractory acute lymphocytic leukemia (ALL) in 90% of patients, with some patients showing durable remissions and persistence of CAR-T cells [104–106]. The principle of CAR-T therapy is to genetically modify a patient's T cells ex vivo to express specific receptors that will recognize selected antigen (e.g., CD19 in B-cell malignancies) independent of the natural T cell's major histocompatibility complex (MHC) restrictions. This feature helps circumvent the immune suppressive signals within the tumor environment, unlike with cancer vaccines and other immune therapies that rely on activating the endogenous immune system, which is often challenging. Vector or non-vector-based delivery is used ex vivo to transfer the genetic materials encoding the intended receptor to the T cells. These novel receptors could also be designed to include additional signaling domains such as costimulatory (e.g., CD28) molecules, which help enhance the immune response. Optimizing the CAR design toward enhanced immune response is an active area of research. The modified cells are expanded ex vivo and infused back to the same patient. These therapies come with severe side effects, such as cytokine release syndrome, that need to be managed. Manufacturing and logistics of these therapies are also complex, expensive, and still in their infancy.

3.6.13 Gene Therapy

Gene therapy typically refers to inserting exogenous genetic materials such as DNA, mRNA, siRNA, miRNA, antisense oligonucleotide, or gene editing systems, such as zinc-finger nuclease (ZFN) and clustered regularly interspaced short palindromic repeats (CRISPR) RNA together with a CRISPR-associated (Cas) protein (CRISPR-Cas9), into specific target cells either ex vivo or in vivo to replace a mutated or missing gene with the correct copy of the gene or to inactivate a faulty gene [107]. Gene therapy is delivered by viral vectors (e.g., adenoviruses, adeno-associated virus, retroviruses) [108] or non-viral synthetic delivery methods (e.g., electroporation, lipid, or polymer-based nanodelivery systems) [109]. Gene therapy is being investigated to treat genetic disorders such as severe combined immunodeficiency (SCID) and hemophilia, as well as acquired diseases such as blood cancers, neurodegeneration, and HIV [110]. Cancer vaccines and adoptive cell therapies discussed before where genetic material is inserted into the patient's cells are also examples of gene therapy.

To date, it has been 26 years since the first human trial with gene therapy [111–113], and there are still no FDA-approved gene therapy products in the USA. Worldwide, there have been three gene therapy products: alipogene tiparv-

ovec (Glybera®), approved in 2012 in Europe to treat lipoprotein lipase deficiency (LPLD); recombinant human adenovirus (Gendicine®), approved in 2003 in China to treat head and neck squamous cell cancer [114]; and autologous CD34+ enriched cell fraction that contains CD34+ cells transduced with retroviral vector that encodes for the human adenosine deaminase cDNA sequence (Strimvelis®), approved in May 2016 by the European Medicines Agency for the treatment of a rare, but fatal childhood genetic disorder called severe combined immunodeficiency (SCID) due to adenosine deaminase (ADA) deficiency (ADA-SCID) (commonly called "bubble boy syndrome") [115, 116]. While most of the children will die within 2 years of the disease, in the latest Strimvelis clinical trial, 18 children were still alive 13 years after the functioning ADA gene was inserted into their bone marrow stem cells. While this one-time treatment is curative, the price tag is very high, and hence GlaxoSmithKline, who markets Strimvelis, has offered a money back guarantee if the patients are not cured [117]. It is anticipated that two decades of active research in this area, which has resulted in several ongoing clinical trials, more than 60% of which are in cancer, will likely result in more gene therapy FDA approvals soon [107, 110].

RNA interference (RNAi) is a natural process in cells used to silence target regions of post transcriptional RNA. RNAi was discovered in 1998 and was awarded a Nobel Prize in 2006 [118, 119]. Short interfering RNA (siRNA) is a therapeutic modality where a short (~20 nucleotide) nucleic acid target sequence is inserted into the cell's RNAi apparatus to silence the intended faulty gene from making the disease-causing protein. Despite its huge potential to treat diseases whose targets are not easily accessible to conventional drugs, clinical translation has been slow due to the challenges of systemic delivery of these charged macromolecules to the cytoplasm in sufficient quantities. Most advanced siRNA clinical candidates are based on lipid nanoparticle (LNP) encapsulated delivery, which by virtue of nanoparticle preferential distribution into the liver target specific liver diseases. A phase 3 clinical trial with a transthyretin (TTR) siRNA product candidate (ALN-TTR02) to treat TTR-mediated amyloidosis was stopped in late 2016 due to the increased number of deaths in the treatment arm [109, 120–122]. SiRNA therapies that target other liver diseases, including infectious diseases HBV and HCV, are in early clinical trials. Alternatives to LNP delivery strategies also have reached the clinic. In cancer, the first clinical evidence of RNAi induced by siRNA therapy was established in 2010 by CLA-001 [123], and currently there are multiple early clinical trials ongoing that target established oncogenes such as MYC, kRAS, and PLK [120].

In addition to these clinical advancements, new gene editing tools are constantly emerging to more precisely manipulate disease-causing genes [124, 125]. Zinc-finger nucleases (ZFNs), transcription activator-like effector (TALE) nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system [126] are nuclease-based gene editing tools that can precisely introduce a targeted, site-specific double-strand break (DSB) in the host DNA and stimulate the endogenous cellular DNA repair pathways. CRISPR-Cas9 is the most promising of these gene editing tools as it is precise, fast, and least expensive of all

current technologies [127]. Inspired from a similar tool in certain bacteria to protect themselves from viral infections, the CRISPR-Cas9 system consists of a guide RNA that is complementary to the gene being targeted in the cell. The Cas9 DNA endonuclease that is attached to the guide RNA recognizes and precisely makes double-strand breaks in the target gene. In the last 2 years, gene therapies that use TALEN or ZFN have entered the clinic and are being evaluated to treat HIV, sickle cell disease, thalassemia, and leukemia and for the manipulation of immune cells in CAR-T therapy for cancer [112]. By mid-2016, a NSCLC clinical trial in China plans to use CRISPR-Cas9 technology to inactivate the checkpoint PD-1 gene from patient's T cells [128]. Similar CRISPR-modified T-cell therapy clinical studies are in the final stages of regulatory review in the USA, and clinical trials are expected in early 2017 [129].

Despite advances in gene therapy, there are still multiple challenges and risks associated with it that need to be managed and overcome. These include potential for severe immune response, targeting the wrong cells, off-target effects, risk of cancer, and successful delivery of the intended gene in sufficient quantities to target cells for a durable correction of the disease without affecting other regulatory functions [124, 125].

3.6.14 Stem Cell Transplantation

Stem cell transplant procedures, or hematopoietic stem cell (HSC) transplantation, restore blood-forming cells in leukemia, lymphoma, or multiple myeloma patients whose own blood-forming cells have been destroyed following high doses of chemotherapy or radiation therapy. Occasionally, they may also be used to treat other cancers, such as testicular cancer, neuroblastoma, or pediatric cancers. The blood-forming stem cells used for transplants can come from the bone marrow, blood-stream (less invasive and therefore preferred), or umbilical cord and may be autologous (from self), allogeneic (from a matched related or unrelated donor), or in special cases, syngeneic (from identical twin). Graft versus host disease (GvHD), a serious problem where white blood cells from the donor (graft) recognizes cells from the host body as foreign and attack them, can more likely develop when peripheral blood is used compared to bone marrow HSCs. While GvHD may not be as big of a concern in cord blood, it can on the other hand take longer to engraft in the patient, leading to a longer time that the patient is exposed to infections. In addition, in contrast to peripheral and bone marrow donors, the cord blood donor cannot be called back for more [130].

With all the tremendous advances and strides in drug discovery for achieving better treatment outcomes for cancer, the 2014 update from the International Agency for Research on Cancer addresses the continued burden of morbidity and mortality of cancer around the world [131] as successful cancer treatment is challenged by factors including ineffective therapeutic drug concentrations reaching the tumor site, life-threatening side effects caused by non-specific tissue distribution, and

acquired resistance to the drug. Acquired drug resistance is a major obstacle to successful long-term outcomes [62, 63]. Resistance emerges due to drug exclusion, drug metabolism, and alteration of the drug target by genetic or epigenetic changes that allow them to escape treatment. Modern and emerging treatments include combination therapies that combine treatment options against multiple signaling pathways that promote tumor growth/survival and therapy resistance.

3.7 Novel Treatments and Drug Delivery Approaches

Nanotechnology-based approaches for drug delivery in oncology have evolved significantly in the last decade, with a focus on enhancing the drug bioavailability and targeted delivery of the drug, while reducing any deleterious or serious side effects caused by related toxicities. Nanotechnology-based drug delivery is used to manipulate drug pharmacokinetics, tissue distribution, and release in the target tumor tissues/cells. Liposomal doxorubicin (Doxil®) and albumin-bound paclitaxel (Abraxane®) represent the first-generation nanotherapeutic drugs with demonstrated improvements in their pharmacokinetics and bioavailability, resulting in improved benefits to cancer patients compared with their non-formulated parent counterparts. Thus, nanotechnology-based solutions have potential for improved delivery of therapeutic agents and are actively being pursued for both diagnosis and therapy.

Various classes/types of nanomedicines (Fig. 3.3), including viral vectors, drug conjugates, lipid-based nanocarriers, polymer-based nanocarriers, and inorganic nanoparticles, are currently being evaluated in solid tumors and are either approved or at various stages of clinical development [132]. To date, approximately, 12 drug conjugates and nanocarriers have been approved for cancer therapy; 20 polymer drug conjugates, 56 lipid-based nanocarriers, 20 polymer-based nanocarriers, and 6 inorganic nanoparticles are currently in various phases of clinical trials. In October 2015, FDA approved irinotecan liposomal injection (Onivyde®). Irinotecan liposomal injection is approved for use in combination with fluorouracil and leucovorin to treat metastatic pancreatic cancer patients who have previously been treated with gemcitabine. The cowpea mosaic virus (CPMV) nanoparticle has been evaluated as an immunotherapy in models of metastatic lung melanoma and other cancers and found to have striking efficacy in mouse models as an in situ vaccination reagent [133].

While cancer nanomedicine represents one of the fastest-growing and promising research areas for cancer treatment, there are substantial obstacles that must be overcome before they can enter mainstream cancer care settings [134]. Some of these obstacles include technical challenges of manufacture and high cost of development, additional regulations on manufacturing standards and process control requirements, surface charges of nanomaterials and their effect on biological outcomes, metabolism and elimination of nanomaterials from the body, and the delivery of nanomedicines to tumors and their subsequent internalization and complex mechanisms of release [135].

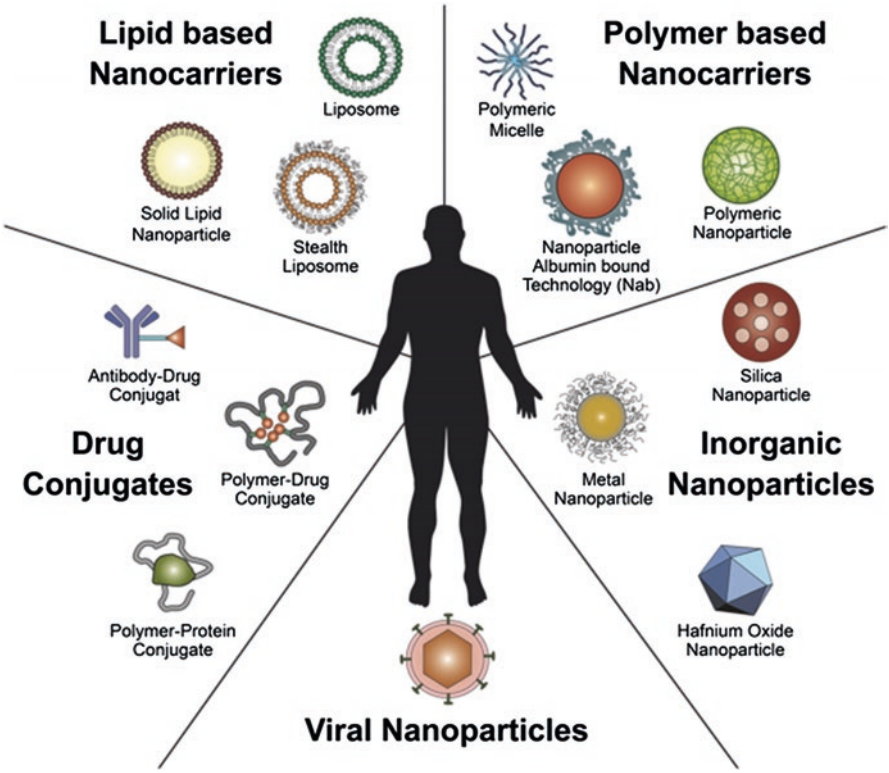

Fig. 3.3 Established nanotherapeutic platforms with specific examples for each category. (Reprinted with permission [132])

3.8 Challenges Associated with the Commercialization of Oncology Therapeutics

Commercially, the oncology space has become quite crowded, especially with agents targeting similar pathways and manufacturers facing fierce competition. Yet, the treatments for cancer are far from adequate, and there is still the need for continuous improvements of new and effective approaches, both for treatment and management of complications associated with cancer care therapy.

With the rapid increase in the influx of innovative new therapies, combination therapies, and increased segmentation of cancer types, so have the challenges associated with commercializing them. Further, because not all new drugs have resulted in significant improvements in patient survival times, medical insurance companies mandate information on a new drug's cost-effectiveness in addition to the safety and efficacy before they agree to reimburse them. Value-based medicine and not just simple evidence-based medicine is now viewed as the emerging paradigm in oncology. From the regulatory perspective, the threshold for determining whether an

oncology product has a clinically meaningful effect is being raised to include more robust endpoints, such as overall survival, to support approval of novel drugs, as opposed to currently used response rates or progression-free survival. Therefore, oncology drug makers now face the challenge to demonstrate the overall economic and clinical value of new products to justify the benefit and cost-effectiveness of a new treatment and its subsequent reimbursement.

While developing innovative platform technologies that show promise in preclinical or in translational models and that aim at novel targets, improved drug delivery, imaging modalities, and diagnostics, increased participation between academic and community research teams, the NCI, FDA, private foundations, and industry would certainly be beneficial. Further, it would help to focus on identifying and developing first in-class, innovative, and unique targets with strong IP protection, increased collaborations between academia and pharma, continued guidance from health authorities on development strategies of the asset, enhanced IND enabling translational studies that better reflect outcomes in the clinic, and conduct of science to global standards; achieving the best outcomes for cancer patients is definitely achievable.

In addition, while not just confined to the oncology indication, it will be important to define the most effective strategies that can overcome barriers to clinical trial accruals which may include barriers associated with physicians, protocol, eligibility and/or patients, etc. Tailoring clinical trial designs should also be considered to improve efficacy, efficiency, and overall costs associated with drug development.

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Chapter 4
Nanomedicine in Cancer

Liang Ma, Phuong Le, Manish Kohli, and Andrew M. Smith

4.1 Introduction

Nanotechnology is a multidisciplinary field focused on engineering structures with sizes between 1 and 100 nanometers, intermediate between microscopic molecules and macroscopic objects [1]. At this scale, materials exhibit new and profoundly useful properties and physically resemble the nanometer-scale components of biology, such as proteins and viruses. The goal of nanotechnology is to develop tiny

L. Ma
Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Micro and Nanotechnology Laboratory, University of Illinois at Urbana-Champaign, Urbana, IL, USA
e-mail: liangma2@illinois.edu

P. Le
Micro and Nanotechnology Laboratory, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA
e-mail: ple9@illinois.edu

M. Kohli
Department of Oncology, Mayo Clinic, Rochester, MN, USA
e-mail: kohli.manish@mayo.edu

A. M. Smith (✉)
Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Micro and Nanotechnology Laboratory, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA
e-mail: smi@illinois.edu

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functional objects, analogous to complex biological structures that originated from eons of evolution. Due to this similarity to biology, it is not surprising that the rapid advancement of this field over the past 30 years has been driven by biological inspiration [2] as well as specific needs in the biomedical sciences and clinical medicine [3]. Because of a unique match between the capabilities of nanotechnology and needs, cancer and oncology have been the primary beneficiaries of these new advances [4, 5].

The most advanced applications of nanotechnology in cancer are in therapeutics, imaging, and diagnostics. For therapeutics and imaging, the standard platform technology is a nanoparticle (NP) administered to the body, with the goal to localize to a tumor, to provide image contrast for detection or monitoring, or to therapeutically treat the diseased tissue. Compared with conventional contrast agents and therapeutics, NPs can give higher image contrast, higher treatment efficacy, and fewer side effects, primarily due to localization to tumor tissues selectively over healthy tissue. For clinical diagnosis and monitoring, NPs are also integrated into devices for in vitro molecular analysis of biospecimens from patients, such as blood or tissue biopsies. Compared with conventional methods, nanotechnology devices can improve sensitivity of detection, increase throughput, and increase speed. NP technologies have rapidly advanced from basic research tools for laboratory studies to successful FDA-approved products, exemplified by liposomal formulations of chemotherapeutic drugs such as doxorubicin (Doxil) [6] and irinotecan (Onivyde) [7] that have become a part of the clinical regimen for late-stage ovarian and pancreatic cancer, respectively. Medical nanotechnology is now a major commercial sector with a global market with projected value of USD 528 billion by 2019, of which cancer nanotechnologies are the largest segment [8].

While first-generation nanomedicines have been effective in exploiting biological barriers to improve the delivery of conventional anticancer drugs and imaging agents, major challenges remain in increasing therapeutic efficacy and targeting efficiency. In the near term, clinically driven strategies are expected to involve the coupling of genome-targeted interventions with NP-based drug delivery, while monitoring of nanomedicine drug and host interactions can be enabled by companion diagnostics through theranostic imaging and assays of the patient's body fluids [9–11].

4.2 Classes of Nanoparticles

NPs used for cancer are colloids: tiny solid particles suspended in aqueous solution. There are usually three distinct structural domains: a core, coating, and targeting agent (Fig. 4.1). The core provides a useful emergent property, with a composition typically classified as metallic, semiconducting, magnetic, or organic, as depicted in Fig. 4.2. A coating surrounds the core and prevents it from chemical or physical damage due to the diverse constituents of biological media such as proteins that can bind to and "foul" the surface, disrupting its intended function [12]. The coating also provides an inert biophysical interface to minimize toxic or

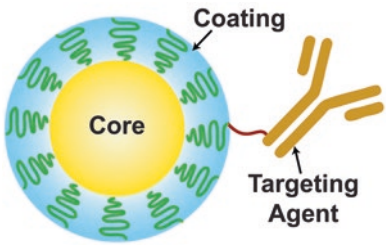

Fig. 4.1 Typical structure of a nanoparticle designed for use for therapeutic or imaging, showing a core, shell, and targeting agent

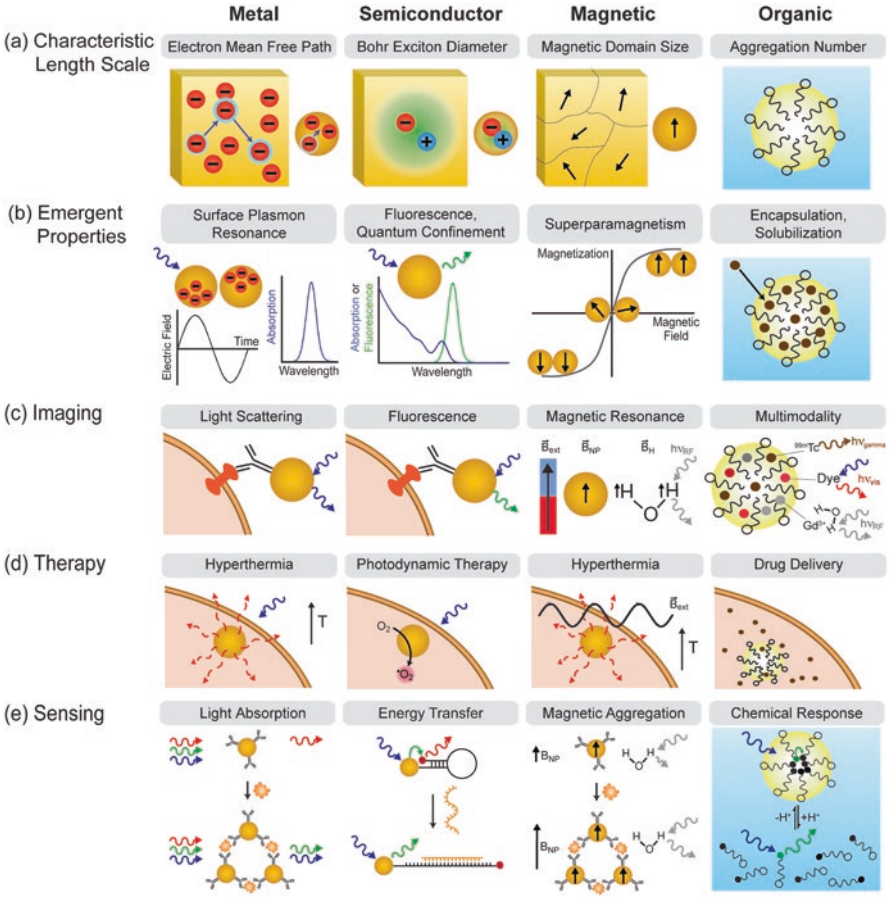

Fig. 4.2 Major classes of nanoparticles and their biomedical applications in cancer. Four major categories of nanoparticles are shown as metallic, semiconducting, magnetic, and organic, with (**a**) their characteristic length scales over which their unique properties arise and (**b**) their primary emergent properties. In the bottom three rows, schematics depict the applications of these materials in (**c**) imaging, (**d**) sensing, and (**e**) therapy. Detailed descriptions are provided in the text

damaging effects toward cells [13]. The most common surface functionalization is polyethylene glycol (PEG) [14], a flexible, linear, and electrostatically neutral polymer tethered to the surface that sterically inhibits protein binding and provides long-term chemical stability. The surface is further attached to a targeting molecule, which is often an antibody or ligand for a cell surface receptor, which functions to either home to a specific tissue in the body or to bind to a specific molecular or cellular target in solution [15]. Note that many nanomedicines may not require a molecular targeting agent to function, and sometimes the core and shell comprise the same entity.

Below we describe each of the four major classes of core materials used in cancer applications, focusing on their unique emergent properties that arise when materials are within a characteristic size range. We also briefly describe how each material is used for therapy, imaging, and sensing. For many of these materials, a wide range of subclasses have been engineered, and each type can be prepared with diverse sizes and shapes, such as spheres, rods, and disks [16]. The vast parameter space can be used to dramatically impact NP function, and multiple components can be fused together into a single NP to engender multifunctionality [17]. A primary example is the incorporation of both imaging and therapy core constituents to provide new possibilities in clinical theranostics.

Metallic NPs Metals like gold (Au) and silver (Ag) conduct electricity due to free mobility of electrons within the material. When shrunk from macroscopic sizes to NPs, a host of new optical properties arise when metals are smaller than the average distance that an electron moves unimpeded (the mean free path, ~10–100 nm) [18]. Smaller than this size range, electrons that absorb energy from light will collectively resonate on the NP surface. This resonance is called a surface plasmon, and its frequency is often in the visible spectrum, allowing excitation by visible light. The result is an exceptionally high intensity of light absorption and scattering, with frequency tunable over a broad range of colors by the NP size, shape, and composition. These optical features provide a high sensitivity of detection, sensitivity to changes in local environment, and the ability to locally increase temperature because absorbed light is converted to heat [19]. The capacity for heat generation has led to clinical trials for use as photothermal ablation agents for solid tumors [20]. Metal NPs are widely used in basic research for sensors and devices and have been explored as injectable therapeutics and imaging agents, but they have not yet received approval for human administration [21].

Semiconductor NPs Semiconductors like silicon (Si) and cadmium selenide (CdSe) exhibit composition-dependent electrical conductivity and light absorption. When these materials are nano-sized, the optical and electronic properties are tunable by size due to the quantum confinement effect [22]. The concept is that light absorption by a semiconductor causes an electron to enter a higher energy orbital, and the empty ground state orbital behaves as a similar but positively charged particle called the “hole.” The electron and hole are electrostatically bound together as the Bohr exciton, which has a characteristic size (~1–100 nm) dependent on the composition. When the NP is smaller than the exciton, the exciton is spatially con-

fined, causing its energy and light absorption frequency to increase. Moreover, the exciton decays through fluorescent light emission, so the emission color can be widely tuned by the NP size. Quantum dots, which are spherical variants, have diverse applications as fluorescence imaging agents and light-emitting sensors and have been explored for therapeutic applications based on photocatalytic generation of cytotoxic compounds [23]. However, prototypical materials used today (CdSe) contain potentially toxic elements, so they are unlikely to be approved for administration in humans and are applied solely for in vitro diagnostics and experimental use.

Magnetic NPs Ferromagnets like iron (Fe) and ferrimagnets like ferrite iron oxide (Fe_3O_4) can be permanently magnetized and exhibit unique properties when they are small [24]. These materials in bulk are composed of many microscopic domains, each with coherent magnetic moment. When shrunk to the size of a single magnetic domain (~5–50 nm), a NP has only one magnetic moment, which induces superparamagnetism. Unlike a bulk magnet, the NP has a net magnetic moment that is nearly zero but has an exceptionally large magnetic field in the presence of an external magnetic field. Because of this magnetic inducibility, these NPs have been widely applied to generate a local magnetic field for magnetic resonance imaging, to apply a localized physical force to manipulate cells, to sense the presence of molecules and cells, and to heat tissue by an oscillating magnetic field [25, 26]. Iron-based magnetic nanoparticles have been approved for use in humans and have been available under trade names such as Feridex and Lumirem, but these were removed from the American market due to safety concerns [21].

Organic NPs Organic materials are most often composed of amphiphilic molecules or polymers and are the primary NP class used for therapeutics [21, 27]. In aqueous solution, amphiphilic molecules self-assemble into larger, quasi-stable particles because the hydrophobic molecular domains aggregate to minimize contact with water. The size depends on the number of molecules in the assembly (the aggregation number), which largely derives from the relative volumes of the hydrophobic and hydrophilic domains, parameterized by the packing parameter [28]. By tuning the amphiphile domains, it is possible to generate micelles with a hydrophobic core or liposomes with a hydrophilic core. The key emergent property for nanomedicine is the capacity to entrap chemical agents within the different NP regions. Hydrophilic drugs and imaging agents can be entrapped in the hydrophilic core of a vesicle, whereas hydrophobic drugs can be entrapped in hydrophobic domains. Doing so can reroute the biodistribution of small molecules in the body and increase the solubility of hydrophobic compounds to allow the use of higher dosages. Moreover, diverse classes of such materials have been approved for use in humans due to the strong record of safety for organic NPs composed of biogenic materials such as lipids and proteins [21]. Synthetic NPs composed of reduced carbon, such as nanotubes, nanodiamonds, and graphene, are also being widely explored [29].

4.3 Nanoparticle Biodistribution

The most attractive capability that nanomedicine has provided to the cancer community is the ability to control the distribution and circulation time of drugs and imaging agents in the body [30]. This effect is primarily due to the relationship between NP size and the size selectivity of different physical barriers in the human body. It is important to emphasize that a majority of cancer nanomedicines are delivered through injection into a vein to allow systemic circulation in the bloodstream, with the goal of delivering drugs or imaging agents to a tumor that either cannot be precisely located (e.g., metastases) or cannot be resected surgically. Other parenteral routes can be used, including local injection for inoperable tumors, but NPs are not commonly administered through enteral routes due to low bioavailability caused by either degradation in the gastrointestinal tract or inefficient transport to the blood compartment [31]. Once in blood circulation, NPs distribute to capillaries of vascularized tissues. Depending on the intended target, NPs may simply bind to endothelial cells (ECs) that line tissue capillaries or may need to penetrate into the tissue interstitium by extravasating across the endothelium. The ability to extravasate selectively in tumors compared to normal tissue is a defining feature of nanomedicine that has elicited wide exploration for drug delivery [32]. This effect derives from the selective permeability of tumor endothelium [33] as well as properties of the interstitium that provide a driving force for transport.

Endothelial Permeability In most normal tissues like the muscle and skin, solutes in the blood can transport across endothelial layers by four different known pathways depicted in Fig. 4.3a [33]. Small hydrophobic molecules can directly permeate due to their solubility in lipid membranes, giving access to any tissue. Hydrophilic molecules like glucose can directly pass through cells by specific transporter proteins on ECs or pass between adjacent cells through paracellular transport. The cutoff for paracellular transport is approximately 5 nm for most tissues, dictated by gap junctions, but this is more restrictive (~1 nm) in tissues like the brain with EC tight junctions [34]. For larger cargo, caveolae-mediated transcytosis is an active ATP-driven process in which ~70 nm endosomes shuttle cargo directly through ECs [35]. However the 5 nm threshold is the most important for nanomedicine, defining the upper limit for efficient access to a healthy tissue from the blood (Fig. 4.3).

The kidney and liver largely determine the clearance of NPs from the blood, partially due to the high blood flow that they receive (together approximately 50% of cardiac output [36]). The kidney glomerulus has a distinctive permeability, reflecting its major role in solute filtration. Glomerular ECs have large holes (fenestrations) through the middle of the cells that allow efficient fluid transport [37]. These fenestrations function as ~15 nm filters due to a mesh of polysaccharides called glycocalyx that spans the pore. The fibrous basement membrane acts as a second filter, followed by underlying cells called podocytes with intercellular slit diaphragms with size cutoffs of ~5 nm. The tiered structure provides clog-free, high-rate filtration, and

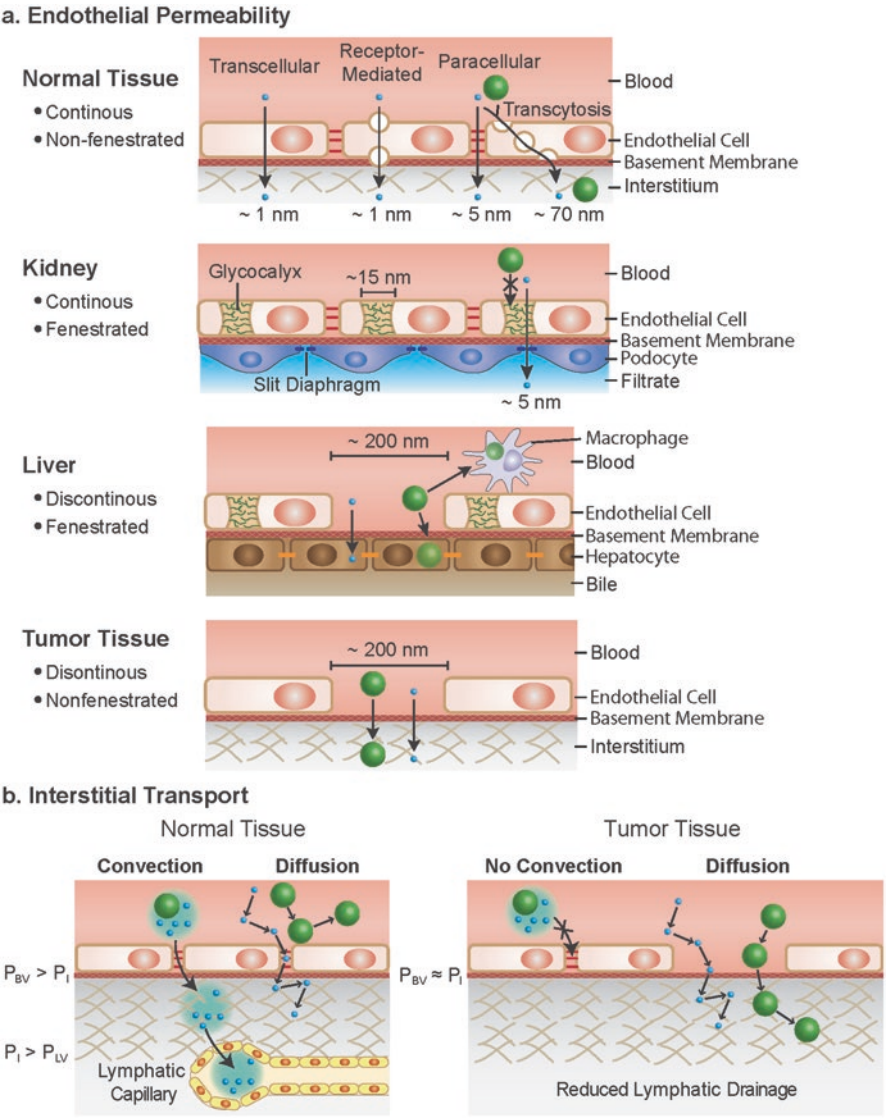

Fig. 4.3 Transport processes of nanoparticles and free drugs from the bloodstream to tissues. (**a**) Endothelial cells that line blood capillaries provide the first major barrier to nanoparticle entry to tissue. Primary tissue examples show "normal" tissue, kidney, liver, and tumor. Solutes in the blood can permeate the endothelium through transcellular, receptor-mediated, or paracellular transport or through transcytosis. In normal tissues, small molecules (blue circles) readily pass through the endothelium, while NPs (green circles) do not. In kidneys, NPs smaller than 5 nm can be filtered from the blood and excreted into the urine. In the liver, most NPs are taken up by macrophages or hepatocytes and can be potentially eliminated through the bile. In tumors, the endothelium is discontinuous, and nanoparticles smaller than 200 nm can penetrate to access the tissue interstitium. (**b**) In normal tissue, small solutes are driven into a tissue through the convective motion of bulk fluid due to the high hydrostatic pressure of the capillary blood compared with the (continued) interstitial fluid. Small molecules also pass through by diffusion if there is a difference in concentration between the blood and tissue. Neither process occurs for NPs because they are too large to pass across the endothelium through paracellular transport. Altered tumor endothelium allows paracellular transport for NPs, enabling diffusive transport. However, the trans-endothelial pressure differential is eliminated due to a lack of lymphatic drainage and fluid continuity between the blood and the interstitium, so convective transport is inefficient

NPs smaller than 5 nm are rapidly removed from the blood into urine and excreted from the body [16]. This excretion pathway is inaccessible to larger NPs, which almost invariably accumulate in the liver. The liver endothelium also has fenestrations but is also discontinuous, with large gaps between adjacent ECs to allow rapid transport to underlying hepatocyte cells that function in metabolism [38]. The liver also harbors a large number of macrophage cells (such as Kupffer cells) that exhibit a high rate of macropinocytosis to rapidly engulf particulate matter [16]. The high transport rate in the liver combined with these cells causes the liver to uptake nanoparticles larger than 5 nm, which is also observed in the spleen, which has a similar capillary microstructure. Depending on the NP type and chemistry, NPs may be taken up by macrophages, ECs, and hepatocytes and remain in the liver and spleen indefinitely or may be degraded and excreted [39].

The primary characteristic of tumor tissue that allows access to cells within tissue is that, like the liver, the endothelium is discontinuous (but not fenestrated). This effect is thought to be a result of rapid angiogenesis occurring in tumors that leaves the capillaries poorly formed and without a consistent structure [40]. The pore size of the inter-EC junction has been reported to be as large as 600–800 nm, and NPs in the size range of 10–200 nm have been widely used to treat and image tumors without the need for molecular targeting [41, 42]. However, wounds and sites of inflammation have similar discontinuities, so they exhibit similar effects, and it further remains uncertain how accurately vascular alterations in specific human tumor types are simulated by tumor models in animals that have been used to evaluate this effect [43].

Interstitial Transport Endothelial pore size and vascular perfusion together are only partially responsible for NP biodistribution. As depicted in Fig. 4.3b, there are two primary transport processes that drive NP efflux from the blood: diffusion and convection [44]. Diffusion occurs by random motion of molecules, allowing net transport from regions of high concentration (blood) to regions of low concentration (tissue interstitium), driven solely by concentration differences. Convection is driven by a pressure difference between two regions such that a bulk mass of aqueous fluid will be transported across the vascular wall into the interstitium. This occurs in normal tissue for any molecule smaller than the endothelial pore size, due to the high pressure of the capillary blood vessel (P_{BV}) compared to the interstitial pressure (P_I). However, mass balance requires a low-pressure sink in the tissue to drain the effluxed fluid. In normal tissue, lymphatic vessels serve this function as a site of low pressure (P_{LV}), whereas in the liver this also includes the bile duct, and in the kidney the urine filtrate. However in tumors, lymphatic ducts are often not present or do not function, and because the blood vessel pore size is large, the interstitial fluid is at high pressure, such that $P_{BV} \approx P_I$. Therefore in a tumor, convective transport is negligible, and diffusive transport is primarily responsible for extravasation, which is inefficient compared with convection. The diffusive transport of nanoparticles less than 200 nm into tumors is called the enhanced permeability and retention (EPR) effect and has been the primary rationale to explain "passive" delivery of NPs to tumor tissue [32, 45].

Once in the tumor interstitium, the interconnected network of extracellular matrix acts as a sieve to trap NPs, and interstitial cells engulf NPs [32]. Notably the total amount of NPs delivered to a tumor is very small, <1% of the injected dose based on a recent survey of the literature of NPs [43], but this is still often sufficient for many nanomedicine applications. However it has been observed that certain anti-angiogenic therapies may be able to further increase the transport of NPs into tumors by restoring the vascular pressure differential to increase convective transport [46]. Moreover, tumor endothelium exhibits the capacity for transcytosis, which has the potential to pump solutes up concentration gradients and thus concentrate exogenous molecules in tissues [47]. Recent discoveries of cancer tissue-specific caveolae targets may soon make this possible [48]. Another widely explored strategy to increase the accumulation of NPs in tumors is to use targeting agents such as antibodies, peptides, and aptamers that bind to tumor-specific cell surface receptors [15].

4.4 Therapeutic Applications

Conventional cancer treatments based on cytotoxic chemotherapeutics elicit serious adverse effects such as impaired immune function, organ failure, and nausea due to the impact of the drugs on healthy tissues other than the tumor. Newer targeted therapies and biologics have much better safety profiles but are still not curative for the vast majority of advanced cancers. The foremost promise for nanomedicine is to further increase the specificity of anticancer pharmaceutical agents toward a tumor to increase localized concentration and reduce the toxic impact on other organs, thus widening the therapeutic window [32, 49]. Nanoparticles can also alter pharmacokinetics of single drugs or drug combinations entrapped by NPs, increase drug solubility, and allow controlled and sustained releasing within blood or tissue compartments.

Tunable Drug Pharmacokinetics The utility of NP-based drugs for therapy derives from tunable biodistribution and tunable pharmacokinetics. NPs administered to the bloodstream can exhibit the EPR effect and largely avoid impacting off-target tissues except for the liver and spleen. However, biodistribution is only a part of the effect, as their circulation time in the blood is also unique compared with their small-molecule counterparts [50] and slow release of drugs from the carrier in circulation may play a major role in their efficacy. Figure 4.4a shows a commonly observed trend for the blood concentration of a free drug (FD) and NP drug after a single bolus injection. Because they are smaller than the kidney filtration threshold, most FDs that exhibit little protein binding will rapidly leave circulation, whereas NPs exhibit sustained circulation, with a duration tunable by properties such as surface coating and size, yielding a typical blood half-life ($t_{1/2}$) in the range of 1 h to several days [51]. This increases the total exposure of a diseased tissue to the drug, quantified by the area under the curve (AUC) of a [drug] versus time plot, which can

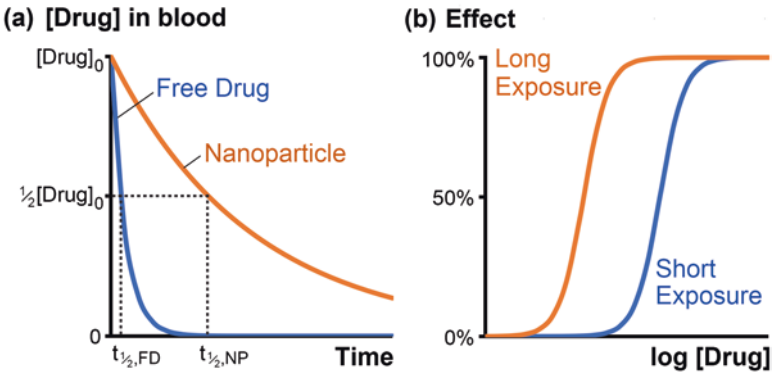

Fig. 4.4 Differences in pharmacokinetics and pharmacodynamics of drugs encapsulated in nanoparticles compared with free drugs. (**a**) Free drugs are often rapidly eliminated from the blood after injection due to excretion through the urine, providing a short half-life ($t_{1/2}$). The half-life for nanoparticles can be much longer and tunable due to their ability to be retained within blood, without kidney filtration. (**b**) Comparison between the impact of a long exposure to a drug and a short exposure on the cellular effect, such as proliferation inhibition. By lengthening the exposure to a drug, an effect is achieved at lower concentration, justifying the utility of nanoparticles for drug delivery

be orders of magnitude greater for a NP compared to a FD. A key rationale for the benefit is plotted in Fig. 4.4b, showing that when the exposure duration is longer, the therapeutic effect occurs at a lower drug concentration (i.e., lower IC_{50}), thus increasing potency. Therefore, the longer circulation time alone could have a dramatic improvement on patient outcome and reduce the dosing frequency. Moreover, the co-encapsulation of multiple drugs within a single NP can equalize the pharmacokinetics and biodistribution of drugs that have dissimilar physical properties [52]. This can increase the efficacy of combination therapies by precise tuning of the ratio between multiple pharmaceutical agents [53].

The amount of drug absorbed in a tissue is also expected to be proportional to the AUC, but this can be misleading, as small molecules rapidly distribute to interstitial sites in both normal tissues and tumors, whereas for NPs, interstitial access requires extended circulation time due to slow extravasation and transport. Moreover the behavior of NPs within a tissue is still poorly understood, and access of the delivered drug cargo to the intended molecular target is impeded by extracellular barriers to diffusion, nontargeted cell types that may uptake the NPs, and physical sequestration in intracellular vesicles [16]. Therefore even if a tumor exhibits enhanced uptake of a NP compared to a FD, the drug bioavailability may not increase, although extended local release may lead to a more sustained therapeutic effect.

Inorganic Therapeutic Agents While translational studies of therapeutic NPs have primarily focused on the use of organic materials due to FDA approval of several carriers and compatibility with conventional pharmaceutical agents, there

have been exciting results with inorganic materials as well. Unlike small-molecule drugs, inorganic materials exhibit unique optical and magnetic responsivity and novel mechanisms of efficacy (see Fig. 4.2d). The capacity to heat tissue with magnetic and metallic NPs using magnetic fields or light provides the ability to localize treatment to specific tissues. Moreover, heat, as a physical mechanism of ablation, could potentially be applied more precisely than drug treatments, surgical resection, or radiotherapy due to the high spatial control of laser light. Theoretically, these therapies could be applied more modularly than pharmaceutical agents due to the universal toxicity of heat. In fact, gold nanoparticles are being investigated in clinical trials at present for ablation of unresectable tumors, following local injection [21]. Similarly semiconductor NPs could be used as sensitizers for the localized production of reactive oxygen species to induce localized chemical damage, with higher efficiency over conventional photodynamic therapy agents that have been used for decades for certain cancer types [54]. However for most inorganic materials, the apparent inability to be biotransformed prevents them from leaving the body after systemic injection, which presents major safety problems for clinical translation [55].

Clinical Perspective The first-generation NP-based therapeutic drugs approved for use in cancer medicine include liposomal doxorubicin in 1995 to liposomal irinotecan in 2015. Table 4.1 summarizes the currently approved nanomedicines in the past two decades [56]. While considerable success has been seen with a variety of nanomaterials for delivering conventional chemotherapeutics, the gains of nanoformulations over conventional chemotherapeutic drug therapy remain incremental. One reason for this is that the majority of these NPs are used for delivery of conventional chemotherapeutic drugs, which are inherently nonselective and do not target specific tumor genomic aberrations. Because the underlying mutational landscape of cancer is heterogeneous between tumor types, and within a tumor type at different stages of progression, specific targeting of actionable mutations within an individual tumor, with NP delivery, has the potential to enhance nanomedicine efficacy and limit unnecessary toxicities, but has yet to be systematically evaluated. Such efforts have begun to emerge in phase I/II clinical trials for delivery of p53-based nanodrugs [57] and with clinical applications of nucleic acid-loaded nanocarriers using lipid-based nanoparticles [58]. Beyond targeting genomic aberrations, clinical applications of NPs that target specific cancer pathways such as angiogenesis pathways through vascular endothelial growth factor and receptors [59] and anti-HER2 using immunoliposomes [60] are other novel approaches being explored. The success of these targeted approaches will require not only a safe and efficacious drug delivery NP vehicle but also a coupled mechanism to monitor downstream drug-host effects based on target nucleic acid profiles and conventional clinical monitoring as is performed during assessment of imaging using the “response evaluation criteria in solid tumors” (RECIST) criteria [61].

Table 4.1 List of currently approved nanomedicines in the clinic. Adapted from Tran et al. [56]

Year approved[a]	Name	Type	Active drug	Diameter	Type of cancer
1994 (Japan)	Zinostatin stimalamer	Polymer-protein conjugate	Styrene maleic anhydride neocarzinostatin (SMANCS)	[b]	Renal cancer
1995 (FDA) 1996 (EMA)	Doxil/Caelyx	Liposome (PEGylated)	Doxorubicin	80–90 nm	HIV-associated Kaposi's sarcoma, ovarian cancer, metastatic breast cancer, multiple myeloma
1996 (FDA)	DaunoXome	Liposome (non-PEGylated)	Daunorubicin	45 nm	HIV-associated Kaposi's sarcoma
1998 (Taiwan)	Lipodox	Liposome (PEGylated)	Doxorubicin	180 nm	Kaposi's sarcoma, breast cancer, ovarian cancer
1999 (FDA)	DepoCyt	Liposome (non-PEGylated)	Cytosine arabinoside (cytarabine)	10–20 μm	Neoplastic meningitis
2000 (EMA)	Myocet	Liposome (non-PEGylated)	Doxorubicin	190 nm	Breast cancer
2005 (FDA) 2008 (EMA)	Abraxane	Nanoparticle albumin-bound	Paclitaxel	130 nm	Advanced non-small-cell lung cancer, metastatic pancreatic cancer, metastatic breast cancer
2006 (FDA)	Oncaspar	Protein (PEGylated)	L-asparaginase	50–200 nm	Leukemia
2007 (South Korea)	Genexol-PM	PEG-PLA polymeric micelle	Paclitaxel	20–50 nm	Breast cancer, lung cancer, ovarian cancer
2009 (EMA)	MEPACT	Liposome (non-PEGylated)	Mifamurtide	[b]	Osteosarcoma
2010 (EMA)	NanoTherm	Iron oxide nanoparticle	None	20 nm	Glioblastoma
2012 (FDA)	Marqibo	Liposome (non-PEGylated)	Vincristine	100 nm	Philadelphia chromosome-negative acute lymphoblastic leukemia
2015 (FDA)	MM-398 (Onivyde)	Liposome (PEGylated)	Irinotecan	80–140 nm	Metastatic pancreatic cancer

[a] *FDA* US Food and Drug Administration, *EMA* European Medicines Agency
[b] Data not available

4.5 Imaging Applications

Contrast-enhanced medical imaging Imaging is a standard component of clinical oncology for detecting primary tumors and metastases, monitoring therapy response, and detecting relapse. Importantly, high survival times require detection before distant spread; for example, the 5-year survival rate for non-small cell lung cancer is 80% when found locally but <15% once it has migrated to distant sites [45]. However the conventional clinical imaging modalities, X-ray computed tomography (CT), magnetic resonance imaging (MRI), radioisotopic imaging, and ultrasound, can typically only detect tumors larger than 1–2 cm in diameter, which often have already spread to lymph nodes and other organs [62]. This insufficient detection sensitivity derives from inadequate image contrast to differentiate tumors from normal tissue, so a major goal of nanotechnology is to use NPs as targeted contrast agents to increase tumor contrast (see Fig. 4.2c).

MRI contrast agents. To date, MRI has been the biggest beneficiary of NPs for imaging. Clinical MRI provides three-dimensional anatomical images and physiological information, with unlimited penetration depth and up to 1 millimeter resolution [63]. Anatomical contrast differences arise from the content of water in a tissue. An external magnet provides a magnetic field B_{ext} that aligns the magnetic moments of hydrogen atom nuclei (B_H), primarily in water. Photons with radio frequency (RF) are then transmitted to the patient and are absorbed by the nuclei, flipping their magnetic moments. The protons then relax to realign with B_{ext}, emitting RF photons in the process that are detected in two orthogonal directions to reconstruct an image. The intrinsic anatomical contrast provided by water in healthy tissue compared with a tumor tissue reflects gross structural changes in late stages of progression. But localized magnetic molecules and NPs can increase image contrast by changing the relaxation rate of nearby water protons, which alters the signal intensity. The clinical standard is Gd^{2+} chelates, which are small molecules that are administered intravenously and rapidly removed from circulation. Magnetic NPs have been explored for these applications, with the new ability to provide contrast through EPR uptake or through molecular targeting, which is lacking in clinical MRI imaging of cancer [64]. However while iron oxide nanoparticles were approved for human use, they have not been approved for cancer imaging but rather for imaging of the liver and vasculature.

Nuclear contrast agents Nuclear imaging is one of the most commonly used modalities in radiology for detecting metastasis and recurrence due to its extremely high sensitivity of detection and molecular specificity. Molecules labeled with an unstable isotope, such as ^{18}F, ^{15}O, ^{13}N, and ^{11}C, decay to emit a high-energy photon (gamma ray) or particle (positron) that can be detected to reconstruct three-dimensional images [65]. The ability to measure tumor uptake of metabolites (fluorodeoxyglucose) indicative of abnormally high metabolic rate has been critical in clinical care. However, the metabolic changes reflected in image contrast may be later-stage carcinogenic events, and higher molecular specificity is needed to

improve detection at earlier stages of carcinogenesis. Therefore molecularly targeted radiolabels have been widely explored, and NPs have been tested with radiolabels to reroute biodistribution and pharmacokinetics [66]. However the use of NPs as imaging agents has not gained traction because their longevity in circulation reduces tumor contrast, while the label is still radioactive. Nevertheless, radiolabeling is widely used to assess the pharmacokinetics and biodistribution of NPs, as described below.

X-ray contrast agents X-ray CT is widely used for detection of cancer of the breast and lungs, due to differential X-ray attenuation through normal and cancerous tissue. Contrast agents such as iodine and barium are clinically used for vascular and gut imaging, and NPs composed of heavy metals have been tested in research settings for targeted imaging of tumors [67, 68]. However NP contrast agents for CT are challenging to apply due to the relatively low sensitivity of CT, requiring administration of large amounts of high-density material.

Ultrasound contrast agents In ultrasound imaging, high-frequency sound waves reflect from interfaces and surfaces in the body and can be detected to reconstruct images. Interfaces between materials with different acoustic impedance, such as bone and water, lead to strong echoes and higher contrast. Micro- and nano-bubbles filled with gas are several orders of magnitude more compressible than water or tissue and provide a high degree of ultrasound contrast. Since their sizes are smaller than the wavelength of the applied ultrasound field, micro- and nano-bubbles undergo volumetric oscillation and produce strong backscatter ultrasonic waves, with enhanced signals [69]. Microbubbles have been used for decades to provide contrast in blood vessels for diagnosis of circulatory disorders, and recent nanobubbles are poised to provide even higher contrast, at a size scale more appropriate to make use of endothelial pores in tumor tissue and for molecular targeting.

Intraoperative imaging Surgery is the main treatment for localized cancers that are accessible and non-life-threatening when removed. In fact, it is estimated that 90% of cancer cures are due to surgery, rather than chemotherapy or radiotherapy [70, 71]. The success of surgery strongly depends on the complete removal of tumors, as small numbers of cancer cells left behind at margins can cause relapse. During resection, surgeons predominantly use palpation and visual inspection to identify tumors and delineate their margins [72]. However, these modes of inspection are often insufficiently specific and rely on gross anatomical changes to differentiate healthy from normal tissue. Therefore, optical imaging has been widely explored for imaging during surgery to assist in the identification of small tumors, assess tumor margins, and inspect tissue following resection [73]. Because light can be easily detected by the human eye, or by cameras, optical imaging has been the primary focus for intraoperative imaging compared with other modalities that require large and expensive equipment [74].

Optical contrast agents For general use in clinical imaging, outside of ophthalmology, optical contrast agents have had little use due to the low penetration depth of light through the tissue. However optical contrast agents are uniquely suited for imaging close to a surface due to the high spatial resolution of optical imaging (200

nanometers) and the availability of many distinct colors for simultaneous detection of multiple molecules. These materials provide contrast in the tissue by light absorption, emission, or scattering. The primary modality is fluorescence, a process by which absorption of light results in light emission at a longer wavelength. Fluorescein and indocyanine green are the most widely used organic dyes for clinical applications, and semiconductor quantum dots provide brighter signals as well as emission at long wavelengths in the infrared where background noise is negligible and penetration depth is enhanced [75]. Scattering and absorption by metal NPs can also provide contrast in tissues containing large quantities of NPs [76], which can be imaged through new techniques such as photoacoustic tomography to provide high-resolution three-dimensional images. Both semiconductor and metal NPs have been conjugated to targeting agents to improve tumor contrast in research applications.

Clinical perspective NP-based contrast agents are particularly needed for early detection of tumor recurrences while monitoring patients after completing primary cancer treatments. Typical follow-up may include traditional radiography with CT scans, standard X-rays, or bone scans, with a recent increasing use of MRI and PET scans. The detection sensitivity and specificity of these imaging techniques are highly variable and often low for early detection of recurrences [77]. Tumor-targeted nanocarriers that can enhance functional imaging and provide molecular contrast for prognostication and monitoring treatment outcomes would have outstanding value but have yet to be tested in the clinic. This will require a next generation of targeted nanomaterials with cellular-level and tumor-specific precision that can provide real-time structure-function imaging assessment of low-volume metastatic disease.

NPs are already a routine part of clinical practice for image-guided detection of cancer-bearing lymph nodes which are below the detection limits of conventional radiography; radiolabeled particles composed of antimony trisulfide, sulfur, and dextran polysaccharides are all selectively taken up by pinocytotic cells in draining lymph nodes after peritumoral injection, allowing identification and resection [78]. Major applications on the clinical horizon are NP-enabled precision mapping of surgical margins, detection of remnant cancer tissue, and assessment of tissue perfusion and function. While great progress has been made toward the development of targeted optical imaging agents based on NPs for intraoperative use in research laboratories, only a small subset will be suitable for clinical translation due to challenges with FDA approvals [79, 80].

4.6 Multimodal Nanoparticles for Imaging and Therapy

It is possible to construct NPs with two or more emergent properties by mixing multiple classes of cores within a single entity. A common practice is to combine metallic, semiconducting, or magnetic components by fusion of separate NPs, through sequential growth as core-shell materials, or by co-encapsulation within organic NPs. A major application is for imaging through multiple complementary modalities. For example, superparamagnetic iron oxide nanoparticles can be

co-encapsulated with quantum dots into organic nanoparticles to provide both high-depth imaging through MRI and high-resolution features through optical imaging [81]. Ultrahigh sensitivities can also be achieved by combining radioisotopic and optical NPs for accurate localization from the whole-body level down to the level of single cells, to discern biodistribution by both tissue and cell type [82]. However, it remains challenging to balance image contrast across different modalities, and different instruments must be used for the different modalities, resulting in image acquisition being sequential rather than simultaneous.

Multimodal NPs also commonly incorporate both therapeutic agents and contrast agents, most commonly by co-entrapment in the same organic nanoparticle. These materials combining imaging and therapeutics have the major advantage of allowing quantitative correlation between a therapeutic outcome and the biodistribution or pharmacokinetics [63, 83]. This strategy is widely used for understanding drug biodistribution at the preclinical stage of development, and some types of materials are also moving toward clinical application [84, 85]. This has been particularly useful for analyzing the complex biodistribution patterns of NPs to quantify their delivery when the composition, size, or shape is tweaked [86]. Clinically one of the most exciting opportunities would be the capacity to correlate an individual's therapeutic outcome with biodistribution, which at present is decoupled, to enable an advanced level of therapy personalization akin to modern pharmacogenomics.

4.7 In Vitro Diagnostics

Laboratory tests on clinical biospecimens play a major role in the clinical care of cancer patients. Blood and other bodily fluids can be screened for molecular cancer markers (e.g., prostate-specific antigen protein) or cells (e.g., cytology smears), and histopathological assessment of solid tumor biopsies is the gold standard of diagnosis [87, 88]. NPs are providing new and exciting capabilities that have not been previously possible, including high detection sensitivity to minimize the amount of specimen needed, multiplexing to analyze numerous markers, and lab-on-a-chip capabilities for low-cost analysis in remote locations or at the point of care. NPs are applied in two different categories of detection platforms, distinguished as *sensors* that intrinsically respond to the presence of an analyte in a solution or *labels* that bind to an analyte captured on a surface.

Nanoparticle sensors in solution Figure 4.2e depicts examples of the different ways in which NPs can be used to "sense" the presence and concentration of analytes like molecules and cells in a complex solution without the need for multistep processing and purification. For metal NPs, mixing with an analyte that can induce cross-linking between multiple NPs, the wavelength of light absorption can shift due to plasmonic coupling between the particles [89]. If this same procedure is

performed with magnetic NPs, the intensity of nuclear magnetic resonance signal can be enhanced upon aggregation [90]. Both effects can induce a measurable signal, either by optical or magnetic resonance, to indicate the concentration of the molecule or cell being analyzed. Quantum dots can also be used as sensors without the need for aggregation by attachment to dyes that undergo energy or charge transfer with the quantum dot to turn on or off its emission [91]. Because transfer only occurs in close proximity, transfer will be attenuated by a molecule that causes the quantum dot and dye to separate physically, e.g., via protease cleavage or nucleic acid hybridization-induced extension, leading to a change in fluorescence intensity. Organic nanoparticles can also function as sensors, by altering the equilibrium of self-assembly or aggregation due to interactions with molecules or chemicals (e.g., protons), causing encapsulated content to release or to alter energy transfer between encapsulated dyes [92].

Nanoparticle labels on a surface NPs can also be used to measure the presence and concentration of molecules or cells after capture to a surface. Surface capture provides the ability to wash away molecular and cellular contaminants so that the analyte can be isolated and measured using multistep processes with higher specificity and accuracy. The standard practice of most clinical laboratory assays today is to capture proteins and cells using antibodies and capture nucleic acids through hybridization with their complementary sequences. After capture, it is possible to detect analytes even without a label if binding induces a measurable change to the substrate itself. If the substrate is a metal, the analyte can alter the local electric field, which changes the angle at which the metal scatters light (Fig. 4.5a) [93]. Similar effects can occur if the surface functions as a diffraction grating (e.g., a photonic crystal), with surface adsorbates changing the wavelengths of light absorbed or reflected by the substrate [94]. If the surface is piezoelectric or piezoresistive, the analyte can cause a mechanical deformation of the substrate, which can be measured by a change in resistance across the surface (Fig. 4.5b) [95]. Also if the substrate is an electrode in an electrochemical cell, analyte binding can alter the measured current or voltage [96]. In clinical laboratories, most molecular assays use labels rather than sensors due to the ability to amplify the measured signal. Most often labels provide an optical signal after binding to the surface-captured analyte (Fig. 4.5c). After washing, the amount remaining, detected through light absorption or emission, can be used to quantitatively determine the concentration of the analyte. This is the same technique applied for molecular analysis of biopsies of solid tumors, for which the analytes are already fixed to a tissue substrate. Quantum dots have played an important role in increasing the number of different types of molecules that can be simultaneously measured on these substrates due to the large number of distinct optical codes that can be employed [97, 98]. Conductive metal NPs can also be used, particularly when using substrates between two electrodes in electrochemical platforms as they both serve to measurably increase conductivity in the cell and allow the specific deposition of metals like silver to physically complete the electrical circuit and drastically increase signal strength (Fig. 4.5c) [99].

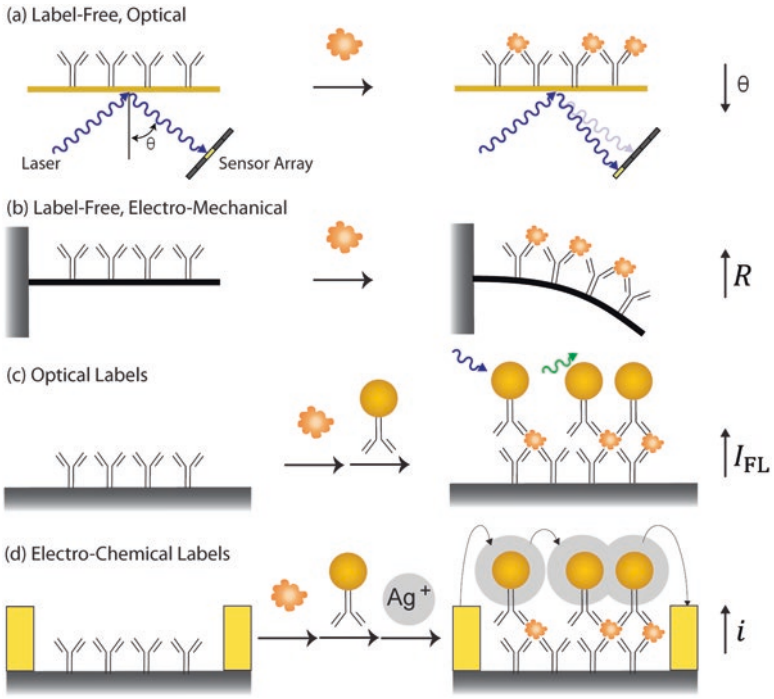

Fig. 4.5 Examples of surface-based in vitro detection platforms, showing (**a**) label-free with optical readout, (**b**) label-free with electromechanical readout, (**c**) optical nanoparticle labels, or (**d**) metallic nanoparticle labels. (**a**) Binding of an analyte can significantly alter the properties of a substrate. In this example, the angle of light reflection by a metallic substrate shifts when analytes like proteins or nucleic acids bind to capture molecules on the surface and alter the local electric field. (**b**) Alternatively, a piezoresistive cantilever can be mechanically deflected by binding to an analyte, which causes the electrical resistance across the surface to change. (**c**) Optical labeling platforms use two-step processes, by which the analyte is first captured, and a light-absorbing or light-emitting label is then used to bind to the surface. After washing away excess, the intensity of absorption or fluorescence is measured. (**d**) Electrochemical platforms can have numerous types of arrangements, whereby the assay surface is between two electrodes, between which the voltage or current can be measured. By using metallic labels, the conductivity can be significantly changed, and additional silver deposition to the metal nanoparticles can further increase the signal

Clinical perspective The current generation of in vitro diagnostic tests for early detection and screening of cancer is largely based on analysis of a single biomarker in tissue or blood at a single point in time and does not take into account the molecular heterogeneity of tumors or the effect of a constantly evolving tumor genome. From solid tumor biopsies or resections, newer multiplexed platforms in clinical use can assess numerous DNA and RNA biomarkers, which can be used to identify therapeutic targets, predict drug efficacy, and prognosticate survival. These analyses can include germline single nucleotide profiling and somatic aberrations, including copy number variations, mutations, and RNA-based profiling. Pharmacogenomic

and pharmacogenetic markers pertaining to individual drugs can also be monitored clinically, although this is currently not offered routinely.

An area in which nanotechnology-enabled in vitro diagnostics can make a rapid impact is through the serial analysis of biological fluids containing molecular components of a primary tumor and metastatic sites. Since the tumor genome following a leading truncal mutation is heterogeneous and constantly evolving, it would be a revolutionary advancement to be able to measure the genomic changes in a patient's cancer and to monitor the effects of treatments after initial diagnosis simply through analyses of blood or urine. The potential for developing and multiplexing candidate genes and low-abundance molecules such as exosomal microRNA, cell-free DNA, and mRNA in circulatory fluids using in vitro ultrasensitive diagnostics has the potential to refine clinical outcomes and can be enabled by nanotechnology [4, 100]. Such in vitro diagnostics in the future are likely to fill a critical gap by offering a real-time serial capture of genomic changes for rapidly adapting therapies (drug and dose) based on an individual's response to drug interventions.

4.8 Outlook

Nanotechnology has provided numerous new capabilities in cancer medicine that are being used to revolutionize screening, diagnosis, surgery, and therapy. NP therapeutics could enable the capacity to precisely dose and control a combination of therapeutics in a single entity for personalized therapy, and NPs combining both therapy and diagnostic capabilities through imaging (i.e., theranostic NPs) can provide direct insight into mechanisms of response as well as real-time monitoring. More broadly, advanced theranostic approaches to clinical oncology stand to revolutionize personalized care in the next decade. While drug selection should ideally be based on the individual host tumor profiles of underlying mutations, unfortunately this is currently impractical in the clinic as individual tumor genomes are heterogeneous and are constantly acquiring mutational and clonal changes during drug therapy. Thus, the increasingly prescribed targeted molecular treatments are not effective in all patients, but only in subpopulations, and for unpredictable durations. Therefore theranostics that enable a combination platform of molecular diagnostics and therapeutics for individualized therapies would be of outstanding clinical value [101]. These tests should differ from traditional tests that simply measure tumor morphology and metabolic rate and instead exploit the clinical specificity of genetics and molecular biology being uncovered by the ongoing genomic revolution in clinical oncology. It is exciting to envision the near-term connection between nanomaterials and this revolution, for which NP agents can specifically report the molecular state of a tumor, rather than simply its presence. In addition, ultrasensitive and portable nanotechnology-based devices can provide needed blood tests to screen for tumor profiles at early stages, to monitor

posttreatment recurrences, to predict a patient's response toward therapies, and to evaluate prognosis at low cost. Integrating such ultrasensitive tools for individual patient genomic and genetic characteristics together with NP-based drug delivery potentially offers a refinement of current management paradigms which may increase the effectiveness of anticancer strategies.

While nanomaterials for clinical therapy and diagnosis provide exciting opportunities, there are numerous challenges in the way for practical and routine clinical use. Most importantly, for in vivo NP approaches to be effective, significant improvements in targeting specificity are needed to reduce the quantity of nanomaterial that distributes to the liver and spleen and to understand mechanisms of nanomaterial-specific toxicity. Rapid translation is more feasible for in vitro diagnostics, as toxicity is not a concern and long-term clinical trials are not needed. However, proof of diagnostic accuracy and reproducibility is challenging for nanomaterials due to their imprecise structures and challenges in synthetic scale-up and quality control. Indeed, ideal NP structures are often highly complex, so design criteria should include not just knowledge of the ultimate clinical application but also manufacturing and customer use insights. Highly integrated teams of experts across diverse disciplines and industries are thus needed to enable these approaches to effectively pair nanotechnologies with the most critical needs in cancer medicine and clinical care and to ensure that the pipeline to practical use is feasible.

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Chapter 5
Theranostics: A Historical Perspective of Cancer Nanotechnology Paving the Way for Simultaneous Use Applications

Christopher M. Hartshorn and Stephanie A. Morris

5.1 Introduction

Imagine a time when our approach to oncology relies exclusively upon weekly intravenous injections that simultaneously treat and immediately report treatment response via the imaging modality of choice. Direct measurement of this response then allows for follow-up treatment to be attenuated – this optimized, personalized treatment regimen ensues with response-guiding treatment. Is this pure speculation or an impending reality that could come to fruition in our lifetimes?

Theranostic medical nanotechnologies have advanced to the point where the first approved products, enabling this dream, will occur in our lifetime and become standard of care by the end of the twenty-first century. It was only 50 years ago that this modern reality was merely science fiction. Indeed, from the view of modern day researchers in the field of cancer nanotechnology, nanotechnologies have always been viewed as multimodality platforms capable of theranostics applications. As with any breakthrough medical technology, the process bringing scientists to this downstream vision has been iterative. It has not been easy nor quick, although this is inherent in the challenges faced in delivering a safe and more efficacious platform to humans for a disease that has only been recently understood at the genetic level.

Over this time, nanotechnologies from the perspective of the clinician have gone from "robots to fix cancer at the molecular level" to a "technology not able to live up to the hype" [1–3]. As such, it is important to understand the context of what has led us to this point. Furthermore, we need to delineate to ourselves the apparent benefits versus the reality, as the clinical community will continue to weigh medical nanotechnologies by the sole metric of, "How will they improve patient outcomes

C. M. Hartshorn · S. A. Morris (✉)
National Cancer Institute, NIH, Nanodelivery Systems and Devices Branch, Cancer Imaging Program, Division of Cancer Treatment and Diagnosis, Rockville, MD, USA
e-mail: morriss2@mail.nih.gov

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over the current standard of care?" [4]. Thus, the goal of this chapter is to convey both *cancer* and *nanotechnology*, their ultimate convergence, and the coming transition to multifunctional use in the clinic as context for the reader.

5.2 Cancer in Historical Context

In over 48 years since the "War on Cancer" was declared (Nixon, Laskar) and 5 years since the "War" was decried (Varmus), there have been numerous life-prolonging innovations [5, 6]. Nonetheless, cancer-related deaths remain the second largest contributor to mortality worldwide (595,000 in the USA alone, representing ~25% of all deaths and 8.8 million worldwide, representing ~17% of all deaths) [7–9]. The lifetime risk of developing cancer remains at ~40%, and death due to cancer is ~20%. Furthermore, 30–50% of cancers are preventable as of 2017 (e.g., cancer caused by infection, smoking, the environment, and more). From merely a socioeconomic point of view, cancer has a global cost of $1.16 trillion annually [7]. Despite all of these facts, cancer has always been a collection of related diseases (>200 types and a multitude more of genetic subtypes) that were identified long before the modern era.

We understand that cancer, by its inherent nature, has been impacting human health since the dawn of mankind. The oldest reports of cancer dating back to ca. 3000 BC were discovered in Egyptian mummies, manuscripts, and fossilized bone tumors of the time albeit these were merely descriptions and evidence that cancer has been with us as long as historically obtainable. Not until 2500 years later were terms even developed to describe aspects of the disease studied by Greeks and Romans in 460–30 BC. Specifically, terms were developed by the physicians Hippocrates (coined the terms of *carcinos* and *carcinoma*, non-ulcer- and ulcer-forming tumors, respectively), Celsus (translated those into Latin for crab, *cancer*), and Galen (used the Greek word for swelling, *oncos*, to describe tumors). Later in the sixteenth century AD, the Greek lexicon was again used to describe a syndrome of the disease, *cachexia* (Greek for bad and habit to describe energy wasting, a syndrome of the disease in ~50% of cases). From the Greeks through to the seventeenth century AD, a myriad of theories as to cancer causes were postulated, although none utilizing modern day scientific method or tools. Hippocrates' humoral theory where an excess of any one humor caused disease remained unchallenged for well over a millennium until several more began to be developed (e.g., lymph, blastema, chronic irritation, infectious disease, and trauma) [10]. The birth of scientific oncology in the nineteenth century, by way of the scientific method (from Newton and Galileo) and the modern microscope, began the pathological path to our current genetic understanding (Fig. 5.1).

The Scottish surgeon, John Hunter, was the first to suggest and offer guidance for surgical resection of tumor tissue in the late 1700s. Additionally, Giovanni Morgagni was the first to perform routine autopsies *posthumous* relating illness to pathologic findings. These two paved the way for "oncologists" of the nineteenth century to

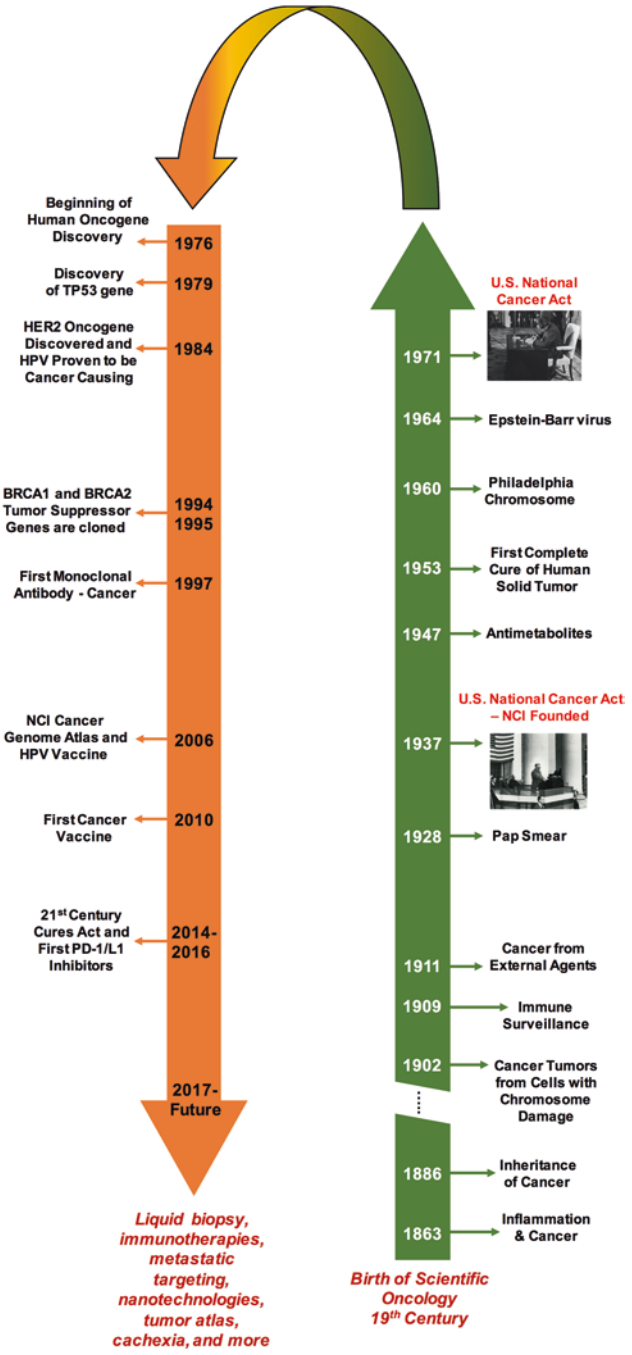

Fig. 5.1 Timeline of modern advances and important historical events in oncology

biopsy, resect, and prognosticate patient cancers via the microscope. Since this time, the total volume of discoveries made, and therapeutics or diagnostics developed, are far beyond the scope of this chapter, although the major biological understanding as to causes are listed here:

- *Inflammation and Cancer (1863)* Rudolph Virchow identifies leukocytes in cancerous tissue, the first known observation connecting inflammation and cancer.
- *Inheritance of Cancer (1886)* The ophthalmologist Hilário de Gouvêa delivers the first documented confirmation that cancer susceptibility can transfer from a parent to a child.
- *Cancer Tumors and Single Cells with Chromosome Damage (1902)* Theodor Boveri proposed that cancerous tumors occur from specific cells after chromosomal damage.
- *First Use of Radiation Therapy to Cure Cancer (1903)* S.W. Goldberg and Efim London use radiation therapy to treat basal cell carcinoma of the skin. Ultimately leading to much research as to the underlying mechanisms of DNA damage and cancer.
- *Immune Surveillance (1909)* A concept that eventually becomes known as the "immune surveillance" hypothesis is originally proposed by Paul Ehrlich. In essence, this is a biological assumption that the immune system suppresses tumor formation and the beginning of research to determine the mechanisms by which tumors adapt to evade this basic principle.
- *Cancer from External Agents (1911)* The virus that causes cancer in chickens is discovered by Peyton Rous, affirming that some cancers are caused by infectious agents; cancer is induced in rabbits in the laboratories of Katsusaburo Yamagiwa and Koichi Ichakawa by smearing coal tar on the skin, offering experimental proof that chemicals can cause cancer (*1915*); and Ernst Wynder, Evarts Graham, and Richard Doll identify cigarette smoking as a significant feature for lung cancer (*1950*).
- *Pap Smear (1928)* George Papanicolaou's initial observation that cervical cancer can be detected by examining vaginal cells under a microscope.
- *Antimetabolites (1947)* Sidney Farber demonstrates treatment with an antimetabolite induces temporary remission in patients.
- *First Complete Cure of a Human Solid Tumor (1953)* The initial comprehensive cure of a human solid tumor with chemotherapy achieved by Roy Hertz and Min Chiu Li.
- *Philadelphia Chromosome (1960)* Peter Nowell and David Hungerford describe the first genetic defect linked to a specific cancer type (chronic myelogenous leukemia) after observations of an unusually small chromosome in cancer patient cells. Ultimately this was found by Janet Rowley in 1973 to be due to a gene translocation for this specific defect.
- *Epstein-Barr virus (1964)* Michael Anthony Epstein and Yvonne Barr identify this virus, ultimately the first virus linked to cancer.
- *DNA of Normal Chicken Cells (1976)* Dominique Stehelin, Harold Varmus, J. Michael Bishop, and Peter Vogt investigate DNA from normal cells of chickens

leading to the discovery of human oncogenes. Subsequently, the most commonly mutated gene (*TP53 Gene, 1979*, also called p53) in human cancers is discovered; the *HER2 oncogene is identified (1984)* and the human version shown to be overexpressed in about 20% to 25% of breast cancers; the *BRCA1 and BRCA2 tumor suppressor genes* are cloned (*1994/1995*), and their mutation shows greatly increased risk of ovarian and breast cancer in women.

- *HPV 16 and 18 (1984)* DNA from human papillomavirus (HPV) types 16 and 18 are identified in a large percentage of cervical cancers, in essence, establishing a link between cervical carcinogenesis and HPV infection. This ultimately leads to the development and approval of multiple HPV vaccines (e.g., Gardasil and Cervarix).
- *First Monoclonal Antibody (1997)* The first approval of a monoclonal antibody for cancer, *rituximab*, which begins a wave of monoclonal antibody drugs (e.g., *trastuzumab* (1998), *ipilimumab* (2011), and *pembrolizumab* (2014)).
- *The Cancer Genome Atlas (TCGA) (2006)* TCGA project supported by the National Cancer Institute begins, resulting in the analysis of genomes for over 33 tumor types from 11,000 patients.
- *First Cancer Vaccine (2010)* The first approval of a cancer treatment vaccine, sipuleucel-T.

Much of this has been driven by societal level efforts to "Cure Cancer." From the US National Cancer Act of 1937, signed into law by the US President Franklin D. Roosevelt, that formulated the NCI and NIH into what they are today—the largest public funder and driver of cancer research in the world [11]—as well as the numerous global public, nonprofit, and private sector funding and research with similar aims, the push to a "Cure" has been a modern medical endeavor. The efforts were reinvigorated ("War on Cancer") with the National Cancer Act of 1971, which was signed into law by US President Richard M. Nixon.

Over this time, therapy and diagnosis developments have developed rapidly following suit with our respective understanding of the disease, as with all of modern medicine. This collective body of knowledge and contemporary molecular understanding were consolidated in 2000 by Douglas Hanahan and Robert Weinberg into the *Hallmarks of Cancer* [12, 13]. They are now working on their third revision in just 15 years (Fig. 5.2) due in part to the acceleration of our genetic, proteomic, and systems biology understanding over this period. This has led to major clinical developments for immunotherapy, imaging, radiotherapy, and ex vivo biopsy in the last several years. The most recent large-scale effort was just initiated in 2015, the Beau Biden Cancer Moonshot Initiative, signed into law under the twenty-first Century Cures Act by US President Barack Obama in 2016.

With all of the advances and efforts of the modern era, we have yet to offer legitimate cures to most cancers. This unfortunate fact mandates that we continue efforts to better understand, detect, and treat cancer. Pioneering treatment and diagnosis paradigms will be required to magnify the current arsenal available to clinicians and caregivers. Nanotechnology offers the ability to contribute to both of these paradigms for the twenty-first century.

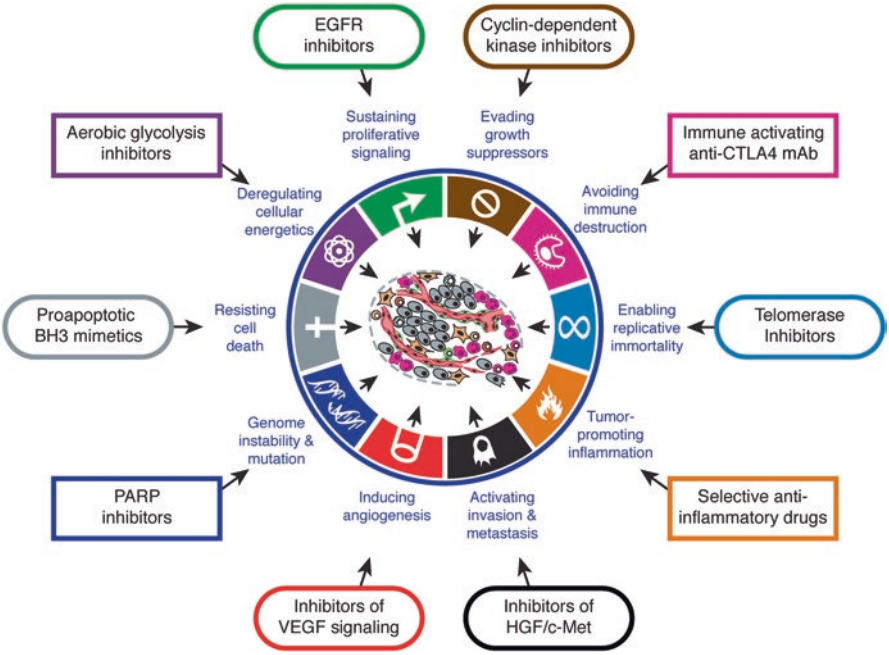

Fig. 5.2 Therapeutics utilizing basic tenants of the "Next Generation of the Hallmarks of Cancer." Focus of drugs and targets relative to our modern day understanding of the acquired capabilities necessary for tumor growth and subsequent progression. (Reprinted with permission from Hanahan and Weinberg 2011. Copyright 2011 Elsevier)

5.3 Nanotechnology in Historical Context

Arguably, the first reported observation of physics at the nanoscale was by Michael Faraday in 1857 [14]. Many of what are now known to be nanoscale materials used (e.g., glasses and metals) in the fourth century AD and beyond are not a topic of this discussion, as they were not synthesized and/or observed as consisting of nanoscale materials until very recently. Faraday reported his observation of a colloidal gold solution to the Royal Society of London to propagate the understanding of light as both wave and particle, a theoretical construct with growing awareness during these and subsequent years. He introduces the reason for his experiments in this lecture by stating "Conceiving it very possible that some experimental evidence of value might result from the introduction into a ray of separate particles having great power of action on light, the particles being at the same time very small as compared to the wave-lengths, I sought amongst the metals for such. Gold seemed especially fitted for experiments of this nature, because… known phenomena appeared to indicate that a mere variation in of its particles gave rise to a variety of resultant colours." Ultimately, John Tyndall completed the theory by which this physical effect was originally observed from Faraday and is now known as the Faraday-Tyndall or

simply the Tyndall effect (i.e., light scattering by particles in a colloid or very fine suspension) in the 1860s. Tyndall showed that the light scattering could only be of particles in the 40–900 nm size range or similar to the visible range of electromagnetic radiation. The thought of materials at the nanoscale was not ended after these observations, although very little research effort was given to them until being revived in our imaginations in the late 1950s.

During these years, many scientists including the preeminent quantum field theory physicist, Richard Feynman, revived our collective scientific machination to the nanoscale. From lectures on the idea of nanoscale to the first use of the current name for the field of nanotechnology (e.g., first coined by Norio Taniguchi, 1974), the research community's vision for the manipulation and application of nanoscale materials transformed [15, 16]. This transformation went from science fiction to scientific reality rapidly. Although much of the fundamental chemistry, materials science, measurement tools, and physics had been rapidly maturing during the 1940s to 1970s, very little could proceed without the enabling tools to "see" below the diffraction limit, as well as to measure and manipulate at the nanoscale. This all changed with the development of the modern electron microscope as well as the scanning tunneling and subsequent atomic force microscopes developed by IBM scientists in the early 1980s then implemented soon after [17, 18]. Furthermore, the first nanoscale material, such as the carbon allotrope known as buckminsterfullerene (C_{60}), was discovered by Kroto et al. during this period, opening the door for a myriad of nanomaterials research and their consequent application as nanotechnologies [19]. In roughly the same period, Ekimov discovered nanocrystalline, semiconducting quantum dots in a matrix of glass, and reported on these findings [20]. After which, by way of these primary discoveries, the field of nanotechnology was off to the proverbial races.

Much of these initial collective efforts were driven by the semiconductor industry and for weapons research. The correlation is more than coincidental but was the underlying premise to the original trend predicted in 1965 by Gordon Moore, cofounder of Intel, as to the basis for what became known as "Moore's law." Specifically, the semiconductor industry's growth would rely increasingly upon nanoscale processes in order to double the number of transistors every 2 years. Parallel to these efforts, centered mostly toward semiconductors, has always existed the opportunity nanotechnology presented for other applications, including nanoenabled therapies or diagnostics. As soon as the mid-1990s, consumer products began to appear that were developed in whole or in part by way of nanotechnology advances. From large-scale manufacturing tools to the actual product, nanotechnology was being used in many sectors of the worldwide economy. Following many of these great advances and consumer-driven nanoscale products, the USA initiated a dedicated effort to fund and coordinate, at a large scale, nanotechnology research and development across the federal government. The US National Nanotechnology Initiative (NNI) was born from this as well as what became the National Nanotechnology Coordinating Office (NNCO), which was first funded in 2001 with a specific task of coordinating federal agency spending for nanotechnology. Since this time there has been a concerted and strategic effort to advance the field at an

accelerated rate, especially in applications to the medical and biotechnology sectors. Applying nanoscale solutions to solve health problems and improve patient outcomes presents a unique set of challenges. With the main exception of liposomal delivery systems, at the beginning of the twenty-first century, nanotechnology still had major hurdles to overcome as far as its basic mechanisms in vivo (e.g., ability to deliver cargo to specific site, toxicity, enhanced efficacy, etc.). Putting this in context after over 30 years of dedicated efforts to advance the field, if nanotechnology was compared to the age of a human being, it would still only be a young adult relative to other well-defined areas of medicine. In essence, only capable of participating in a collaborative with other older sciences and fields of the medical community, yet not ready to lead. However, there are many examples and reasons to trust that nano-centric science and technology will continue to advance rapidly into clinical care in the near future. Much of these current and future successes have been strategically enabled by way of new approaches to traditional research and development within science, engineering, and medicine now utilizing the principles of science and technology convergence.

5.4 Nanotechnology for Cancer: An Iterative, Generational, and Convergent Process Toward Theranostics

Cancer goes back as far as human history can be recorded, while the ability to control matter at the molecular scale and process it in such a way as to regulate its length scale and physical properties is a modern advance. The notion of using nanoscale materials to target cancer seems like a paradox in scale. Nanoscale materials as defined by the US National Nanotechnology Initiative (NNI) range in size between 1 and 100 nm, while a typical solid tumor ranges between 1 and 4 cm in diameter. To understand this at volume scales we can understand, a comparison of throwing a rock at the moon is most appropriate. Furthermore, the complexity of delivering nanoparticles (passively or actively) to the tumor while maintaining their novel functionalities (e.g., stability, etc.) and avoiding quick removal by the immune system and other clearance mechanisms seemed a daunting task.

Nanotechnology had been utilized in therapeutics to target cancer as far back as the mid-1990s (Fig. 5.3). A handful of nanoscale delivery systems for the chemotherapy drugs paclitaxel or doxorubicin were approved by the US Food and Drug Administration (FDA) over the first 15 years. Nanometer size, liposomal delivery systems (Doxil™, approved in 1995; DaunoXome™, approved in 1996; and DepoCyt™, approved in 1999), a polymer-protein conjugate (Oncaspar™, approved in 2006), and albumin-bound nanoscale particles (Abraxane™, approved in 2005) rely on the delivery of their therapeutic loads directly to multiple cancer indications via passive delivery (e.g., enhanced permeability and retention (EPR) effect) [21, 22]. In both cases, they have expanded beyond their original use cases to other tumor types and indications as well as paved the way for a myriad of similar delivery

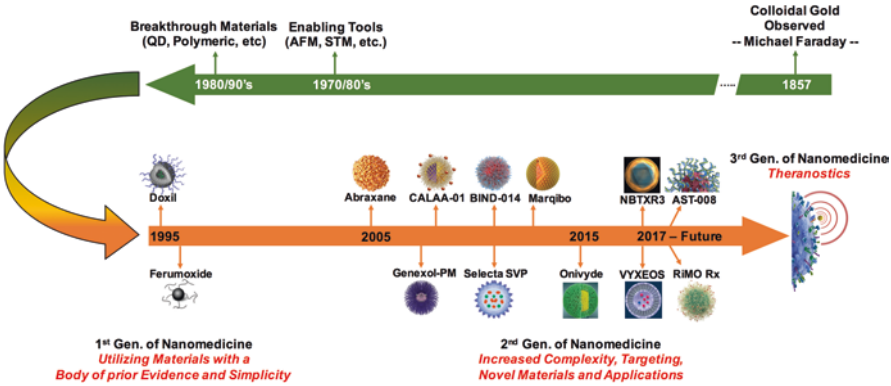

Fig. 5.3 Timeline of nanotechnology and subsequent use in cancer applications. The listed nanomedicines are a representative sample of the bulk of currently approved or in clinical trials

platforms (e.g., liposomal). While these are appropriate in reduction to patient toxicity relative to the free drug, their respective efficacy does not offer an improvement. Furthermore, relative to the potential that nanocarriers do have for multicomponent cargos, targeting, and more, they represented only the initial offering to display the potential impact of nanotechnology in cancer.

Discussion as to these initial first-generation offerings is best considered by way of an iterative process that has been requisite to advancing nanotechnologies for human use. The first aspect of this process can be formulated under the guise of unifunctional nanosystems for cancer. Both of these above cases, as well as many through to 2010, can be classified as novel systemic in vivo transporters of single traditional small-molecule therapies, albeit at the nanoscale. By no means does this detract from their impact to the field, although the benefits have been a reduction in toxicity and not an increase in efficacy over other drugs. Thus, the real impact was derived from fewer side effects for the patient during their dosing regimens. Furthermore, for a long period of time, these systems were much costlier and often not covered by insurance. Most of these latter points have been rectified as costs have decreased and can be covered by some insurance companies. Both were to be expected for the initial offerings of any drug which requires manufacturing and R&D costs for systems of higher complexity than ever before. But this is entirely the point that this has been an iterative process, beginning with what is less complex (e.g., liposomes carrying a single cargo) and building to more complex systems. The second aspect of the iterative process over the last 30 years can be discussed in terms of prior understanding of in vivo performance for nanotechnology, again, highlighting the above examples that were approved earlier on through to today. These systems already had a body of evidence to indicate their ability to be effectively used in human. Liposomes had been known and studied for many years (~1960s). Liposomes are already similar to biological species present in the human body and thus were more compatible to begin with, than other nanoscale materials. In essence, the hurdles to translation and clinical use were fewer. Simply stated, the

iterative process has been necessary and until more recently had not revealed the true potential nanotechnology, envisioned at the birth of the field, could offer to improve patient outcomes. With this understanding of the iterative process that started as far back as the early 1990s for delivery platforms that had a prior body of knowledge, one can then understand any perceived stagnation as to the progress in the field.

If we then look at this in historical context of a stepwise iterative process needed to deliver the platform technologies (e.g., materials, cargo, targeting moieties, etc.), by which we had very little in vivo knowledge of prior and/or are multifunctional in their purpose, the timeline can instead be begun in 2004. The task at hand required the interaction and strategic collaboration of multiple medical, scientific, and engineering disciplines. Although not a wholly new concept, this multidisciplinary approach was aided by models and programs that brought the concept of science and technology convergence for this common goal forward. The framework of this was defined by foundational principles of convergence integrating into various funding efforts [23]. Specifically, "Convergence is a transformation model in the evolution of science and technology (S&T) that unites S&T fields with society. It provides a framework and approach for advancing not only science and engineering but also business and policies. Convergence is a deep integration of knowledge, tools, and all relevant areas of human activity to allow society to answer new questions, to create new competencies and technologies, and overall to change the respective physical or social ecosystems." In 2004, the National Cancer Institute (NCI) formalized a large-scale funding effort, *the Alliance for Nanotechnology in Cancer*, dedicated to developing the next generation of nanotechnology-based therapeutics as well as in vitro and in vivo diagnostics. This ongoing initiative relies on a multidisciplinary community of researchers whom represent diverse disciplines covering the spectrum of clinicians to chemists. All were tasked with the mission of solving challenges in contemporary oncology by way of utilizing nanoparticles and/or nano-devices to overcome these challenges. It continues to be a holistic translational approach to drive the field to the next level. Included in the Alliance are large cancer nanotechnology centers of excellence (CCNE), individual basic research projects (CNPP/IRCN), and training centers (CNTCs) across multiple institutions with a fundamental requirement of synergy between all components within each individual center or project, as well as across the Alliance. Included in this initiative is the Nanotechnology Characterization Laboratory (NCL, https://ncl.cancer.gov), which acts as public resource offered by NCI. It provides researchers and private entities a uniform set of assays to measure in vivo aspects of their platform technology by way of an objective body of tests while providing feedback to both developer and regulators. The NCL co-exists as a formal collaboration of the NCI, the National Institute of Standards and Technology (NIST), and the FDA to develop and apply a consistent set of standardized characterization assays of nanomedicines to facilitate successful clinical translation and subsequent commercialization. The NCI effort has been quite successful of which the fruits of much of the Alliance's efforts are just starting to ripen. The other components include an external and internal steering committee, a data sharing public resource (Cancer Nanotechnology Laboratory data

portal, https://cananolab.nci.nih.gov/caNanoLab/), and other resources aimed at translation and strategic guidance [24–27].

From purely a numerical perspective, scientific output of the initiative has resulted in over 3700 peer-reviewed publications (cited over 88,000 times) and over 100 start-up companies formed or utilized as commercial outlets to more than 220 patents and disclosures (Fig. 5.4) (https://www.cancer.gov/sites/ocnr). The NCI Alliance program established a successful model of employing government funds for initial stages of development and translation of research in nanomedicine. Its principal investigators have successfully leveraged these public monies to obtain millions of dollars from venture capital and philanthropic sources driving these technologies to even more mature stages. The Alliance has been viewed as a success story from the perspective of the research and technology produced as well as the perspective of science and technology convergence. This model for coupling the rapid development of novel technologies and their eventual implementation into clinical practice can be recognized through a myriad of examples including many in this book and elsewhere [23, 28].

Fig. 5.4 NCI Alliance for Nanotechnology in Cancer program scientific and translational output from 2005–2017. (Copyright 2017 National Cancer Institute)

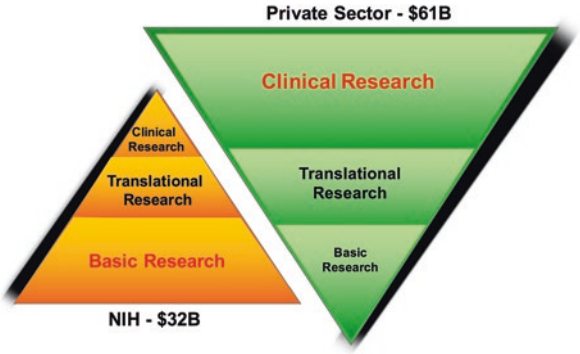

Fig. 5.5 Generalized structure of NIH public funding versus private sector funding for basic, translational, and clinical research. US dollar values represent FY2016 funding levels

It would be disingenuous to impress that the public funding of nanoscale research for cancer applications have been the sole driving force. Indeed, it has been an accelerating aspect of a field that was already under way within the private sector. The acceleration operating in the very traditional sense of the National Institutes of Health (NIH), which has been to deliver public funding for expanding the knowledge base in medical and associated sciences enhancing fundamental discoveries and innovative research strategies. This effort is targeted often in an inverse manner as to the biotech and pharmaceutical industries, as displayed in Fig. 5.5. Furthermore, it would be misleading to ignore the many major advances to the clinic that have been made over the last decade that were not driven directly by public funding [29]. Undeniably, many platforms have advanced through clinical trials and/or just recently beginning to see the light of day for clinical approvals. These recent successes display the maturity of the field as it extends into the real clinical potential of nanotechnologies for cancer and is showing signs of critical mass. There are several examples of these, which include:

(i) *Vyexos CPX-351*, *formally owned by Celator and now Jazz Pharmaceuticals*, is a liposomal formulation of cytarabine:daunorubicin (5:1 molar ratio). They have successfully completed Phase III/IV clinical trials, and Vyxeos™ (cytarabine and daunorubicin) was approved by the FDA in August 2017 for the treatment of acute myeloid leukemia (AML). The novelty again that is presented in this nanomedicine is the safe and effective delivery of a drug combination.

(ii) *NBTXR3*, *by Nanobiotix*, is a first-in-class radio-enhancer that could be applicable to most solid tumors. The platform is a hafnium oxide nanoparticle that relies on intratumoral injection and is currently in multiple clinical trials in several countries. It has had several successful outcomes to date and represents one of very few inorganic (e.g., non-liposomal)-based nanomedicines in the clinic. Furthermore, the company had recent revelations that included enhanced downstream abscopal effects delivered with the addition of their platform versus conventional radiotherapy.

(iii) *MM-310*, *by Merrimack Pharmaceuticals,* relies on the lessons learned from their previous successful approved drug, Onivyde, for pancreatic cancer. MM-310 is an antibody-directed nanotherapeutic (ADN) which delivers a

novel prodrug of the chemotherapy, docetaxel, encapsulated within an ephrin receptor A2 (EphA2)-targeted liposome. EphA2 receptors are shown to be overexpressed in several solid tumors, including prostate, ovarian, bladder, gastric, pancreatic, and lung cancers. Albeit they have just begun Phase I trials (March 2017), this platform will rely on their previous lessons learned from a recent Phase III clinical trial for MM-302 (HER2-targeted liposomal doxorubicin). Thus, MM-310's benefits will remain as the next clinical demonstration of a targeted cancer nanomedicine.

5.5 Segue into the Future for Nanoscale Platforms, Multifunctionality

Now in 2017, the field itself has begun to expand to multiple sub-disciplines as shown by the diversity of the target applications above. The platform technologies in late-stage clinical trials have begun to increase in their complexity as of late (i.e., second-generation platforms in Fig. 5.3). Additionally, when comparing to other novel therapeutic modalities, two points must be remembered beyond the scope and challenges presented for translation of nanotechnologies for cancer: (1) investments of up to $2.6 billion and 20 years of development often accompany high impact drugs prior to approval [30, 31]; and (2) correlations can be made to the development and first approvals for monoclonal antibodies (Fig. 5.6). Publications for these

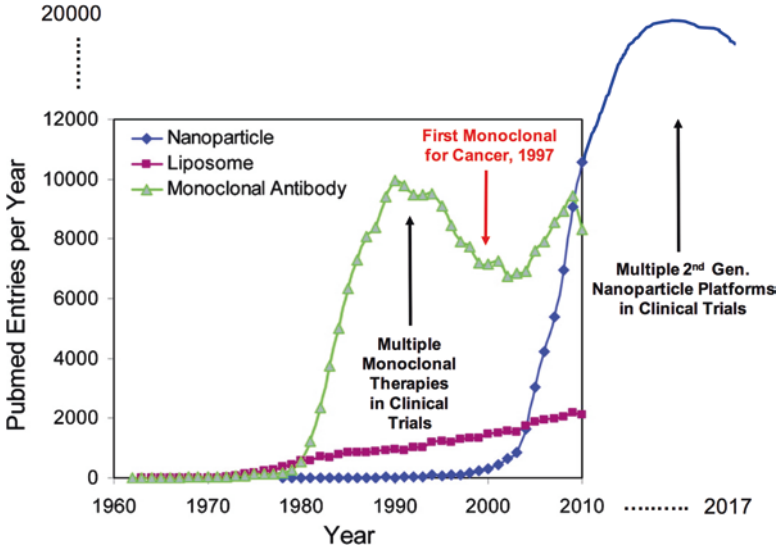

Fig. 5.6 PubMed entries for nanoparticles, monoclonal antibodies, and liposomes with extension of nanoparticle entries post-2010. (Adapted with permission from Shi et al. 2011. Copyright 2011 American Chemical Society)

game-changing therapeutics spiked in the 1990s, and a critical mass was achieved soon after (e.g., first for cancer, rituximab, in 1997). The time from initial body of research and development to approved product was >20 years [32]. Nanoparticle platforms publications have recently peaked and plateaued, coupled with several successful clinical trials and approvals of second-generation platforms. Furthermore, one can argue the complexities and challenges faced at translating non-liposomal nanotechnologies are much greater and that the body of in vivo evidence was close to zero in the late 1990s. Albeit an argument based off anecdotal information, it does give hope that the field is starting to mature enough to push forward its own game-changing therapeutics and diagnostics. Though, to date, no theranostic platforms are available for clinical use, this iterative process will continue, and the platform technologies will evolve as such with fewer hurdles than their predecessors.

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Part II
Cancer Nanodiagnostics and Nanotherapeutics

Chapter 6
In Vitro Cancer Diagnostics

Jung-Rok Lee, Chin Chun Ooi, and Shan X. Wang

6.1 Introduction

It has been estimated that the human body consists of about 37 trillion cells [1]. These cells each have their own metabolic and proteomic profile, and yet, multiple types of cells with vastly different functions can remain highly coordinated in ensuring homeostasis and organ-level or system-level viability. Thus, understanding neoplasia in this highly complex environmental system is very difficult but necessary. For example, the interaction of tumor cells with neighboring cells such as stroma cells within the tumor microenvironment is extremely significant to understanding tumor growth and development. Therefore, to precisely understand tumorigenesis, it is highly desirable to investigate tumors in the body, via so-called in vivo studies. In vivo studies refer to the testing of biological hypotheses within or on a living organism, and a good example of in vivo studies is imaging, via techniques such as magnetic resonance imaging (MRI) and X-ray computed tomography (CT) scans. While more representative of the complex cancer biology, in vivo research on humans is largely restricted due to ethical issues. The many regulations required to ensure the safety of various reagents or interventions can complicate in vivo studies,

J.-R. Lee
Division of Mechanical and Biomedical Engineering, Ewha Womans University, Seoul, South Korea
e-mail: jungrok@ewha.ac.kr

C. C. Ooi
Department of Chemical Engineering, Stanford University, Stanford, CA, USA
e-mail: ooicc@stanford.edu

S. X. Wang (✉)
Department of Materials Science and Engineering and Electrical Engineering, Stanford University, Stanford, CA, USA
e-mail: sxwang@stanford.edu

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but considerable efforts have still been made to achieve progress, and they will be further discussed in the next chapter (Chap. 7). As an alternative to human in vivo studies, researchers in oncology have successfully used mouse models to more easily manipulate and test tumorigenesis by alterations and therapeutic interventions, thus furthering our collective understanding of the fundamentals of tumorigenesis [2]. Although mice and other animal models can be established to approximate humans in terms of their genetic, biological, and behavioral characteristics, there remain species-specific issues that impede findings in mouse and animal models from being directly translatable to humans.

Relieving researchers from ethical issues, ex vivo studies are another approach in oncology. Ex vivo indicates biological processes are tested outside a living object and is often synonymously used with in vitro, which literally means in glass. While the definitions of in vivo and ex vivo are based on the location of the test sample, the term in vitro is rooted more in how experiments are conducted, especially since studies with microorganisms, cells, or biological molecules have traditionally been done in glassware such as test tubes, flasks, or petri dishes. In vitro studies in oncology mainly include (1) studies involving biological molecules like DNA and proteins, such as in genomics and proteomics, (2) experiments with human cancer cell lines, and (3) tissue-level research. All of these studies are intimately related to cancer diagnosis because their findings from patients' clinical samples can and are frequently used to define biomarkers.

A biomarker refers to a measurable indicator of a particular biological condition or process. For example, skin color can be a biomarker for jaundice, but it will be very non-specific because skin color can be affected by other factors. There are many different forms of cancer biomarkers, including physiologic measurements, images, molecular signatures such as gene or protein signatures, and cell-based markers [3]. In the context of cancer, cancer biomarkers are critical for identifying the presence of cancer (diagnosis), predicting the right drug, and monitoring therapeutic response. Since the survival rates of cancer patients can be greatly improved when they are diagnosed earlier [4], researchers in oncology have been searching for highly specific and sensitive biomarkers for cancer diagnosis and prognosis over the past several decades. Critically, the specificity of these biomarkers can vary tremendously between cancer types, as they are mainly determined by specific biological processes in neoplasia or activated biological pathways. Conversely, the sensitivity of measurement tools can often define how distinctly the biomarkers are expressed in cancer patients compared to healthy controls and, hence, the sensitivity of these biomarkers. If a new technology can precisely differentiate a subtle difference in a biomarker, the biomarker becomes more promising and reliable. As detection of biomarkers is usually technology-driven, nanotechnology has been successfully utilized in cancer diagnosis because these nanostructures are on similar length scales to, and thus can effectively interact with, cellular and sub-cellular components and even molecular entities [5]. The ultimate goal of diagnosis technique based on nanotechnology would be that in vitro tests can be routinely performed to predict or diagnose multiple types of cancer in regular checkups as easily as pregnancy tests.

Indeed, a commonly cited motivation for nanotechnology-based approaches is the potential for miniaturization and automation to reduce the need for a laboratory and hence enable point-of-care cancer diagnosis [6, 7]. For example, the use of point-of-care breath tests for breast cancer diagnosis could resolve difficulties with proper access due to poverty while also reducing radiation exposure due to the typical mammography [8].

In this chapter, we will review the basic concepts of diagnosis and nanotechnology-based in vitro diagnostic modalities.

6.2 Diagnostic Tests

6.2.1 Sensitivity and Specificity

Since biomarkers are measurable quantities, they can be represented by numeric values. For instance, CA-125 is a protein biomarker of ovarian cancer, which is widely used to monitor cancer recurrence [9], and its concentration in serum is a quantitative entity. Thus, its concentration can be used for diagnostic tests of cancer recurrence. However, the concentration of a protein biomarker is not always proportional to tumor size or indicative of disease stage or progression because of heterogeneity between individuals (e.g., different expression levels or shedding rate into blood circulation), within the same individual (e.g., different nutrient levels or metabolic changes), or measurement variations (e.g., temperature fluctuation and device variations). In addition, if the same protein is simultaneously being produced by healthy cells, there will be a distribution of background levels, which makes it extremely difficult to detect subtle changes induced by a small mass of tumor. An ideal biomarker should be generated only by cancer cells, like in the case of the protein biomarker, HBsAg (surface antigen of the hepatitis B virus), which is present in the sera of patients only upon viral hepatitis B infection [10]. In such instances, it can be relatively easy to determine a cutoff or threshold to separate patients (positives) from healthy (negatives) as shown in Fig. 6.1a, c. Since cancer cells originate from our own cells, however, the biomarkers produced by cancer cells are typically also generated by healthy cells but with minor differences. In such cases, due to all the sources of variation discussed above, both healthy individuals and cancer patients will have distributions of biomarker quantities with significant overlaps, as shown in Fig. 6.1b, d. Therefore, the selection of any discrimination threshold inadvertently results in undesirable subpopulations of false positives (FP, healthy people misdiagnosed as cancer patients) and false negatives (FN, cancer patients misdiagnosed as healthy). Thus, while we endeavor to find ideal cancer biomarkers with perfect separation between cancer patients and healthy controls, researchers and clinicians typically have to deal with determining an optimal discrimination threshold for biomarkers with overlap between healthy and patient populations, so as to maximize true positives (TP, patients diagnosed as positive) and true negatives (TN, healthy diagnosed as negative) and minimize FP and FN.

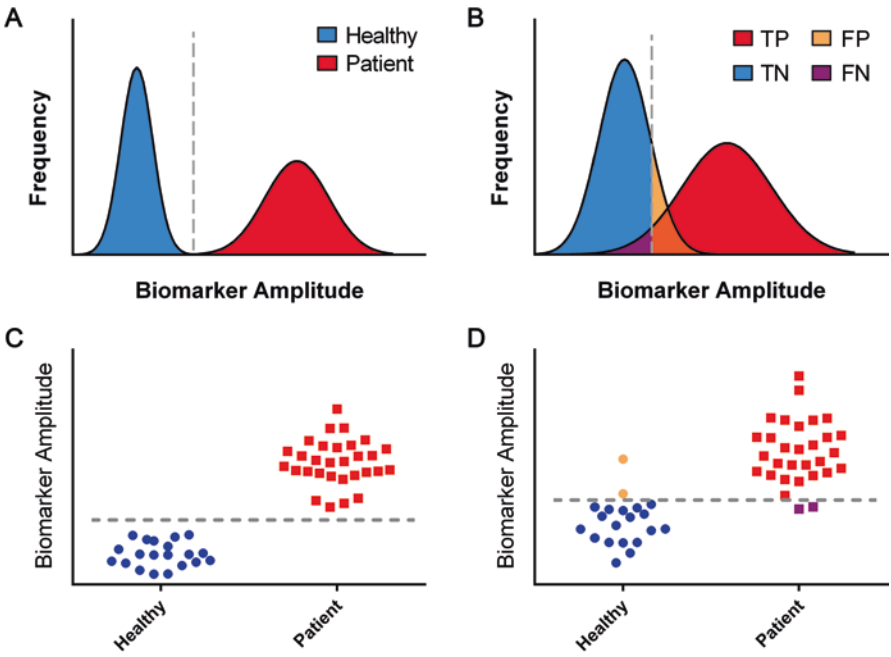

Fig. 6.1 Distributions of biomarker amplitude and discrimination threshold. (**a**) Ideal distributions of biomarker amplitude in patients and healthy controls. (**b**) Distributions of biomarker amplitude in general. TP true positive, FP false positive, TN true negative, and FN false negative. (**c**) Example of dot plots of an ideal case. (**d**) Example of dot plots of a general case. The gray dashed lines represent discrimination thresholds

To discuss these aspects more quantitatively, we can introduce two definitions widely used in diagnostic tests using biomarkers. Sensitivity and specificity (often called clinical or medical sensitivity and specificity because they are different from the sensitivity of an assay, i.e., signal output per input) are basic terminologies that define the probabilities of patients being tested as positive and healthy people being tested as negative, respectively, for a given test. Thus, higher sensitivity and specificity are better, and an ideal test achieves both sensitivity and specificity of one.

$$Sensitivity = \frac{TP}{TP + FN}$$

$$Specificity = \frac{TN}{TN + FP}$$

Although higher sensitivity and specificity are always desired, it is quite difficult to estimate the societal costs of a diagnostic test from these values alone. For example, a test with sensitivity of 1 (100%) and specificity of 0.95 (95%) might be promising because it can identify every single patient and generates false positives at a rate of

only 5%. However, if the prevalence (the proportion of living people estimated to have the disease) of the disease is too low (true for most cancer cases), a considerable number of healthy people will be misdiagnosed as positive, which leads to substantial societal and medical costs. To evaluate these aspects, we need to introduce two more terms, positive predictive value (PPV) and negative predictive value (NPV). While sensitivity and specificity are intrinsic properties of the test for a given threshold, PPV and NPV are dependent on the prevalence of the disease. PPV and NPV can be interpreted as the probabilities of tested positives being correct and tested negatives being correct, respectively. In the case of a high specificity (more than 95%) diagnostic test for a low prevalence (less than 0.1%) disease, PPV is still extremely low [11], because it is mainly determined by the prevalence, with many instances of FP from the test.

$$\text{PPV} = \frac{TP}{TP + FP}$$

$$\text{NPV} = \frac{TN}{TN + FN}$$

6.2.2 Receiver Operating Characteristic (ROC) Curve

Sensitivity, specificity, PPV, and NPV can provide overall insight into how good a diagnostic test is, but they can be used as a basis to evaluate the test only after a discrimination threshold is selected for the test. How then can we determine an optimal discrimination threshold (or cutoff) for a test? There is a graphical way to visualize the performance of a binary classifier system. A receiver operating characteristic (ROC) curve plots *Sensitivity* of the test, also known as the true positive rate (TPR), versus $1 - Specificity$ (the false positive rate [FPR]), as the discrimination threshold of the test varies from the minimum of measured biomarker values to the maximum. As an example, a ROC curve is plotted in Fig. 6.2a for the data shown in Fig. 6.1d. The most common way to interpret the ROC curve is to calculate the area under the curve (AUC). This single number is a fair indicator of the overall goodness of the test, and a perfect test yields an AUC of 1 with 100% sensitivity and specificity. Flipping a coin corresponds to the diagonal line with an AUC of 0.5. Since a ROC curve shows all pairs of sensitivity and specificity for various thresholds, an optimal threshold can be graphically selected with the ROC curve. Simply, the best threshold maximizes both sensitivity and specificity, which is equivalent to maximizing $Sensitivity + Specificity$. This term is closely related to Youden's index ($Sensitivity + Specificity - 1$), which corresponds geometrically to the length of a vertical line between the ROC curve and no-discrimination line (diagonal line). However, sensitivity and specificity might not be weighted equally for certain diagnostic tests and diseases. For example, sensitivity should be heavily weighted when

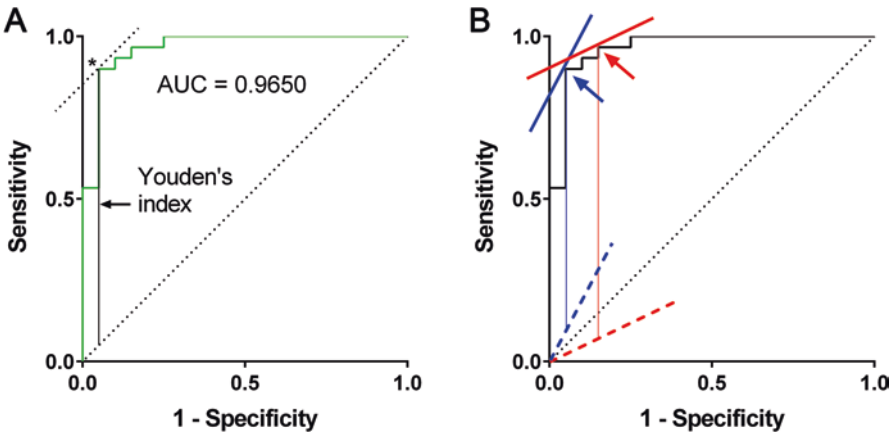

Fig. 6.2 Receiver operating characteristic (ROC) curve. (**a**) ROC curve of data shown in Fig. 6.1d. AUC area under curve. The asterisk indicates sensitivity and 1-specificity at an optimal discrimination threshold. (**b**) Determination of optimal discrimination thresholds. The blue arrow indicates a specificity-weighted discrimination threshold, while the red indicates a sensitivity-weighted threshold

screening for highly contagious and lethal diseases as it is critical to catch every single carrier, even if the test produces more false positives. On the other hand, as we discussed earlier, specificity can be prioritized for diseases with a low prevalence to reduce related costs for follow-up examinations of FP cases. Hence, for every disease, even though it is not easy to precisely estimate the appropriate weighting factors (m,n), a point that can maximize $m \bullet Sensitivity + n \bullet Specificity$ will be the optimal threshold for the test. If sensitivity is more important ($m > n$), a point on a line with a slope of n/m will be the optimal threshold, as indicated by a red arrow in Fig. 6.2b.

Cancer biomarkers approved by the Food and Drug Administration (FDA) are summarized in Table 6.1. To date, for most forms of cancer, we have not found a single biomarker that can precisely diagnose cancer on its own. Hence, it is extremely common to combine multiple biomarkers to further increase sensitivity and specificity and will probably continue being so in the future, e.g., multiple protein biomarkers in the blood or multiple forms of biomarkers including a specific gene and/or protein signature and images [12]. This approach requires multivariate analysis, and ROC curves can still be used if a score (multivariate index) is calculated based on multiple biomarker quantities. A recent FDA-approved blood test, OVA1 test, utilizes five ovarian cancer biomarkers (CA-125, beta-2 microglobulin, transferrin, apolipoprotein A1, and transthyretin) to help evaluate the risk of ovarian cancer in patients with an adnexal mass planned for surgery. This test generates an OVA1 score by combining the levels of those biomarkers, and it has been reported that the OVA1 test has better sensitivity than CA-125 alone [13]. In addition to protein biomarkers, counting circulating tumor cells (CTCs) or circulating cancer stem cells (CCSCs) have been studied, and DNA and microRNA (miRNA) are also

Table 6.1 List of FDA-approved cancer biomarkers

Biomarker	Clinical use	Cancer type	Year first approved or cleared
Pro2PSA	Discriminating cancer from benign disease	Prostate	2012
ROMA (HE4 + CA-125)	Prediction of malignancy	Ovarian	2011
OVA1 (multiple proteins)	Prediction of malignancy	Ovarian	2009
HE4	Monitoring recurrence or progression of disease	Ovarian	2008
Fibrin/ fibrinogen degradation product (DR-70)	Monitoring progression of disease	Colorectal	2008
AFP-L3%	Risk assessment for development of disease	Hepatocellular	2005
Circulating tumor cells (EpCAM, CD45, cytokeratins 8, 18+, 19+)	Prediction of cancer progression and survival	Breast	2005
p63 protein	Aid in differential diagnosis	Prostate	2005
c-Kit	Detection of tumors, aid in selection of patients	Gastrointestinal stromal tumors	2004
CA19–9	Monitoring disease status	Pancreatic	2002
Estrogen receptor (ER)	Prognosis, response to therapy	Breast	1999
Progesterone receptor (PR)	Prognosis, response to therapy	Breast	1999
HER-2/neu	Assessment for therapy	Breast	1998
CA-125	Monitoring disease progression, response to therapy	Ovarian	1997
CA15–3	Monitoring disease response to therapy	Breast	1997
CA27.29	Monitoring disease response to therapy	Breast	1997
Free PSA	Discriminating cancer from benign disease	Prostate	1997
Thyroglobulin	Aid in monitoring	Thyroid	1997
Nuclear mitotic apparatus protein (NuMA, NMP22)	Diagnosis and monitoring of disease	Bladder	1996
Alpha-fetoprotein (AFP)	Management of cancer	Testicular	1992
Total PSA	Prostate cancer diagnosis and monitoring	Prostate	1986
Carcinoembryonic antigen	Aid in management and prognosis	Not specified	1985
Human hemoglobin (fecal occult blood)	Detection of fecal occult blood	Colorectal	1976

Adapted from Ref. [11]

promising targets for cancer diagnosis [14, 15]. In fact, there have been multiple studies discussing the ability for miRNA profiles to distinguish between different cancers and for diagnosis of different carcinomas such as lung cancer or prostate cancer [15–17]. Thus, they can be considered as one of the entities in multiple biomarker tests.

As new and powerful analytical technologies emerge in genomics and proteomics, more cancer biomarkers are continually being identified. As a result, more tests with various combinations will be available in the future. To evaluate each test accurately, there are several precautions that researchers need to take [18]. First and foremost, studies should be well-designed to avoid a bias in selecting patient population and healthy controls. In addition, the measurement of biomarkers and related protocols, including post-processing, analytical methods, and sample collection processes, should be robust and reliable. As new promising nanotechnologies are developed and applied to cancer diagnostics, these aspects become more critical for successful clinical translation of these techniques.

In the next section, various in vitro modalities currently available for cancer diagnosis will be discussed, along with a brief description of their embodiments in various novel nanotechnologies and their potential for clinical translation.

6.3 Diagnostic Modalities

6.3.1 Molecular Diagnostic Tools

It took about 50 years after Watson and Crick discovered the molecular structure of nucleic acids in 1953 [19] to finally obtain the first complete human genome in 2001 [20, 21]. However, barely a decade after the Human Genome Project was completed, the 1000 Genomes Project Consortium completed the sequencing of more than 1000 reference human genomes to provide a foundation for identifying variants related to diseases [22]. Now in 2016, it has become feasible for a group of researchers to initiate challenging projects to construct or synthesize large-scale genomes beyond editing and manipulation [23]. These great achievements in genetics relative to biology and medicine, since the human genome was first sequenced, were accelerated largely by substantial advances in technology development, especially in nanotechnologies.

Modern DNA sequencing began with the Sanger method developed in 1975 [24] and was subsequently boosted by the introduction of the shotgun strategy [25]. With improvements to computational algorithms and techniques for attachment of fluorescent dyes, the first automated DNA sequencing machine was developed in 1987 and subsequently became a successful and popular tool for sequencing genomes at various scales until replaced by next-generation sequencing (NGS) technologies such as sequencing by synthesis (Fig. 6.3). Enabled by these technological advances, oncologists have identified mutated genes that cause cancer, so-called oncogenes,

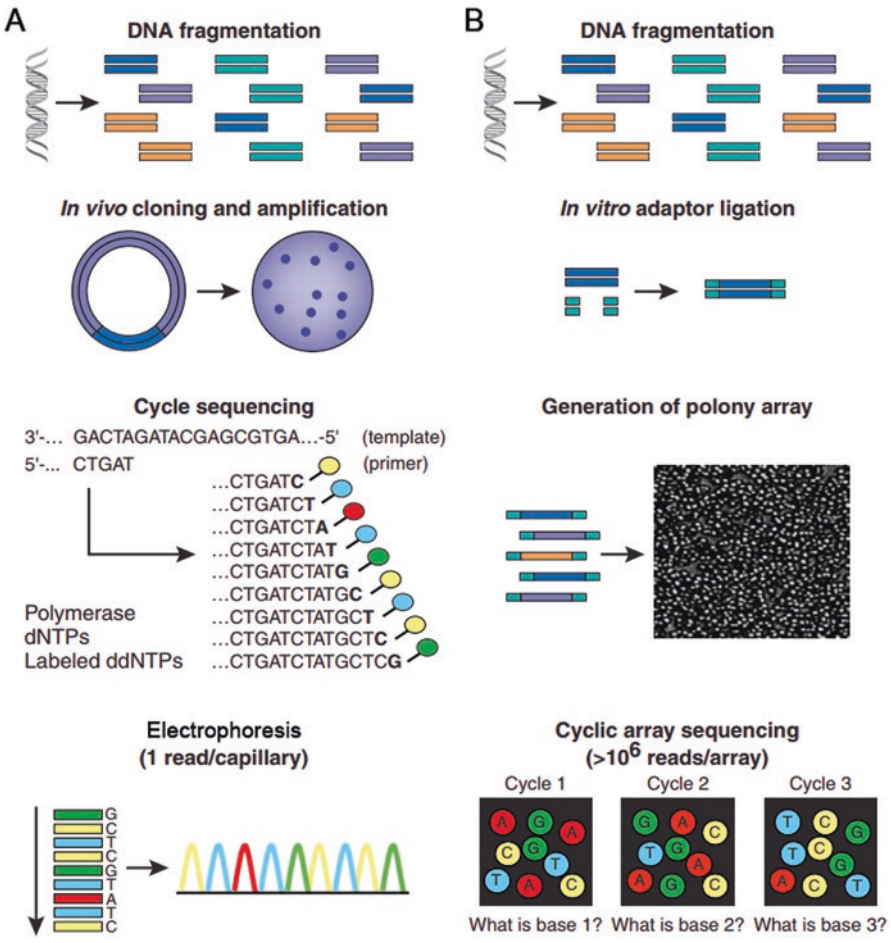

Fig. 6.3 The Sanger method and NGS. (**a**) Work flow of shotgun Sanger sequencing. Fragmented DNA is cloned to a plasmid vector and transfected into *E. coli*. Each cycle of sequencing generates fragments terminated with fluorescently labeled dideoxynucleotides (ddNTPs). With electrophoretic separation, each fragment is read via the four-channel emission spectrum. (**b**) Work flow of NGS. Fragmented DNA is ligated with adaptors and then immobilized on a surface. Each immobilized DNA fragment is amplified via bridge amplification technique to form PCR colonies. Through cyclic array sequencing, fluorescence signals are monitored upon incorporation of fluorescently labeled reagents to the spatially separated PCR colonies. (Adapted from Ref. [37])

and their pathways [26]. However, only a small fraction of the genes had been analyzed until 13,023 genes in 11 breast and 11 colorectal tumors were sequenced in 2006 [27]. Currently, there are two main projects to compile comprehensive cancer genomes: The Cancer Genome Atlas (TCGA) and the Cancer Genome Project (CGP). Although whole genome sequencing for cancer can reveal genetic alterations responsible for tumorigenesis including sequence variants like mutations, insertion, and deletion as well as structural changes such as chromosomal transloca-

tions, fusion genes, and copy-number variations, researchers have also had success with much more targeted approaches such as whole exome sequencing (WES) and transcriptome sequencing (mRNA-seq) for individual patients [28, 29].

In addition, sequencing has also been applied to the study of epigenetics as the relationship between DNA modifications and cancer becomes more apparent [30, 31]. For example, recent work has demonstrated a clear link between epigenetic changes in circulating tumor DNA and hepatocellular carcinoma [32] and the effectiveness of nanotechnology-based platforms for such epigenetic analysis [33, 34].

However, conventional NGS techniques, especially Illumina-based sequencing, remain heavily reliant on fragmentation, amplification, and computational assembly and can only read short fragments under 1000 base pairs. Consequently, deep sequencing is required to ensure a high degree of statistical confidence, and it is impractical for whole genome or transcriptome sequencing to be implemented for diagnostic tools. However, targeted sequencing of specific genes (or biomarker genes) can be very efficient for the purposes of screening and diagnosis. For example, the identification of a BRCA mutation (in BRCA1 and BRCA2 genes) alone is sufficient to screen for women at higher risk of ovarian and breast cancer, while other RNA-based panels have shown promise in cancer diagnosis [35, 36].

In recent years, alternative nanotechnology-based sequencing techniques have shown great potential for reducing the material and time costs of sequencing and improving the ability to obtain long reads, which would greatly enhance the attractiveness of sequencing as a routine tool for diagnosis and therapy selection and evaluation (Fig. 6.4). The first method is single-molecule real-time (SMRT) sequencing [38] where a single polymerase molecule is attached in an optically designed nano-chamber, called zero-mode waveguides (ZMW) [39], and polymerization signals are monitored in real time. The read length of this technique is more than 1000 base pairs (1 kb). The second approach is nanopore-based sequencing [40]. In this approach, an electrical bias is applied across a membrane with a nanoscale hole, and as a strand of DNA passes through the gap, the current will vary with each unique nucleotide. The beneficial features of this technique include the removal of amplification, labeling, and polymerization steps from the sequencing process and the ability to obtain longer read lengths (more than 1 kb). In addition, due to its inherent portability based on electrical readouts, a USB-memory-stick-sized sequencer based on this technique is commercially available and has been used for cancer research [41].

For proteomic biomarkers, analytical tools and diagnostic techniques can be extremely varied and specialized. Typically, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS) have been predominantly used to identify biomarkers, while immunoassays are the key technique in detection of biomarkers [42]. Immunoassays are biochemical tests that measure the presence or concentration of a biomolecule of interest by using antibodies to specifically recognize the target biomolecule and labels attached to the antibodies to produce quantitative signals. The first immunoassay was developed with radioactive labels in 1960 [43]. Due to concerns about radioactivity, the radioimmunoassay was supplanted by the enzyme-linked immunosorbent assay (ELISA) developed in 1971

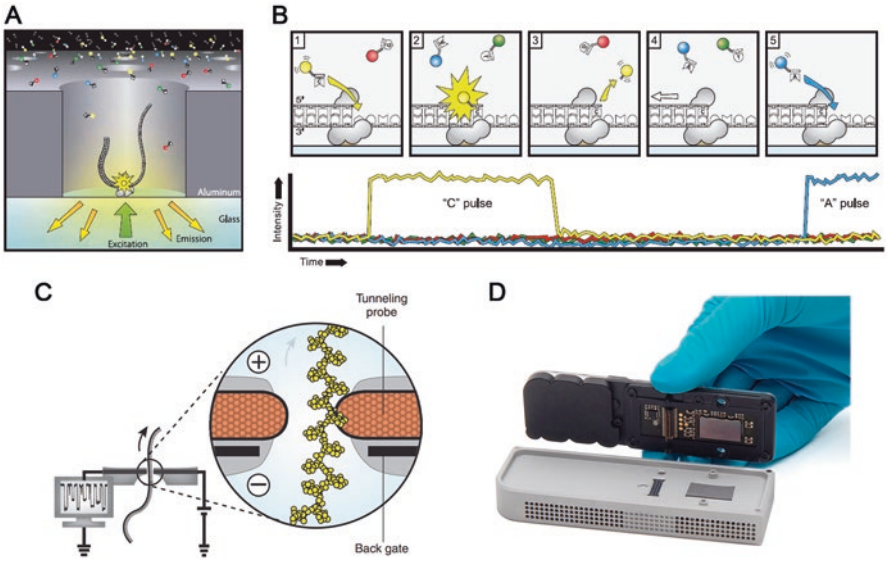

Fig. 6.4 Nanotechnology-based sequencing techniques. (**a**) Schematic of single-molecule real-time (SMRT) sequencing. A single DNA polymerase is tethered at the bottom of a zero-mode waveguide (ZMW). The ZMW is illuminated from below, and confined excitation produces local signals from fluorescently labeled nucleotides upon incorporation into the DNA strand. (**b**) Sequence of SMRT sequencing. A phospho-linked nucleotide is incorporated into the template DNA strand and produces fluorescence signals. Phosphodiester bond formation releases the dye-linker-pyrophosphate product. This product diffuses out of the ZMW, which results in a drop in fluorescent signal. The template DNA strand is translocated to the next position, and the next nucleotide is incorporated. (Adapted from Ref. [38]). (**c**) Nanopore-based sequencing technique using transverse electrodes. The tunneling current through the nucleotides varies as the DNA strand is driven through the nanopore by electrophoretic force. (Adapted from ref. [40]). (**d**) Miniaturized nanopore-based sequencing device. A picture of MinION. (Adapted from https://www.nanoporetech.com)

[44]. In ELISA, enzymes, typically horseradish peroxidase (HRP), are used as labels and produce observable color changes by reacting with substrates or reagents, with these color changes being proportional to analyte concentration. Immunoassays are widely used in numerous clinical tests, including cancer biomarkers, and are the most common technique of measuring proteins, albeit in many variants with different labels depending on their transduction mechanisms. For example, in fluorescent or electrochemiluminescent assays, the labels are fluorophores like green fluorescent protein (GFP) or phycoerythrin (PE) and ruthenium complexes, respectively, with correspondingly different transduction mechanisms from ELISA. In addition, protein microarrays, in the style of DNA microarrays [45], have also been developed to improve multiplexing capability and have been successfully applied toward cancer [46]. Furthermore, immunoassays can be employed in different formats such as forward-phase assays and reverse-phase assays (Fig. 6.5). The forward-phase assays are typically used to detect one or more analytes in the same sample, while

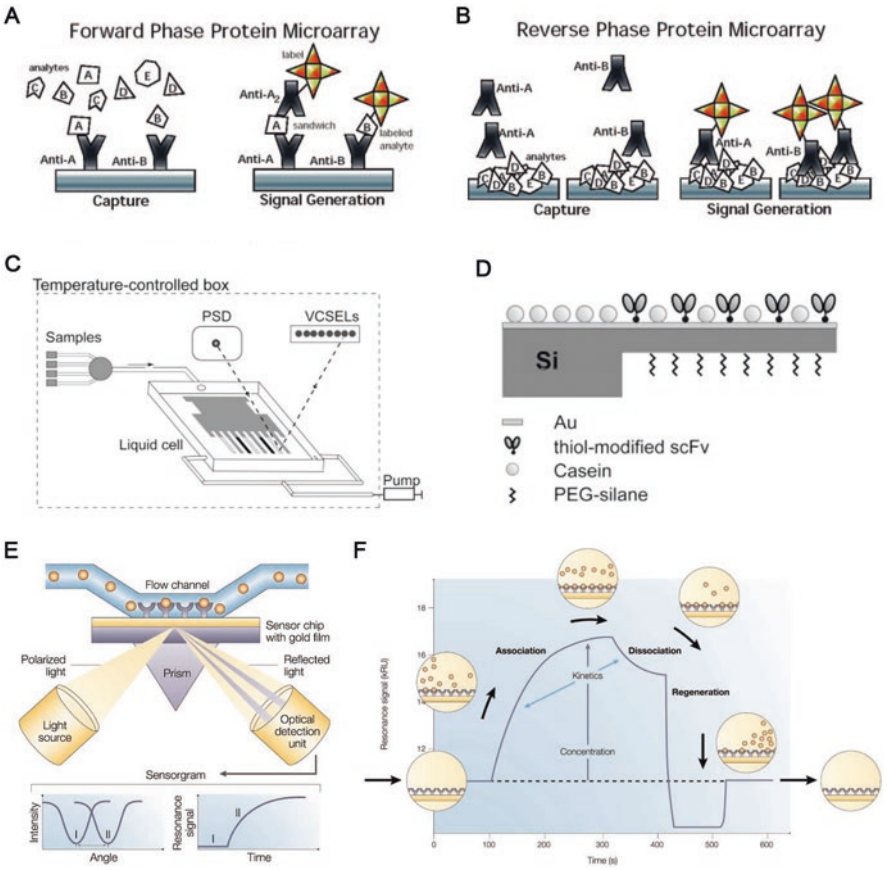

Fig. 6.5 Formats of immunoassays and label-free assays. (**a**) Forward-phase assays. Different recognition molecules, typically antibodies, are immobilized on the surface, and a single sample containing multiple analytes is incubated with them. Signaling labels can be brought to the analytes by either adding another recognition molecule conjugated with the labels or directly conjugating the labels with the analytes before the incubation. (**b**) Reverse-phase assays. Multiple samples are printed on the solid phase, and then recognition molecules are added. Signaling labels can be conjugated with the recognition molecules. (Adapted from Ref. [49]). (**c**) Measurement setup of microcantilever-based biosensors. The laser from vertical-cavity surface-emitting lasers (VCSELs) is aligned on the surface of the microcantilever to position the reflected laser to a position-sensitive detector (PSD). An eight-microcantilever array is immersed in a liquid cell where samples are introduced using a syringe pump. (**d**) Functionalization of microcantilever. A gold layer on the top surface of the cantilever is functionalized with single-chain Fv (scFv). The cantilever is further blocked with casein and PEG-silane. (Adapted from Ref. [47]). (**e**) Schematic of SPR measurement. The analytes of interest are flowed into the flow channel in contact with a layer of gold film on the chip. The gold film is functionalized with recognition molecules. Upon binding of analytes to the recognition molecules, the resonant angle in the reflected light from the bottom side is shifted from I to II. The light intensity measurement at a fixed angle produces resonance signals which are proportional to binding events. (**f**) A typical SPR sensorgram. The gold surface is functionalized with recognition molecules, and analytes of interest are introduced at t = 100 sec. An association curve is obtained as analytes bind to the recognition molecules. After t = 300 sec, a buffer solution without analytes is flowed over the surface, and a dissociation curve is recorded as bound analytes are released from the recognition molecules. After a proper duration of the dissociation curve is acquired, remaining bound analytes are stripped off through regeneration steps. (Adapted from Ref. [48])

the reverse-phase assays normally measure one analyte in multiple samples. There is another class of immunoassays that do not require labels, the so-called label-free immunoassays. Upon binding of analytes to antibodies on their surfaces, microcantilever-based biosensors utilize magnitude of deflection of the microcantilever (Fig. 6.5) as a detection mechanism [47], while surface plasmon resonance (SPR) detects changes in refractive index [48].

Nanotechnologies also play important roles in improving the sensitivity and multiplexing capability of assays for protein-based cancer diagnostics (Fig. 6.6). The most common technique employed is microfluidic or nanofluidic scaffolds that can compartmentalize or accommodate multiple assays or samples on a single platform [50]. While most immunoassays are based on optical techniques, magnetic immunoassay biosensors have been developed which take advantage of bio-samples naturally being non-magnetic and the consequently low background noise [51]. In the magnetic immunoassay, the label is typically magnetic nanoparticles (MNPs), and magnetic biosensors are implemented to detect these bound MNPs. In addition, there is another type of label-free assays based on nanowires, which manifest changes in conductance upon binding of proteins to them [52].

Another branch of nanotechnology in cancer diagnostics is nanoparticle-based protein detection [53]. By combining magnetic particles and gold nanoparticles encoded with DNA, highly sensitive detection of prostate-specific antigen (PSA) has been demonstrated [54]. Another study has demonstrated that gold nanoparticles can be clustered via a protein corona and its binding to relevant autoantibodies, and the test using this technique is able to detect early-stage prostate cancer [55]. Formation of protein corona has also been employed to detect pancreatic cancer with lipid nanoparticles to collect proteins from patients [56]. Furthermore, quantum dots (QDs) have been used to measure lung cancer biomarkers as fluorescent labels instead of traditional organic dyes due to their excellent optical properties such as larger molar absorption coefficients and longer fluorescence lifetimes when compared to organic dyes [57].

6.3.2 Cellular Diagnostic Tools

Optical microscopy is the conventional technique commonly used for cell analysis in the medical field. Traditionally, a biopsy is taken from the tumor mass, dissociated and stained for their nucleus and cytoskeletons. Tumor cells in the biopsy are then histologically identified and classified based on any abnormality in physical characteristics such as shape, as observed under a light microscope. The advent of immunohistochemistry (IHC) has further led to improvements in diagnostics, as the presence and/or expression levels of certain membrane or intracellular proteins on cancer cells can be estimated by labeling the proteins with fluorophore-tagged antibodies, similar to IHC for lineage markers or cluster of differentiation (CD) markers of hematopoietic cells. The invention of flow cytometry has further facilitated automation of work flow such as cell counting, cell sorting, and biomarker detection, which previously had to be done manually under a microscope.

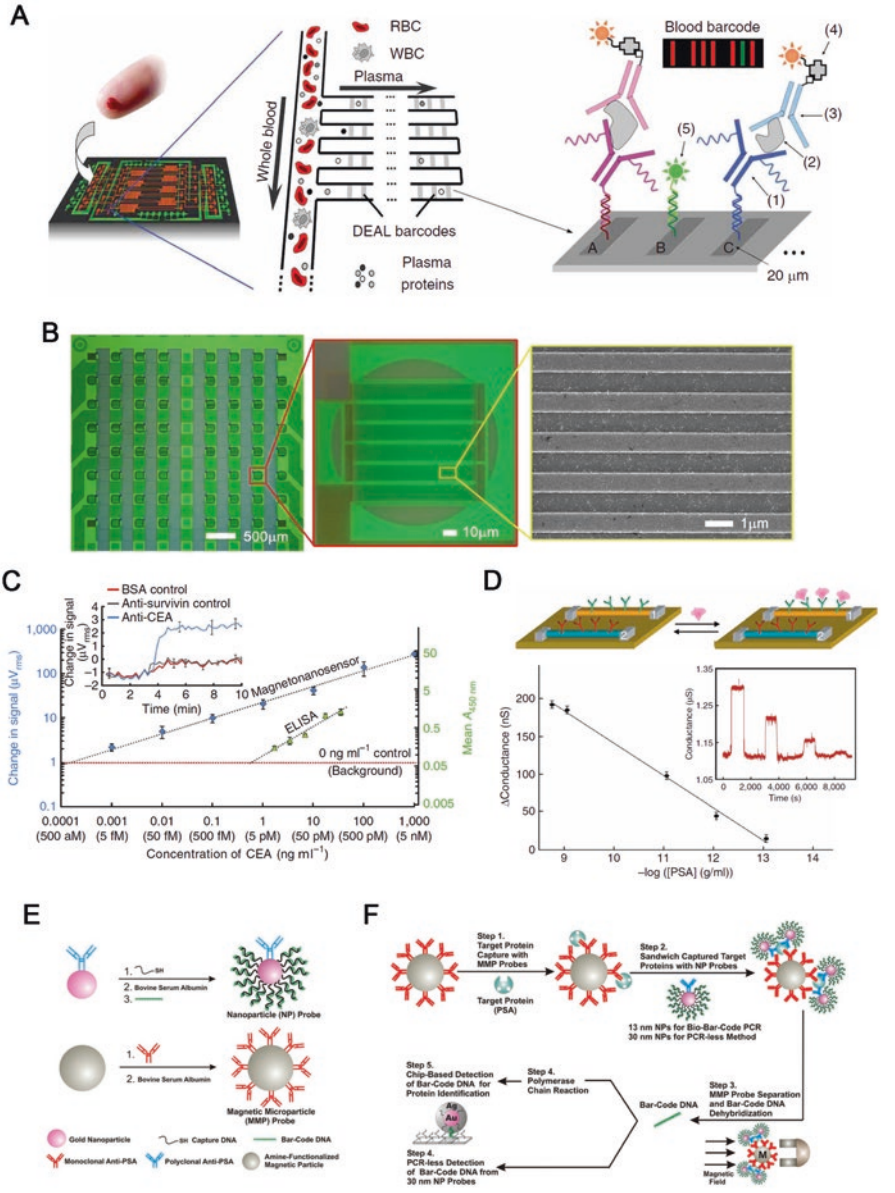

Fig. 6.6 Nanotechnology-based proteomic diagnostic tools. (**a**) Schematic of integrated blood barcode chip using microfluidic chips. Plasma proteins are separated from whole blood and routed into DNA-encoded antibody library (DEAL) barcode plasma channels. In each barcode channel, different DNA codes are immobilized as stripes and linked with antibodies. (1)–(5) represent DNA-conjugated antibody, plasma protein, biotin-labeled detection antibody, streptavidin-Cy5 fluorescence tag, and complementary DNA-Cy3 reference probe, respectively. (Adapted from Ref. [50]). (**b**) Images of an 8 × 8 giant magnetoresistive (GMR) biosensor array. (Adapted from Ref. [58]). (**c**) Titration curves of carcinoembryonic antigen (CEA) measured by ELISA and GMR

In oncology, the enumeration and identification of circulating tumor cells (CTCs) proved to be an independent predictor of progression-free and overall survival in patients with metastatic breast cancer [59]. It has been shown that metastatic breast cancer patients with more than or equal to five CTCs per 7.5 mL of blood have shorter median progression-free survival and overall survival than those with less than five CTCs. Another example of the importance of cellular analysis in cancer is the examination of PD-L1 expression level for cancer immunotherapy. PD-L1 is one of the ligands that bind to PD-1 (programmed cell death protein 1) and is known to regulate immune response upon binding. The recent study showed that immunotherapy using monoclonal anti-PD-L1 antibodies is more effective for patients with high expression levels of PD-L1 on the tumor cells than those with low levels [60], suggesting that anti-PD-L1 therapy recovers antitumor response of T-cells by disrupting the binding of PD-L1 to PD-1 on the T-cells. Therefore, the expression level of PD-L1 on the cell surface is a biomarker that can predict whether anti-PD-L1 therapy will work for the patient.

Nanotechnology-based approaches have also facilitated the cellular analysis of cancers via improving CTC sorting and enumeration techniques (Fig. 6.7). Flow cytometry is a fully automated cell sorting system based on optical techniques, but it processes labeled cells serially, which requires long analysis time proportional to the number of cells being analyzed, and can make the detection of rare tumor cells in complex matrices like whole blood impractical. To improve the throughput of cell sorting, a parallel method using magnetic sifters has been developed [61]. Whole blood obtained from lung cancer patients were flowed through a magnetic sifter with several thousand micropores, and CTCs labeled with MNPs were captured at these pores under an external magnetic field. Upon removing the external field, the captured cells can be eluted for further analysis such as screening for specific therapy-relevant mutations.

The analysis of these cells captured via nanotechnology-based devices can further provide information into the gradual evolution of resistance to cancer therapies in time, such as to androgen receptor inhibitors in prostate cancer [29] and EGFR-related therapies in lung cancer [62]. Sequencing of these cells can alert a doctor to the development of resistance to the therapy the patient is currently being treated with and, hence, facilitate a switch to alternative second- or third-line treatments.

Fig. 6.6 (continued) biosensors using magnetic immunoassays. (Adapted from Ref. [51]). (**d**) Nanowire-based biosensors. Two nanowire devices (1 and 2) are functionalized with different antibodies. Binding of a cancer biomarker protein to the antibody on the first device produces a change in conductance. Conductance changes with varying concentration of PSA. (Adapted from Ref. [52]). (**e**) Preparation of bio-barcode particles for nanoparticle-based detection. Gold nanoparticles are conjugated with anti-PSA antibody and loaded with multiple copies of barcode DNA. Magnetic microparticles (MMPs) are conjugated only with anti-PSA antibodies. (**f**) Work flow of the bio-barcode assay measurement. A sample containing PSA is mixed with prepared MMPs, and the MMPs capture PSA. Then, the complexes are labeled with prepared gold nanoparticles to form a sandwich assembly. The assembly is separated using magnetic field, and the barcode from the assembly (from gold nanoparticles) is isolated for subsequent analysis. The isolated barcode can be amplified via PCR, and gel electrophoresis or scanometric DNA detection is then used to identify the barcode. (Adapted from Ref. [54])

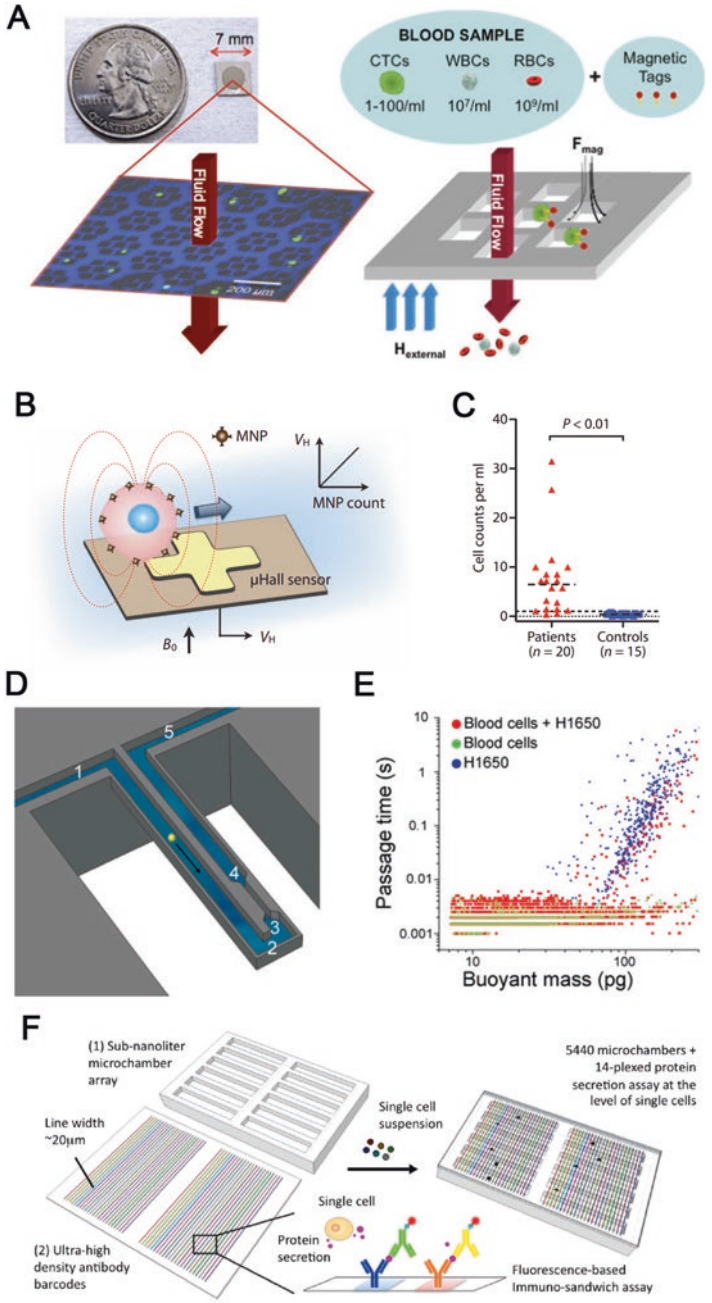

Fig. 6.7 Nanotechnology-based cellular analysis tools. (**a**) Magnetic sifter. MNP-labeled CTCs are captured at the pores under an applied magnetic field. (Adapted from Ref. [61]). (**b**) MicroHall sensors. A microHall sensor produces voltage signals proportional to the number of MNPs on a cell under an external magnetic field when the cell passes over the sensor. (**c**) The number of CTCs per mL

In addition, MNPs have been used to count CTCs in combination with magnetic Hall sensors [63]. When MNP-labeled ovarian cancer CTCs pass over the micro-Hall sensors, the sensors generate electrical signals proportional to the number of MNPs on the cell. Furthermore, other optical nanoparticles such as quantum dots [64], upconversion nanoparticles (UCNPs) [65], and silica nanoparticles with encapsulated fluorescent dyes [66] have been used to identify cancer cells. A label-free technology that does not require nanoparticles is the suspended microchannel resonator (SMR), which has been successfully used to count and characterize lung cancer CTCs [67]. SMR basically measures buoyant mass in the channel, but the addition of a narrow path in the channel allowed monitoring of passage times of CTCs through the path. Thus, instead of surface protein markers, physical properties of single cells such as deformability or surface friction can be used to identify cancerous cells. Nanotechnology has also enabled single-cell analysis and studies into the secretomic signature of cancer cells [68, 69].

6.4 Summary and Future Outlook

While the focus in this chapter has been on detection techniques, nanotechnology can also help improve peripheral techniques required for more accurate tests. The matrix of samples and sample collection and handling protocols have been recognized as key factors that can affect test results, yet they are often overlooked. Using nanotechnology, a reproducible, user-friendly, and operator-independent sample collection method can be developed.

Furthermore, most cancer biomarkers currently are proteins. Unlike DNA, it is impossible to amplify proteins with existing tools or techniques. Thus, the pre-enrichment of protein biomarkers from complex samples could be beneficial for sensitive and specific detection. Nanotechnology is expected to greatly foster future development of enrichment techniques for various cancer biomarkers.

Despite substantial studies worldwide, cancer is still one of the major causes of death. This is because cancer is such a highly complex system that it is difficult to understand its underlying mechanism and the effect of therapeutic intervention. This difficulty results mainly from the similarity between cancer cells and healthy cells (cancer cells emerge from our own cells) and heterogeneity between individuals. However, a clear lesson learned through decades of research is that the mortality rate can be greatly reduced if cancer is detected at the early stages. Therefore, a

Fig. 6.7 (continued) measured by microHall sensors between ovarian cancer patients and healthy control. (Adapted from Ref. [63]). (**d**) Schematic of SMR with a constriction pass. A cell passing through the channel is deformed as it enters into the constriction pass. (**e**) Measurement of buoyant mass and passage time of blood cells and lung cancer cell line (H1650) using SMR. (Adapted from Ref. [67]). (**f**) High-throughput multiplexed single-cell secretomic assay. Combination of subnanoliter microchamber array and high-density antibody barcode array enables measurement of secretomic signature from a single cell. (Adapted from ref. [68])

major goal in cancer diagnosis has been early detection and will indubitably remain so in the future. Nanotechnology has been accelerating the momentum toward this goal over the past decades and is anticipated to play an even more essential and critical role in the future.

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Chapter 7
Translational Nanodiagnostics for In Vivo Cancer Detection

Christina H. Liu, Pushpa Tandon, and Luisa M. Russell

7.1 Introduction

The global burden of cancer has been increasing steadily, and according to the International Agency for Research on Cancer (IARC), projections for 2030 have increased to 21.7 million new cancer cases and 13 million cancer deaths worldwide [1]. Most of this increase is attributable to the aging population and the lack of early diagnosis in large parts of the world. Overall cancer mortality has declined in the USA since the early 1990s, mostly due to screening and early diagnosis [2, 3]. These trends emphasize the importance of early detection, leading to better outcomes and long-term survival. Current tools have made cancer diagnostics easier, but better tools are needed to improve cancer statistics around the world. Techniques that can detect precancerous and cancerous lesions, identify their metastatic potential, and provide information about the disease stage are critical. Cancer diagnostic techniques have improved significantly and can diagnose small lesions; however, the ability to detect precancerous lesions, including molecular and cellular changes leading to cancer or malignancy, is lacking. Tools that can detect molecular changes occurring in the body well before the physical manifestation of a diffuse disease like cancer is visible are needed to improve cancer-related morbidity and mortality [4].

The development of novel imaging technologies has helped detection at the anatomical and functional level; the development of specific and novel imaging probes and contrast agents can enable greater utilization and full potential of these technologies to improve detection, monitor treatment, and improve outcomes. With the help of specially designed nanoparticles, imaging technologies can detect biological

C. H. Liu (✉) · P. Tandon (✉) · L. M. Russell
National Cancer Institute, National Institutes of Health, Rockville, MD, USA
e-mail: christina.liu@nih.gov; tandonp@mail.nih.gov; luisa.russell@nih.gov

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and physiological systems at the molecular level, making disease detection and characterization possible at early stages. Diagnostic tools at the nanoscale are selective and sensitive and have the capability of detecting small adverse changes at the cellular and molecular level occurring in the tissue before the physiological manifestation of the disease occurs [5]. This could allow for even earlier stage detection, provided the right biomarkers for specific diseases are identified.

Nanoparticles have been used to detect disease markers, virus fragments, antibodies, specific proteins, and other disease indicators, and nanodiagnostic tools are being routinely used for in vitro testing and diagnostics [6]. The last decade has seen the development of nanoprobes and imaging agents for in vivo use; the earliest MR receptor imaging was done by Weissleder et al. [7]. As the field matures, nanoparticles being explored are those targeted to specific receptors for drug delivery using monoclonal antibodies, multiplexed for detection of specific tumor biomarkers, activated in the presence of proteins or changes in pH to release agents or therapeutics (theranostics), and designed for multimodal imaging, to name a few. With capabilities for use in multiple imaging modalities and the ability to be functionalized, nanomaterials are ideally suited to provide access to biological functions and can be used for sensing, imaging, and treating disease. Nanoparticles have been used in conjunction with instruments or devices in biopsy-based assessments for studying characteristics in tumors with considerable heterogeneity. Nanoscale devices have also been used for detecting and treating solid tumors because of their ability to selectively accumulate in tumors based on the enhanced permeability and retention (EPR) effect [8].

Nanodiagnostics for in vivo use are now being developed for early detection and staging to direct and monitor treatment, not only in cancer but also in other diseases. Most nanodiagnostic tools are in the preclinical stage and must address issues of toxicity, biodistribution, and clearance mechanisms before moving to clinical trials and beyond. This chapter outlines the current status of nanodiagnostic tools being developed for various detection modalities, examples of the applications in clinical and preclinical research, as well as the challenges and potential in clinical translations. Due to their versatile nature and unique capabilities, nanodiagnostics and associated imaging techniques have the potential to change the landscape of cancer detection and management, reducing morbidity and mortality as well as lowering the cost of cancer care, which increases significantly with late-stage detection.

7.2 Nanoparticles as Imaging Agents for In Vivo Imaging and Cancer Diagnosis

One of the essential components of early cancer care is medical imaging, which is routinely used to evaluate anatomical and functional states of tumor progression, and nanoparticles (NPs) have been developed to serve as imaging contrast

agents to improve the image quality. Examples of the utility of NP-based contrast agents in preclinical studies include gold nanostars and silver nanoclusters for surface-enhanced Raman spectroscopy, gold nanorods and dye-containing NPs for photoacoustic imaging, iron oxide NPs for MRI, gold NPs and iodinated liposomes for CT, chelator-based or intrinsically radiolabeled NPs for PET/SPECT, quantum dots for optical imaging, and gas-filled nanobubbles for ultrasound imaging [9]. While NPs can serve as contrast agents for traditional medical imaging modalities, NPs also enable new imaging methods such as magnetic particle imaging (MPI), in vivo Raman imaging [10], or multimodality imaging. To be used in living subjects, NPs are coated with polymers or carbohydrates [11, 12] to enhance the in vivo stability, biocompatibility, and safety. NPs can also be made to exhibit different sizes and shapes, resulting in preferential accumulation in specific organs after systemic injection. NPs can also be designed to change their properties when encountering specific physiological condition or linked to targeting moieties to molecular signatures to reveal molecular or metabolic information of premalignant, early-stage tumors or tumors under treatment.

7.3 Status of In Vivo Nanodiagnostics

Although a few NP-based drug delivery vehicles have been FDA-approved (e.g., Doxil [13] and Abraxane [14]), the clinical applications of NPs for diagnostic imaging are very restricted. NP-based imaging agents have struggled in the FDA approval pipelines due to health, marketing, and scientific challenges; as such, most of these NPs remain preclinical. For example, Feridex I.V.® (an iron oxide NP-based injectable solution) was approved in 1996 as a magnetic resonance imaging (MRI) contrast agent for detecting liver lesions but later with warnings on potential adverse reactions [15]. Consequently, all iron oxide agents developed for imaging purpose were pulled out of the market due to lack of sales, adverse reactions, and lack of sufficient evidence of clinical utility [16]. The following sections are presented based on the imaging modality and how NPs have augmented these imaging techniques in preclinical or clinical in vivo diagnostics. NPs can also be designed to have both therapeutic and diagnostic capabilities, a concept collectively known as "theranostics." By incorporating imaging agents into the same structure that carries therapeutic molecules, theranostic NPs can reveal drug distribution after the injection and monitor treatment response. Because some liposomal small-molecule drug formulations have already received FDA approval, the use of liposomes as biocompatible particles may expedite the process of clinical translation as drug carriers [17] and as theranostic agents [18, 19], but many other NP types have also been developed with theranostics in mind. Some of these formulations are described below, but for more information, readers are directed to the many recent reports on theranostic NPs [18, 20–24].

7.3.1 Nanoparticles for Magnetic Resonance and Magnetic Particle Imaging

The most known NPs for magnetic resonance imaging are iron oxide nanoparticles. Since the 1990s, researchers have been testing iron oxide NPs such as superparamagnetic iron oxide NPs (SPIONs) in animals. These SPIONs are primarily applied as T2 contrast agents for MRI, which serve as a negative MR contrast agent, producing dark regions in MR images. Because of their ability to produce strong image contrast and lower toxicity to biological systems, there have been extensive studies in the last decade to make SPIONs suitable for translation [25]. Several SPIONs are in the clinical trial pipeline, including ferumoxytol [26], ferumoxtrans [27, 28], and ferucarbotran NPs [29]. Thus far, the clinically approved iron oxide NPs in the USA and EU have sizes ranging from 17 to 150 nm and are coated with carbohydrate such as dextran (under trade names Feridex, Endorem, Sinerem, Comidex), carboxydextran (under tradenames Resovist, Cliavist), or polyglucose sorbitol carboxymethylether (Feraheme®) [30]. Currently, 20 clinical trials focus on ferumoxytol as an "off-label" MRI contrast agent, and ferumoxtran-10 is being tested clinically in Europe (NCT02751606, NCT03223064, NCT02549898, NCT02997046) [31]. Ferumoxytol is also under active research as an alternative contrast agent for magnetic resonance angiography, thereby avoiding the known renal toxicity that comes with conventional gadolinium contrast MRI; however, progress is slow [32, 33]. That said, ferumoxytol has been shown clinically to perform at least as well, if not better than gadolinium-based agents routinely used in the clinics. Figure 7.1 compares brain MRI images of patients with metastatic brain disease after the infusion of either gadolinium (Gd)-based contrast agent or iron oxide-based contrast agent. In this study, ProHance® (containing a small-molecule gadolinium complex with a molecular weight of 558.7 Da) MRI served as the current gold standard in clinical care for delineating brain tumors immediately after injection. With an average hydrodynamic size of around 30 nm [34], ferumoxytol (Feraheme®) has a plasma half-life of about 15 h, which can help differentiate metastatic brain disease from meningioma (benign brain tumors on the meninges, the membranes surrounding the brain and spinal cord) under MRI 24 h after injection [35]. The hyper-intense lesions revealed by Gd-enhanced MRI at early time points could have been diagnosed as meningioma. Without further studies with iron oxide-enhanced MRI at a later time point, multiple pancreatic carcinoid tumor dural metastases would not have been diagnosed in this patient. In the preclinical space, ferumoxytol has been used to evaluate the enhanced permeability and retention (EPR) effect in the tumor microenvironment and could serve as a companion diagnostic particle for predicting therapeutic efficacy [36].

Theranostic nanoparticles with MRI contrast activity have also been developed, such as thermo-active liposomes containing both doxorubicin and Gd-DTPA. These liposomes allowed MRI visualization and monitoring of the

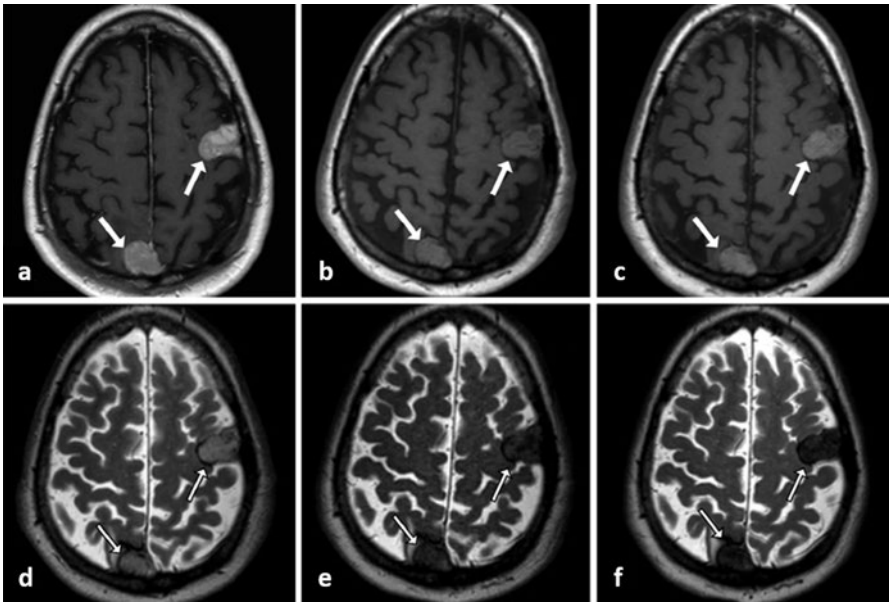

Fig. 7.1 ProHance®-enhanced T1-weighted MR images in a patient with multiple pancreatic carcinoid tumor dural metastases reveal two masses (**a**, white *arrows*) that dissipate in 24 h for meningioma. On the other hand, the same masses exhibit mild enhancement (*arrows*) in T1-weighted images immediately after Feraheme® injection (**b**) that increases to 24 h (**c**). Axial T2-weighted MR images show a heterogeneous mild hyperintensity in the dural-based masses (*arrows*) on precontrast MRI (**d**) with new hypointensity in both masses (white *arrows*) immediately post ferumoxytol injection (**e**) that progressively darkens at 24 h (**f**). (Adapted with permission from [35])

drug release from the liposome in the tumor environment; the study showed that heat-induced doxorubicin therapy resulted in T_1 relaxation enhancement within tumors [37]. Though theranostic nanoparticles are in preclinical development, magnetic nanoparticles are still the overwhelming NP contrast agent for MRI. The use of magnetic NPs as MRI contrast agents has also led to the development of magnetic particle imaging, a related form of preclinical imaging.

Magnetic particle imaging (MPI) is a relatively new imaging technique which directly detects signals from SPIONs without the susceptibility artifacts observed in MRI [38]. MPI was first developed as an alternative vascular imaging technique to subtraction angiography with CT, which requires the use of iodine-containing agents [39]. Recently, SPION-labeled stem cells or macrophages have been used with MPI to assess injuries and disease progression [40]. In this case, the NP is a Janus NP (with a radius of 42.3 nm by transmission electron microscopy (TEM)) made by encapsulating two or more iron oxide nanoparticles in a semiconducting polymer to have both optical and magnetic properties. These were shown in preclinical work to display seven times better MPI signal than the MRI contrast agent Feraheme®, with the ability to image as few as 250 cells after implantation.

7.3.2 *Nanoparticles for Optical Imaging*

Optical imaging techniques offer high image resolution but with limited depth penetration into tissue. As such, optical imaging for in vivo applications is limited to superficial detection or intraoperative imaging. Some NPs are inherently fluorescent and are suitable for optical or near-infrared (NIR) imaging [41]. Not only that, but they often do not display the significant photobleaching as the small-molecule fluorescent agents. NPs can also serve as targeted fluorescent tags by functionalization with active targeting ligands to tumor cells for tumor localization or lymph node mapping and, because of the number of fluorescent molecules they can hold, are more easily detectable than a targeted single dye molecule would be. Silicon (Si) QD NPs can be made to have different sizes (e.g., from 1 to 4 nm) to emit different wavelengths of light [42]. These NPs can be used in multiple biomedical imaging applications after encapsulation in ethylene glycol micelles [42, 43]. Specifically, by incorporating a biocompatible coating with surface modification to target tumor cells, these Si QDs exhibit efficient NIR emission, far away from the green autofluorescence of surrounding tissues. Therefore, this allows for lymph node mapping and multiplexed imaging (Fig. 7.2). Silicon dioxide or silica-based core-shell NPs can be loaded with fluorescent dye to make extremely bright optical imaging probes, known as Cornell dots or C′-dots (≤8 nm in size), for image-guided tumor resection [44–46] which will be described in more detail in a later section entitled "Image-Guided Surgery." Although quantum dots are bright with versatile emission colors well suited for optical imaging, toxicity remains an issue for their approval and use in the clinic. More examples and discussions about the current use of quantum dots described in the section entitled "Quantum Dots and Engineered Nanoparticles for Diagnostics."

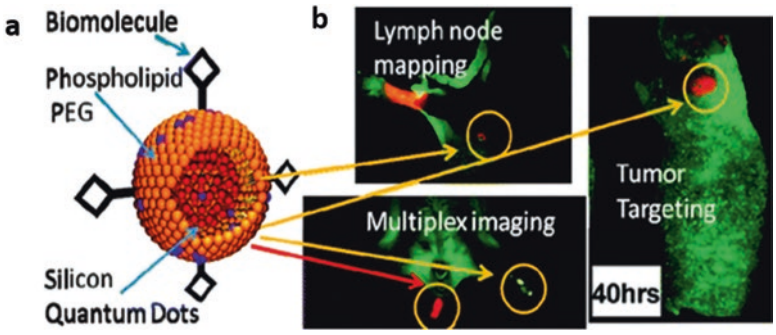

Fig. 7.2 Ethylene glycol micelles encapsulating NIR-emitting Si QDs can be used for targeting and imaging of tumor and lymph nodes. The spectra of these Si QDs can be tuned by the size of the QDs (1–4 nm). (Adapted with permission from [43])

7.3.3 Nanoparticles for Nuclear Medicine Techniques

Single photon emission computed tomography (SPECT) and positron-emission tomography (PET) are nuclear medicine techniques that provide higher detection sensitivity than imaging approaches [47]. Radiolabeled NPs can be utilized to establish drug uptake profiles for predicting treatment response [48] and for molecular imaging of atherosclerosis [49], as well as many other diseases [50]. Several SPECT imaging agents based on ^{99m}Tc-labeled colloids have been clinically approved for cancer diagnosis in the USA or EU (e.g., Technecoll, Nanocoll®, Nanocis, Hepatate, and Senti-Scint [30]). Three of these colloids were deemed suitable for sentinel lymph node (SLN) localization based on the stability of their particle sizes during the ^{99m}Tc-labeling process (with mean diameters of 8 nm, 24 nm, and 72 nm for Nanocoll, Nanocis, and Hepatate, respectively [51]). ^{99m}Tc-labeled Nanocoll® (EU-approved nanocolloid albumin particles with medium sizes from 7 to 14 nm based on dynamic light scattering (DLS) [52]) was used in a prospective multicenter International Atomic Energy Agency Sentinel Node Trial for the added value of SPECT/CT over clinical planar lymphoscintigraphy (PL) in sentinel lymph node (SLN) detection. PL after local injection of radiocolloids can occasionally miss the SLN when the SLN is too close to the injection site or too deep under the tissue. Results from breast cancer studies showed that PL failed to visualize SLNs in 12.3% of patients; SPECT/CT revealed one or more SLNs in 3.7% among these patients, and some were determined to be metastatic after axillary lymphadenectomy. The outcomes of this trial resulted in a recommendation by the agency to include SPECT/CT in all breast cancer patients with no SLN visualized on PL [53]. A recently FDA-approved ^{99m}Tc-nanocolloid with the tradename Lymphoseek® for lymphatic mapping (in 2014) has shown efficacy in SLN mapping and intraoperative procedures in cutaneous melanoma, breast cancer, and oral cavity squamous cell carcinoma (OSCC). Figure 7.3 compares the PL and SPECT/CT in evaluating OSCC and the relationship of SLN location to anatomical landmarks [54]. The adoption of SLN biopsy for head and neck cancer sites has been challenging because of multiple concerns in the unpredictable watershed lymphatics and nearby vital organs. Using added anatomical information as imaging landmarks, ^{99m}Tc-nanocolloid-aided SPECT/CT can enhance the feasibility and accuracy of SLN biopsy and resection of OSCC lesions.

7.3.4 Nanoparticles for X–Ray Imaging and Computed Tomography

Computed tomography (CT) and X-ray imaging are widely used for cancer screening due to their accessibility and low cost. Clinical CT contrast agents are often iodine-based, but these small-molecule formulations often have short circulation half-lives and unfavorable toxicity. There are currently several commercially

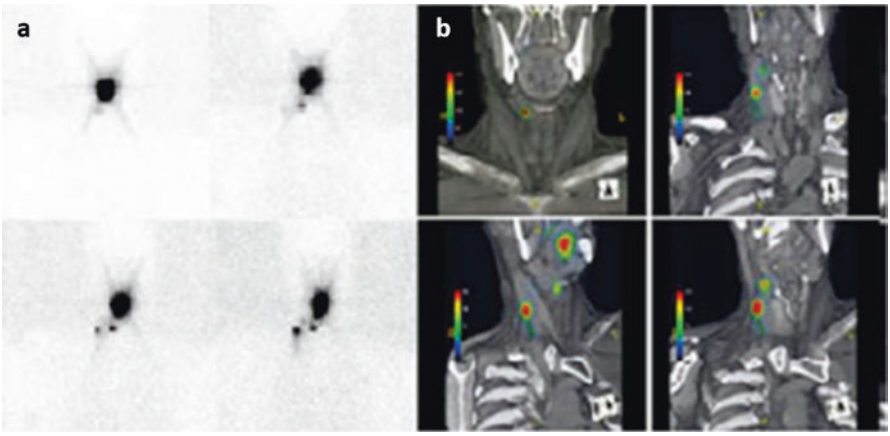

Fig. 7.3 Comparisons between conventional planar lymphoscintigraphy (PI, **a**) and Lymphoseek® SPECT/CT (**b**) of a patient with floor of mouth carcinoma in relationship to SLN locations. The exquisite anatomical information provided by SPECT/CT can enhance the surgeon's view of the operating field, as well as help develop new intraoperative identification methods. (Adapted with permission from [54])

available small-molecule iodinated contrast agents as described by Lusic and Grinstaff [55]. By incorporating iodine, gold, or bismuth, NP-based CT contrast agents are more stable and less toxic and have longer blood circulation time than their free contrast agent counterparts [56]. Within the preclinical space, NP-based CT contrast agents for oncological imaging include iodine- and bromine-containing liposomes [57, 58], gold nanorods, shells, dendrimers [59], and lanthanide oxide NPs [60]. In one example, sequential injections of gold nanoparticles and iodine nanoparticles increased tumor contrast in dual-energy CT scanning and enabled the measurement of fractional blood volume, permeability, as well as morphological information about the tumor mass [61]. Several other elements have been used in constructing NP-based CT contrast agents, including platinum, gadolinium, ytterbium, yttrium, and tantalum [58].

7.3.5 Nanoparticles for Ultrasound and Photoacoustic Imaging

Ultrasound imaging (US) is another common modality for detecting suspicious cancerous masses, particularly useful because of its low cost, non-radiative nature, and real-time viewing ability. Although not considered NPs, microbubbles and their variations (1–8 μm) are used widely as US contrast agents in cardiovascular imaging but with limited usefulness in oncology because of restricted depth penetration by microbubbles into the tumor mass [62]. Using SonoVue (sulfur hexafluoride-based microbubbles with a mean diameter of 25 μm [63]), researchers

demonstrated an improved efficacy of contrast-enhanced ultrasound (CEUS) in detecting transplant renal artery stenosis (TRAS) than the conventional Doppler ultrasound, similar to computed tomography angiography (CTA) but without exposing patients to radiation, as shown in Fig. 7.4 [64]. There are ongoing research efforts to produce gas-filled nanobubbles for oncological applications [65]. Theranostic nanoparticles with US capabilities have also been developed. In addition to mapping microvasculatures, cisplatin-carrying ultrasound-active liposomes released the drug after focal high-frequency ultrasound pulses at the tumor site and inhibited tumor growth by restricting the intratumoral vessel area without affecting the angiogenesis ratios in the tumor [66]. Further development of the technique of ultrasound is also a promising field of research, from which a related novel preclinical modality (photoacoustic imaging) is being explored in preclinical research laboratories.

Photoacoustic (PA) imaging is an extension of ultrasound (US) imaging, which detects the pressure waves ("sound-out") with a US transducer from irradiation of NIR or laser ("light-in") to the tissue [67]. PA can provide information about the physiological, optical, and mechanical properties of tissues, in addition to anatomy and flow rates. Several NP-based contrast agents for PA imaging have been developed to allow for better delineation between healthy and diseased tissue [68]. NP-based contrast agents such as gold nanoparticles [69], semiconducting or engineered nanoparticles (i.e., QDs, upconverting NPs [UCNPs]) [70–72], and carbon NPs (usually single-walled carbon nanotubes) have been used in preclinical PA imaging studies to visualize cancer lesions [73]. NIR-absorbing small molecules or dyes can also be incorporated into nanoparticles to improve quantum yield, blood half-life, and in vivo stability for PA imaging [73, 74].

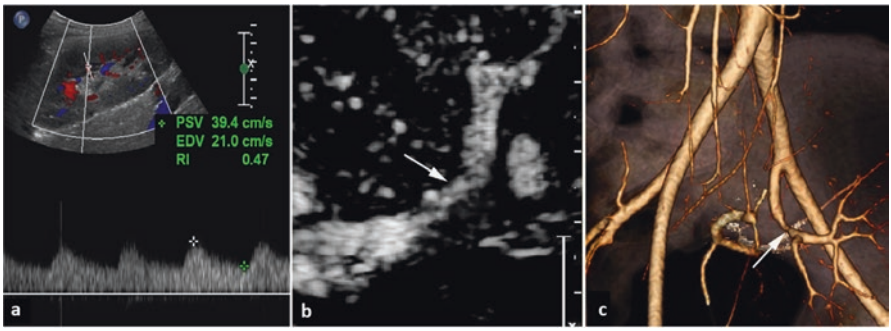

Fig. 7.4 Doppler ultrasound shows potential stenosis of a patient with transplant renal artery stenosis (TRAS) (**a**). Microbubble-enhanced ultrasound shows the narrowing of the renal artery (**b**, arrow), which was confirmed by computed tomography angiography (**c**, arrow). (Adapted with permission from [64])

7.3.6 *Nanoparticles for Surface-Enhanced Raman Spectroscopy*

Raman spectroscopy was initially developed for in vitro application with low sensitivity [75]; however, recent developments in engineered NPs have shown their utility for in vivo detection [10, 76]. By adding a noble metal surface (such as gold or silver) with high curvature, NPs enable a phenomenon called surface-enhanced Raman scattering (SERS) resulting in significant signal enhancement [77–79]. SERS is a newer technique made possible by the development of these nanoscale agents. Figure 7.5 shows that trimodal SERS NPs (average size about 50 nm by TEM) and multimodality imaging can aid in complete tumor resection in vivo [80]. Sequential tumor resection can be done by serial SERS imaging to reveal residual tumor tissue at each resection step (top panels). After the gross resection, persistent SERS signal in tissue near the margin can be indicative of residual tumor, which was later confirmed by tissue immunohistochemistry (bottom panels). Without incorporating SERS imaging in intraoperative resection, the microscopic residual tumor could be missed, leading to an increased risk of future metastasis.

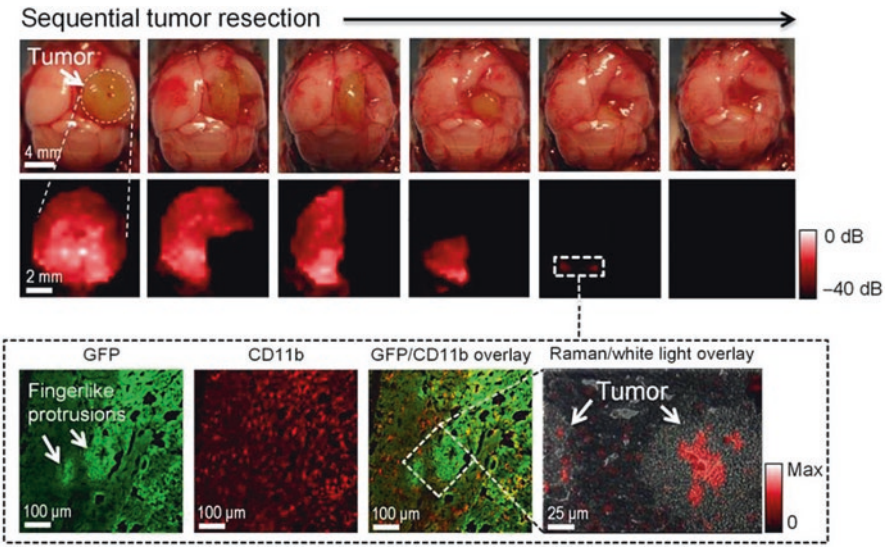

Fig. 7.5 Intraoperative SERS imaging. The top row is the visible surgical field of view, the second row is Raman spectroscopy of the tissue and tumor, and the third row shows staining of the tissue from the residual Raman signal in the white dotted box. (Reproduced with permission from [80])

7.3.7 *Nanoparticles for Multimodal Imaging and Theranostics*

The developments of nanoparticle-based imaging contrast agents have gone together with the development of novel multimodal imaging contrast agents in recent years. Nanoparticles have a large surface-to-volume ratio and can be easily modified to accommodate different contrast agents for multiple modalities, by adding chelators or encapsulating small imaging molecules. Multimodal imaging confers a tremendous clinical advantage, as patients can undergo several different types of scans in succession without worrying about possible interference from a variety of contrast agents. For example, Gd-conjugated gold-silica nanoshells can be used for MRI/X-ray [81], radioarsenic-labeled SPIONS can be used for PET/MRI [82], and liposomes loaded with ^{64}Cu and NIR dyes can be used for PET/fluorescence imaging [83]. Interestingly, some nanoparticles have inherently multimodal contrast, such as QDs for optical and PA [84]; gold NPs for CT, PA, and SERS [85]; and SPIONs for MRI and PA [86]. Theranostic liposome systems with more complexity have also been developed for multimodal imaging and drug release by incorporating Gd-DOTA chelates, IR-dyes, ^{64}Cu-DOTA chelates, and doxorubicin [87].

7.4 Strategies to Improve Signal Sensitivity and Specificity of Nanodiagnostics

NP platforms are well suited to tackle many shortcomings of conventional imaging contrast agents because of their multiplexing capabilities. However, in addition to in vivo stability, biocompatibility, and safety of nanoparticles, NP-based imaging and diagnostic techniques should provide sufficient signal quality. Are these signals specific to the biological events, biomolecules, or physiological conditions of interest? Are these signals strong enough to be detected by the instruments? These challenges are currently being addressed in a research setting by applying active targeting ligands to increase specificity and/or developing activatable structures to reduce background signal, thereby increasing sensitivity. This section highlights some emerging imaging techniques and associated nanoparticle-based imaging contrast agents, which could have a significant impact on nanodiagnostics.

7.4.1 *Active Targeting*

Nanoparticles can be linked to targeting ligands such as antibodies, peptides, antisense oligonucleotides, aptamers, small-molecule drugs, and carbohydrates on their surface to give localized signals with information about cells and molecules of interest for nanodiagnostics. Several cell surface receptors (or antigens) are

highly expressed in disease or cancer cells and make them perfect choices for targeted imaging. For example, nanobubbles designed to target cancer antigen 125 (CA-125) offered a better diagnosis of epithelial ovarian cancer by enhancing tumor accumulation, increasing peak signal intensity, and slowing washout rates [62]. The targeted nanobubbles increased the signal ratio of OVCAR-3 xenografts in mice up to fivefold as compared to the signal from nontargeted nanobubbles. Liposome- or micelle-based CT contrast agents were constructed to target surface receptors or proteins such as transferrin for the detection of gliomas in rats [88] or fibrin for imaging thrombi [58]. ^{124}I-labeled, integrin $\alpha_v\beta_3$ targeting Cy5-encapsulated C′-dots are used for tumor detection using PET [89]. ^{99m}Tc-etarfolatide nanoparticles target folate receptors, which are highly expressed in ovarian cancer, and are used as a SPECT contrast agent to evaluate the efficacy of Vintafolide therapy [90].

7.4.2 Activatable or "Smart" Nanoparticles

"Smart" NPs are engineered NPs which can change color or turn on through conformational modifications induced by the surrounding environment. These NPs have pushed the detection limits of clinical in vitro assays to femto- and picomolar levels, primarily through the reduction of background signal [91].

To achieve the maximal tumor-to-background imaging ratios, these activatable NPs would work best if their signals are detectable at the presence of cancer-specific markers. For example, in vivo evaluation of the tumor microenvironment using PA imaging can be done by albumin-NIR pH-responsive self-assembled NPs [92] or ligand-targeted mesoporous silica NPs [93]. Bioinspired calcium phosphate NPs doped with Mn^{2+} could be "turned on" in hypoxic regions of the tumor and its microenvironment for detection of the tumor using MRI [94]. A caspase-responsive nanoparticle-based MRI contrast agent was used to reveal tumors with doxorubicin-induced apoptosis [95]. Another strategy for this is to build self-quenching nanoparticles that only fluoresce after cell entry [96] or nanoparticles that can only be detected when they aggregate in the tumor microenvironment [97, 98]. Unlike active targeting, in vivo diagnostics using "smart" NPs are not biased by background noise. However, the activated forms of these NPs need to exhibit sufficient signals for in vivo detection, which requires signal quality as well as accumulation of enough nanoparticles in the target tissue.

7.4.3 Novel Signal Types or Co–Localization of Multimodal Signals

The other way in which nanodiagnostic agents can improve clinical imaging through specific contrast is by possession of a unique characteristic unseen in the rest of the body. For example, Raman spectroscopy enables specific identification of

nanoparticle accumulation because of the detection of specific Raman signals. Raman-active nanoparticles have layers with distinct Raman fingerprints, meaning that there is no background signal from the body. Another strategy is to create nanoparticles designed for multimodal imaging. In this case, being able to detect the nanoparticle via two or more different imaging modalities reduces the likelihood of false detection due to the ability to verify specific signal across several imaging modalities. Both of these strategies are discussed in more detail in the sections entitled "Nanoparticles for Surface-Enhanced Raman Spectroscopy" and "Nanoparticles for Multimodal Imaging and Theranostics."

7.5 Emerging Translational Diagnostic Nanomedicines

In the preclinical research space, many new nanoparticle-enabled techniques are developed for diagnostics. These include nanoelectromechanical sensors, the use of semiconductor NPs, and the increasingly translatable field of image-guided surgery.

7.5.1 Nanoelectromechanical Sensors (NEMS)

Electrochemical properties and electroanalytic activities of nanomaterials have made enhanced signal amplification possible through the construction of ultrasensitive electrochemical biosensors. Biosensors have been designed for the detection of viruses like HPV, cancer biomarkers, and a host of other biochemicals, proteins, amino acids, and DNA sequences [99–101]. Very rarely does a single biomarker have the sensitivity and specificity to be predictive of disease. A panel of biomarkers is much more reliable in predicting disease and also of identifying disease states. Nanotechnology-based detection devices like biosensors offer the capability of multiple measurements from multiple targets with high sensitivity and selectivity. This multiplexing ability can be crucial in cases where one biomarker measurement is not specific enough and can reduce the time and cost of running multiple separate assays. These biosensing devices contain recognition elements for biological molecules linked to nanoparticles that can detect the presence of "biomarkers" like molecules, antigens, antibodies, proteins, and RNA or DNA fragments. A transducer in the device then converts the biochemical change into a signal that is measurable and quantifiable. One of the first biosensors developed was a nanoparticle-based bio-barcode device for ultrasensitive protein detection. A magnetic microparticle probe containing antibodies that bind specifically to the antigen (PSA), nanoparticle probes functionalized with DNA unique to the protein of interest (PSA), and detection antibodies to sandwich the target PSA was shown to capture and detect protein analytes at very low levels [102]. Similar biosensors have now been developed for the detection of other cancer-specific biomarkers [103, 104]. Although carbon nanomaterials are highly advantageous as electrochemical

sensors due to their high adsorption of molecules, increased electroactive surface area, and enhanced electron transfer potential [100, 101], other nanomaterials like zinc oxide, gold, and graphene are also being used as elite nanomaterials for the fabrication of nanobiosensors [99]. Nanoparticles, nanotubes, nanowires, and nanocantilevers are other examples of nanobiosensor systems that are being developed for the detection and diagnosis of pancreatic, breast, lung, prostate, and brain cancers over the past few years [105]. Although all these nanobiosensors are still in the developmental phase, they will transition into clinically validated tests as the field of nanobiosensors matures and experimental and engineering techniques advance.

7.5.2 Quantum Dots and Engineered NPs for Diagnostics

Quantum dots (QDs) are engineered photoluminescent NPs with high quantum yields and versatile spectra and are therefore well suited for fluorescent imaging of tissues and cells. Quantum dots (QD), unlike other nanostructures, have the advantages of photostability, narrow emission spectra, lack of photobleaching, broad absorption spectra, and high quantum efficiency. The ability to manipulate the size and shape of QDs allows the intensity of fluorescence in the imaging of targeted tumor cells to be controlled. These properties make the use of QDs for tissue and in vivo imaging and diagnosis of cancer highly desirable [106, 107]. Unfortunately, traditional QDs have limited clinical use because these NPs contain cadmium or other toxic heavy metals [108, 109]. Their small size contributes to this toxicity, with some unfavorable accumulation in healthy tissues. More recently, researchers have attempted to construct cadmium-free QDs to improve biocompatibility, yet these newer formulations still face challenges [110].

QDs have been used for biomarker detection [111–113], tumor imaging [114], and cancer diagnosis [115], for mapping of axillary lymph nodes [116], to detect metastases in breast cancer and study tumor heterogeneity, and for radiation treatment [117] and monitoring [115, 118]. Development of QD-based multiplexed imaging has enormous potential for revealing the interactions of different molecules simultaneously [119]. Based on QD imaging on collagen IV, various patterns of tumor invasion have been identified showing the interaction between cancer cells and the microenvironment during tumor progression [120]. It is recognized that the tumor microenvironment exerts stimulating factors for cancer invasion and metastasis [121]. QD-based multiplexed imaging has also helped in the understanding of the dynamic interactions between biomarkers in the tumor microenvironment (collagen IV, tumor angiogenesis, and infiltrative macrophages) and cancer cells (matrix metalloproteinase-9).

Due to the successful multiplexing ability of QDs, both QDs and novel upconverting nanoparticles (UCNPs) are not only being developed for diagnosis but also for cancer theranostics [107, 122–124]. In addition to the NIR-range absorbance allowing for deeper tissue penetration, QDs and UCNPs have potential in photodynamic therapy. As such, several new UCNP-[125] or QD-based [126, 127]

agents are in preclinical trials. These engineered NPs can exhibit additional imaging contrast and other therapeutic efficacies [107, 128]. Tungsten sulfide (W_2S) QDs developed by Yong et al. were efficient contrast agents for both CT and PA imaging. Not only were these W_2S-QDs applicable in multimodal imaging, but they also served to enhance radiotherapy and photothermal therapy in a synergistic manner [126]. Functionalized graphene QDs (GQDs) are being used for targeting, drug delivery, and imaging [122, 127]. Due to their specific and tunable properties, QDs have the potential to improve our understanding of cancer significantly, changing cancer diagnostics and treatment, despite the significant regulatory challenges they face. The most prevalent issue with QDs is in vivo toxicity due to the involvement of cadmium (Cd) during the manufacturing process, though QD toxicity can also depend on multiple factors, such as QD size, charge, outer coating bioactivity (functional groups), and oxidative, photolytic, and mechanical safety along with contaminants (cadmium) during the manufacturing process [110]. New approaches are being pursued in addressing these toxicities and generating contaminant-free QDs and Cd-free QDs [110].

7.5.3 Image-Guided Surgery

One of the many challenges faced in surgical cancer therapy is the high probability of metastasis because of residual disease. Even with high-level clinical imaging with increased resolution, it can be challenging to translate scans to actual patient tissue in a surgical setting. Because of this, techniques to enable image-guided surgery have been developed to delineate tumor margins in real time, leading to better patient outcomes post-surgery [129]. These include radioactive, NIR, or optical probes whose signal can be detected during surgical removal of cancer, significantly increasing the surgeon's ability to resect all diseased tissue.

Though some small molecules such as dyes or radioactive tracers have been developed for this application, ultimately NP agents have been shown to have numerous advantages including active targeting ability, decreased photobleaching, and enhanced specific accumulation in tumor tissue by the EPR effect [130]. NPs incorporating dyes or radioactive tracers enable more precise visualization of tumor margins and better identification of malignant tissue in a surgical setting. There are several NPs that are currently undergoing clinical trials as image-guided surgery contrast agents, for example, carbon NPs for detection of small central metastatic lymph nodes in the neck (NCT02724176) [131] and fluorescent silica NPs (C′-dots, about 6 to 7 nm before functionalization). In combination with clinical optical imaging tool, this technique can help reveal cancerous sentinel lymph nodes with strong green signal to avoid potential damage to the facial nerve during surgery, as depicted in Fig. 7.6 (NCT02106598) [89, 132]. Both carbon and silica NP-based image-guided platforms have shown impressive promise, with many possibilities for clinical translation.

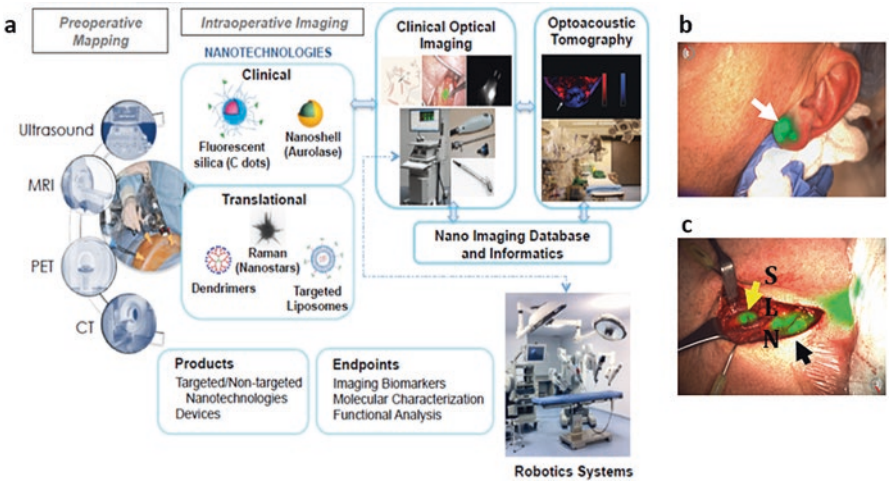

Fig. 7.6 (**a**) Describes a state-of-the-art nano-oncology image-guided surgical suite. Preoperative conventional imaging tools are used to screen for disease and inform optically driven minimally invasive and open surgical procedures. Such procedures currently utilize clinically available fluorescence agents, including particle-based probes, in conjunction with a portable multichannel fluorescence camera system. The operating surgeon, along with the nanomedicine team, can select specific probe-device combinations for key intraoperative indications and acquire real-time structural-, functional-, and/or molecular-level data regarding tissue status for further treatment management. One key indication, sentinel lymph node mapping (**b**, **c**) uses fluorescence imaging guidance to localize optically avid nodes, in this case, following local injection of an integrin-targeting ultra-small (sub 8-nm diameter) silica nanoparticle (i.e., C′-dots) about a primary lesion within the lobule of the patient's left ear (**b**, arrow). A submandibular (level I) sentinel lymph node (SLN, yellow arrow) is identified (**c**) within the exposed nodal bed, in addition to adjacent normal lymphatic channels (black arrow). (Courtesy of Dr. Michelle Bradbury of MSKCC)

7.6 Challenges and Future Perspectives for In Vivo Nanodiagnostics

7.6.1 Regulatory Challenges for Nanodiagnostics: In Vitro Vs. In Vivo Diagnostics

The most significant hurdle in the commercialization of nanoparticle-based imaging agents is their high development costs and low returns [133]. The profit margins (and therefore potentially the incentive for development) on NP-based imaging companion diagnostics are not as high as those for their counterpart drugs, since the companion diagnostic agent is only administered a few times for patient stratification or efficacy monitoring, compared to the longitudinal need for a drug. As such, the development of NP solely for in vivo diagnostic purposes is not a high investment priority for pharmaceutical companies.

In Vitro Diagnostics The development of in vitro assays as companion diagnostics has received a significant boost from the Food and Drug Administration (FDA). In 2014, the FDA issued "Guidance for Industry: In Vitro Companion Diagnostic Devices," to recommend earlier stage co-development of drugs and companion diagnostic tests. In this guidance, pharmaceutical companies are encouraged to develop the in vitro companion diagnostics by a partnership with outside research facilities or in-house. As a result, several companion diagnostics were developed and approved with the drug and increase the efficiency of nanomedicines through a more informed patient administration. For example, an early-stage compound under development by Janssen now has a developing companion diagnostic test, through a collaboration of Siemens Healthcare Diagnostics and Janssen Pharmaceutica NV. IsoPlexis Corporation developed the IsoCode chip, which, in conjunction with their IsoLight Platform, allows precise functional patient profiling to help predict and understand the complex response of patients to cancer immunotherapies. IsoCode was used by Kite Pharma to analyze CAR T-cell therapy produced for 20 patients with non-Hodgkin lymphoma and predicted complete or partial patient response to the product. The list of approved or cleared companion diagnostic devices can be found at the FDA website [134].

In Vivo Diagnostics In contrast to in vitro diagnostics, in vivo nanodiagnostic agents are administered to the patient, and are thus subject to the same full premarket regulatory approval that nanotherapeutic agents and other drugs might be subjected to. Unfortunately, as mentioned previously, this contributes to the already low incentive pharmaceutical companies have to develop companion diagnostics, in addition to low demand. Research partnerships between pharmaceutical companies and academic institutions could fast-track the development of companion diagnostics since many NP-based imaging agents, methods, and protocols come from academic institutions originally. There has not been a major method of imaging-based diagnostic development because of intellectual property issues, but a priori intellectual property agreements could minimize such issue [135].

7.6.2 Animal Models vs. Human Disease

Though cancer is colloquially thought of as a disease in itself, in reality, the human disease is incredibly varied, not just among cancer sites but even at the cellular level. Because of this variability and complexity, researchers have struggled to produce models that are sufficiently complex to recapitulate disease progression and test drugs in, even using small animal systems such as mice or rats. Most commonly used for this purpose are xenograft models, where populations of immortalized cancer cells are injected subcutaneously in mice and allowed to grow into tumors there. However, these models have been shown to be too simplistic, often overestimating the effect of EPR and underestimating the interactions of many cell types with each other. Many other approximations of the actual human disease have

been attempted and developed, including orthotopic models [136], xenografting of cancerous biopsies into mice (patient-derived xenograft, PDX), genetically engineered models (GEMMs), humanized mouse models, and induction of cancer using carcinogens [137]. These more advanced models may provide more accurate clinical insight than basic xenograft models; however, they have been shown to vary significantly regarding reported nanoparticle performance between systems. For example, NP biodistribution has been shown to vary across animal species, where larger NPs tend to accumulate in the liver in humans, mice, rats, monkeys, chickens, and rabbits; they accumulate primarily in the lungs of sheep, pigs, goats, and cats [138]. Selecting preclinical animal models for the evaluation of NPs should consider these confounding factors.

7.6.3 Identification of Imaging Biomarkers

While NPs can significantly increase biomarker detection in vitro because of the ease of conjugation to strong, specific binding agents, in vivo applications have not been shown to be as reliable due to interactions of NPs with components of the body. Because of this, quantitative NP-based tools for clinical imaging do not yet exist, despite the rising importance of quantitative imaging. Quantitative clinical imaging is a relatively new field that is starting to be established, with the establishment of the Quantitative Imaging Biomarkers Alliance (QIBA) in 2007 and the Quantitative Imaging Network (QIN) in 2008. These teams of investigators work together to identify quantitative imaging problems to be solved and develop tools for clinical validation. The ability of NPs to selectively accumulate in tumor tissue and to actively target specific biomarkers could hold significant advantages for clinical imaging. However, NP signals in vivo have not yet been calibrated for observations in larger animals or humans, specific biomarkers still need to be validated, and protocols need to be standardized for human applications.

7.6.4 Safety Concerns and Mitigation Strategies

7.6.4.1 Batch-To-Batch Consistency and Scale-Ups

There are many challenges for the development and approval of nanodiagnostics, and the most critical component of successful clinical translation of NPs for in vivo diagnostics is the ability to consistently manufacture these particles for clinical use [139] with morphological uniformity (size and shape), consistent surface charge, and consistent drug loading. In fact, approval of some NP agents has been revoked because of the issue of reproducibility in all these aspects [140]. Every additional moiety increases the NP's complexity, and reproducibility can become impossible. Although reproducibility during small batch production for the sole purpose of

laboratory research does not warrant reproducibility in scale-up batch preparation required for clinical translation, there have been success stories of reproducible, large-scale manufacturing methods for NPs used in early clinical trials [141–143].

Another hurdle is the lack of characterization method consistency for NPs [144]. For example, there are at least five separate measurement techniques one could use to determine NP sizes: dynamic light scattering, electron microscopy, atomic force microscopy, and X-ray diffraction. Another typical example is zeta potential, a critical measurement of surface charge that is often inconsistently reported. In this case, though measurement methods are similar, the conditions of the measurement (such as pH of the medium or ionic strength) can have a significant impact on the final reading and are often not reported. Overall, even though inconsistent reporting creates a known problem for reproducibility and comparison across synthesis methods, the field still lacks a recognized set of standard physicochemical characterizations. To address this issue, the Nanotechnology Characterization Laboratory (NCL), part of the NCI's Alliance for Nanotechnology in Cancer Program, has developed a series of assays to physicochemically characterize NPs. Through this effort, the NCL has developed standardized protocols for physicochemical characterization as well as preclinical efficacy and toxicity testing to facilitate the progress of nanodiagnostics through the regulatory approval [145–147]. This kind of standardized characterization methodology and reporting that is agreed upon by the field of nanomedicine, particularly for cancer applications, is critical to the future maturation of the NP biomedical field.

7.6.4.2 Nano–Bio Interactions and Biodistribution

Other barriers to the application of in vivo nanodiagnostics are systematic characterizations of potential toxicity, in vivo interaction before reaching the target sites, and biodistribution patterns [148]. Therefore, a complete evaluation of these critical parameters is crucial to robust in vivo applications. In addition to in vitro safety assays, additional parameters are needed such as in vivo dosimetry, the injection and imaging regimens, excretion and accumulation profiles of these NPs, acute metabolic response, as well as long-term and acute effects of the administered agents. Evaluation of these parameters can be tedious and complicated but needs to be done in different animal species as we move from mouse models to larger animals and eventually to humans, as the correlations are not linear across species. A good example illustrating the importance of nano-bio interaction and biodistribution of NPs is iron oxide nanoparticles. Although ferumoxytol is considered safe as an "off-label" MRI contrast agent in several clinical trials, there have been several reports of life-threatening anaphylactic reaction to intravenous administration of ferumoxytol [149, 150]. As such, the FDA issued a black box warning on potential adverse reactions [151]. In addition to carefully monitored dosage and infusion regimens, some nano-bio interactions should not be ignored such as potential immune response [152], long-lasting skin discolorations surrounding the injection site, and iron oxide-induced chronic inflammation and reactive oxygen species productions [31].

7.7 Conclusions

Nanomedicine stands to revolutionize the field of in vivo cancer diagnostics using almost every modality clinically available and to enable new imaging modalities previously not possible. They can increase specificity and sensitivity over traditional contrast agents with active targeting and nanoparticle-specific signals and push the limits of current biomarker detection. These include nanoparticles with relatively immediate clinical application ability, such as biocompatible lipid and polymer-based nanoparticles, as well as other types of nanoparticles that require more basic science and toxicity work to reach the clinic. Many novel nanoparticles incorporating designs mimicking the nano-bio interface have been developed for in vitro studies, and these can provide insights into disease states and tumor morphology while also acting as a form of treatment. They can also serve to detect and amplify minute quantities of biomarkers, thereby aiding in early diagnosis and disease staging. As most imaging techniques for in vivo detection of biomolecules evolved from in vitro molecular assays, it is a matter of time that these nanotechnology-based in vitro assays will be adapted for in vivo imaging applications.

Nevertheless, we anticipate tremendous hurdles in the path for clinical translation of in vivo nanodiagnostics, especially in cases involving novel nanoparticles. Multiple challenges outlined in the last section demonstrate that these challenges are complex and intertwined. Consequently, ways to overcome these challenges are far from straightforward and require interdisciplinary efforts in research and development. While it is impossible to address all these challenges at once, emerging awareness and remediating efforts for the individual challenges presented by different disease pathologies are obvious as nanoparticle-enabled applications in biomedicine are becoming more mainstream. A possible stumbling block in the clinical translation of nanodiagnostics could hinge upon the way research and development of nanotechnology or nanoparticles has been conducted, i.e., designing new tools first and then finding the use for these tools later. With the clinical translation of nanodiagnostics in mind, it makes practical sense to first assess the challenges in clinical diagnostics before pursuing further research and development of a promising nanotechnology platform to address these clinical challenges. Despite regulatory and industrial challenges, there is no doubt that the field of in vivo nanodiagnostics is an active area of research with significant positive progress toward clinical translation.

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Chapter 8
Delivery of Cancer Nanotherapeutics

Bomy Lee Chung, Joseph Kaplinsky, Robert Langer, and Nazila Kamaly

8.1 Introduction

Spanning half a century of research and development, cancer nanotherapeutics describe the incorporation of therapeutic agents in nanoparticles (NPs) that are capable of selectively delivering these toxic payloads to tumors and cancer cells (via passive or active targeting) [1, 2]. This highly multidisciplinary approach to drug delivery has been driven by our greater understanding of manipulating matter at the nanoscale and by discoveries in nanomaterial design and engineering [1, 3–6]. Furthermore, our increased understanding of biological mechanisms, coupled to the fast pace of development in biotechnology and nanotechnology, and the emerging successes of cancer therapeutics in the clinic have spurred an extremely high level of interest and investment in this field [1, 6, 7]. Using biocompatible, bioeliminable, and degradable NPs, the selective accumulation of chemotherapies within tumors has led to improvements in therapeutic index, increased efficacy, and decreased toxicities [2].

Without changing the structure of existing chemotherapies, cancer nanotherapeutics can improve their pharmaceutical properties by facilitating their solubility, increasing circulation half-life and tumor accumulation [1, 2]. Furthermore, NP delivery of chemotherapies enables payload versatility whereby anticancer small molecules, macromolecular polyamino acids, or nucleic acids can be delivered [7].

B. L. Chung · R. Langer
David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Department of Chemical Engineering, Cambridge, MA, USA
e-mail: bomy@mit.edu; rlanger@mit.edu

J. Kaplinsky · N. Kamaly (✉)
Technical University of Denmark. Department of Micro and Nanotechnology, DTU Nanotech, Lyngby, Denmark
e-mail: jkap@nanotech.dtu.dk; nazk@nanotech.dtu.dk

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The spatiotemporal release of the therapeutic payload can also be tuned and controlled (controlled-release) [8]. Simultaneous therapy and diagnosis capabilities are possible (theranostics), as well as the ability to deliver multiple therapeutics (combination therapy) [9–18]. Although considerable nanotechnological successes have been achieved up to now, the dramatic therapeutic outcomes envisaged have not been observed, in part due to complex biological barriers. These include opsonization of the NP surfaces once administered into blood, which leads to their sequestration by immune cells, non-specific biodistribution, and complex tumor biology. The scale-up and manufacturing of NPs also present challenges. Physicochemical properties of NPs such as size, geometry and/or shape, composition, surface charge, surface chemistry, hydrophobicity, roughness, elasticity, and rigidity influence their behavior in vivo and are related to these aforementioned challenges. These NP design parameters are now an active area of research in the field of preclinical cancer nanotherapeutics research and development. Careful design of cancer nanotherapeutics can overcome these barriers, and it is critical to address these inadequacies of cancer nanotherapeutics so that their full clinical potential can be achieved. In this review, we discuss cancer nanotherapeutics in clinical development, in addition to providing insights into their routes of administration and biological barriers.

8.1.1 Cancer Nanotherapeutics

Cancer nanotherapeutics are composed of NPs with either a solid or hollow aqueous core, which commonly entrap poorly soluble drugs (Fig. 8.1a). The development of cancer nanotherapeutics follows the same rigorous approval protocols as small molecule investigational new drugs (IND) and requires similar bench-to-bedside development time frames (Fig. 8.1b).

The surface of the NP can be rendered hydrophilic with the use of polyethylene glycol (PEG) [19]. This grafting of PEG molecules onto the surface of NPs is termed PEGylation. PEGylation can act as a protective cover of NPs, “masking” them from the immune system and thus mitigating immunogenicity, further enhancing the efficacy of the NPs, and it is currently the standard technique for improving the pharmacokinetics, plasma half-life, and biodistribution of NPs in vivo [20].

NPs accumulate at specific sites in the body through blood hemodynamic forces as well as diffusive mechanisms and, in particular, become trapped within tumors through the enhanced permeation and retention (EPR) effect [21]. This theory was proposed following the discovery in the 1980s that colloidal polymer-drug conjugates accumulate in tumors because of fenestrations in tumor vessel endothelia as a result of excessive branching and chaotic vasculature, leading to breakdown of tight junctions and disruption of the basement membrane [22, 23]. As a result, oncology applications of nanomedicines have been widely studied, resulting in a huge research drive in the design of NPs of different sizes, shapes, and surface characteristics—with the aim of improving tumor accumulation [24]. Currently the majority of cancer therapeutics in clinical use are PEGylated first-generation “passively

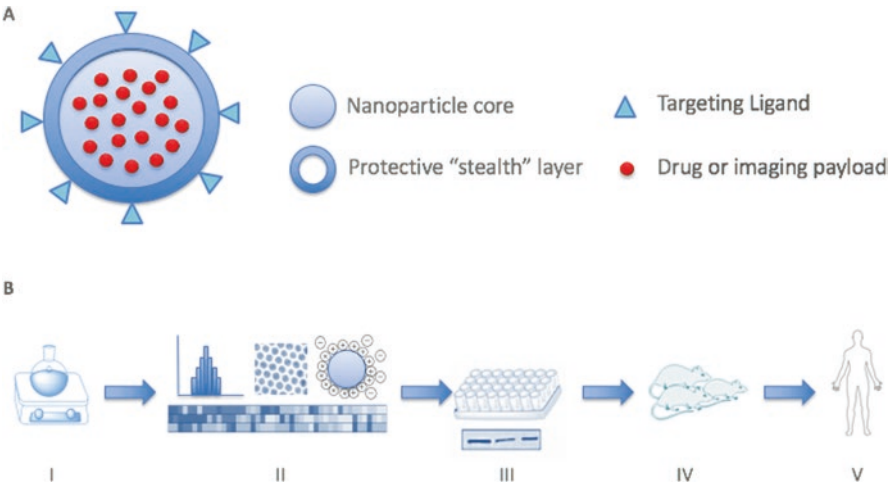

Fig. 8.1 Overview of nanomedicine development. (**a**) A generic structural representation of common cancer nanotherapeutics. (**b**) The overall process of nanomedicine development from bench to bedside. (I) NPs can be synthesized using a variety of techniques involving reverse-solvent precipitation, emulsification, sonication, or extrusion. (II) Physicochemical properties of NPs (including size, hydrodynamic size, polydispersity, surface charge, shape, ligand characteristics, drug loading and release rate, solubility, stability, storage, sterility, and batch-to-batch reproducibility) are measured and compared between various NP formulations. (III) Once in vitro biological assays measuring NP toxicity and biocompatibility, cell viability, efficacy, and cellular uptake are investigated, this screening stage identifies top leads. (IV) Lead NPs are screened in vivo for toxicity, pharmacokinetics, biodistribution, and efficacy studies in numerous species. (V) First-in-human or phase I trial translation upon successful robust preclinical data and investigational new drug (IND) filing (e.g., with US FDA) follows

targeted" NPs that can accumulate in tumors due to the EPR effect [25]. The ability to graft hydrophilic neutral polymers of PEG onto the surface of clinical stage liposomes and polymeric NPs goes hand in hand with EPR discoveries and led to increased blood circulation times of NPs and subsequent improvements in tumor accumulation [19, 26, 27].

Even though many cancer nanotherapeutics are passively targeted and exploit the EPR effect for tumor penetration and accumulation, preclinical features of the EPR model often do not hold true in patients [28]. Although several studies have examined the pharmacokinetic behavior of NPs across species and different tumor models, these data have not been effectively correlated with efficacy in patients [29–31]. Preclinical research has begun to address the limitations of EPR by investigating the normalization of tumor vessels prior to NP administration, in addition to utilizing companion diagnostics with approved colloidal imaging contrast agents to assess EPR prior to treatment [32–34]. Tumor hallmarks such as hypoxic gradients, increased interstitial pressure, tumor heterogeneity, and lack of or predisposition to the EPR effect in individual patients merit further investigations to ascertain this theory as the main mode of NP tumor accumulation [2, 28].

Small molecules such as folic acid, antibodies, antibody fragments, antibody mimetics, proteins, peptides, and nucleic acids (e.g., aptamers) can be conjugated to the surface of NPs to achieve "active targeting" to cancer cells [24]. Targeted NPs also accumulate within tumors due to the EPR effect, although active targeting can lead to the intracellular uptake of cancer therapeutics. Only a handful of targeted cancer therapeutics are in clinical development [35]. Among the limitations that have delayed the translation of targeted cancer therapeutics are lack of validation of specific and robust disease targets for targeting, inter-patient variability of receptor expression, undesired recognition and sequestration of targeted NPs by immune cells, lack of correlation between in vitro and in vivo findings, and difficulties in scale-up and manufacturing [2].

Liposomes are the oldest platform to have been investigated and therefore the first NPs to enter the clinic, followed by polymer-drug conjugates (colloidal nanosized particles) and polymeric micelles. As a result, liposomes represent a large portion of clinical-phase nanocarriers [36]. Polymeric NPs, protein-based NPs, silica NPs, and dendrimers follow on from these platforms (Fig. 8.2).

A recent study estimated that over 1,500 clinical trials involving nanomedicines had been conducted in the USA (up to 2015), 88% of which were cancer-related [37]. Approximately 250 nanomedicine products were investigated in clinical studies; this includes over a dozen nanomedicines, nanocarriers, and polymer-conjugates that are currently approved and marketed (Table 8.1). Specifically, cancer nanotherapeutics based on liposomes (for Kaposi's sarcoma, ovarian and breast cancers, multiple myeloma, osteosarcoma, and acute lymphoid leukemia) [27, 38–48], albumin (for pancreatic and non-small cell lung cancers and advanced metastatic breast cancers) [49–54], polymeric micelles (for breast, lung, and ovarian cancers) [55–61], and nanosized polymer-drug conjugates (for liver cancer) [62–65] have been approved by the FDA.

A few targeted cancer nanotherapeutics are also undergoing clinical development and include Her2 scFv-targeted liposomes (MM-302) [75], a targeted controlled-release polymeric NP BIND-014 [29], and a targeted siRNA NP CALAA-01 [76]. Additionally, further cancer nanotherapeutics based on dendrimers [77–79], gold [80–82], silica [83–85], iron oxide [84, 86, 87], and hafnium oxide [88, 89] are currently under clinical investigation (Fig. 8.2) [90]. Cancer nanotherapeutics are also in clinical translation for gene therapy [91], RNA interference [76, 92–95], and immunotherapy delivery [96, 97]. Viral NPs have also found utility in the specific delivery of a range of therapeutics, including genetic material to tumors [98, 99]. Nanodiamonds [100–102] and nanographene [103, 104] are receiving attention in drug delivery applications as well. The versatility of NPs (where antigens, adjuvants, and targeting moieties can be incorporated into single NPs for immunotherapies) has also led to their development into synthetic vaccines [105, 106].

Although many cancer nanotherapeutics have been shown to improve the therapeutic index of drugs, as yet only CPX-351 has exhibited an overall survival benefit to cancer patients (as a measure of hematologic toxicity) and reduced the risk of early death when directly compared with the conventional parent drug. CPX-351 is a liposomal formulation of cytarabine and daunorubicin in clinical development for

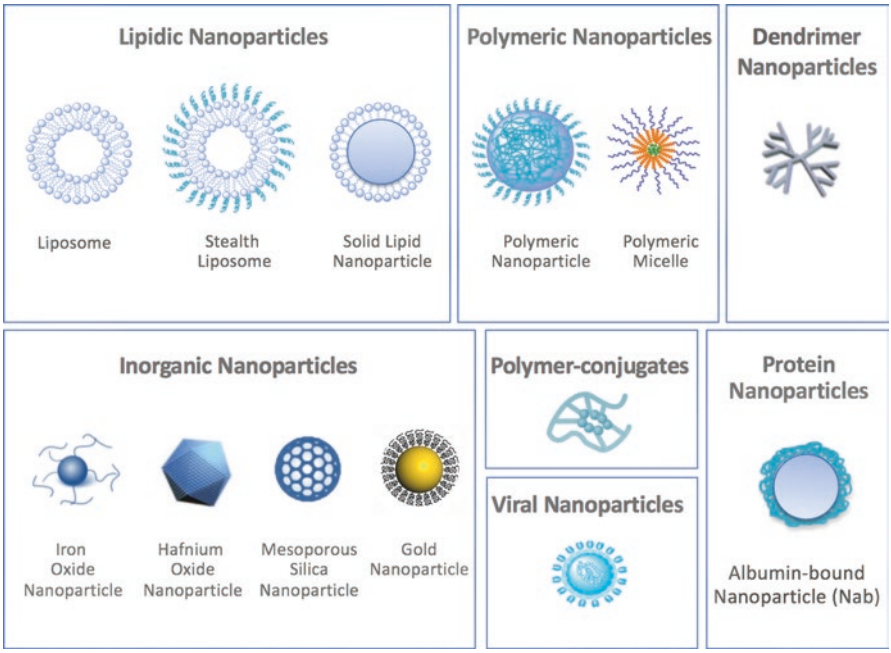

Fig. 8.2 Nanoparticles (NPs) approved or undergoing various stages of clinical trials [37]. *Lipidic NPs*: Liposomes are spherical NPs created from the self-assembly of amphiphilic lipids that form vesicle bilayers enclosing an aqueous compartment used for delivering small molecule drugs, nucleic acids, or proteins (sizes ~100–200 nm). Stealth liposomes incorporate a polyethylene glycol (PEG) shell on their surface for enhanced in vivo circulation and immune system shielding. Solid lipid NPs (SLNs) have a solid lipidic core (may include lipid acids) capable of solubilizing lipophilic drugs and are also used for nucleic acid delivery; SLNs are stabilized using emulsifiers. *Polymeric NPs* are solid-core nanomedicines formed from the self-assembling of polymers, generally biodegradable. Polymeric micelles are small NPs formed from lipids or polymers that have hydrophilic-hydrophobic core-shell structures and are used for drug delivery (size range 10–100 nm). *Dendrimer NPs* consist of symmetrically branched polymeric macromolecules synthesized using polyamides (with small sizes ~10 nm). The most common types of dendrimers are polyamidoamine (PAMAM) and poly(propylenemine) (PPI) based; these molecular structures can be loaded with hydrophobic drugs, either covalently attached or as a result of hydrogen bonding interactions. *Inorganic NPs*: Iron oxide nanoparticles (IONPs) are metal oxide-based small sub-100 nm NPs that are either used as MRI T_2contrast agents leading to increased darkening of images or in hyperthermia-based therapy. Hafnium oxide NPs are inorganic NPs used as a radiosensitizer. Mesoporous silica NPs have a porous structure and can either entrap or covalently bind various drugs. Gold NPs are used with lasers to achieve localized thermal ablation. *Polymer-drug conjugates* are nanosized colloidal drug delivery systems (5–20 nm) consisting of the covalent conjugation of drugs or therapeutic proteins to pendant groups on the polymer backbones; the first examples were drugs conjugated to poly(hydroxypropyl methacrylamide) (PHPMA) polymers. *Viral NPs:* poxviruses with tumor-homing properties have been engineered; JX-594 expresses the granulocyte colony-stimulating factor (G-CSF) to aid increase of the immunological antitumor response. Herpes simplex virus (HSV) type 1-derived, GM-CSF-expressing virus (T-Vec), and adenoviruses have been tested in patients. *Protein NPs:* Nab is an albumin protein-based nanocarrier for drug delivery and nab-paclitaxel; Abraxane® is currently approved

Table 8.1 Examples of approved injectable nanotherapeutics and polymer-conjugates

Nanomedicine	Class	Administration route	API	Indication	Approved	Company	Ref
Zinostatin®	Polymer-protein conjugate	Transcatheter arterial injection	Styrene maleic anhydride	Liver cancer	1994	Yamanouchi	[62, 63]
Doxil®/ Caelyx®	Liposome	i.v. infusion	Doxorubicin	Kaposi's sarcoma Ovarian cancer Breast cancer Multiple myeloma	1995 1999 2003 2007	Johnson & Johnson	[27, 38, 39]
DaunoXome®	Liposome	i.v. infusion	Daunorubicin	Kaposi's sarcoma	1996	Galen	[40, 41]
Lipodox®	Liposome	i.v. infusion	Doxorubicin	Kaposi's sarcoma Ovarian cancer Breast cancer	1998	Taiwan Liposome	[42]
Myocet®	Liposome		Doxorubicin	Breast cancer	2000	Cephalon	[44]
Oncaspar®	PEG-protein conjugate	i.m. or i.v. infusion	L-Asparaginase	Leukemia	2006	Enzon/Sigma-tau	[66]
Genexol-PM®	Polymeric micelle	i.v. infusion	Paclitaxel	Breast cancer Lung cancer Ovarian cancer	2007	Samyang Biopharm	[55, 56]
Mepact®	Liposome	i.v. infusion	Mifamurtide MTP-PE	Osteosarcoma	2009	Takeda	[67]
NanoTherm®	Iron oxide NP	Intratumoral injection	Iron oxide	Thermal ablation of glioblastoma	2010	Magforce Nanotech	[68]
Marqibo®	Liposome	i.v. infusion	Vincristine	Acute lymphoid leukemia	2012	Talon	[45, 46]
AmBisome®	Liposome	i.v. infusion	Amphotericin B	Fungal infections	1997	Gilead Sciences	[25]
DepoCyt®	Liposome	Intraventricular or lumbar puncture	Cytarabine	Lymphomatous meningitis	1969	Upjohn	[69]

Abraxane®	Albumin NP	i.v. infusion	Paclitaxel	Breast cancer, NSCLC, pancreatic cancer	2005, 2012, 2013	Abraxis BioScience	[70, 71]
Visudyne®	Liposome	i.v. infusion	Benzoporphyrin	Abnormal blood vessels in the eye	2000	Bausch + Lomb	[72, 73]
DepoDur®	Liposome	Epidural	Morphine sulfate	Analgesia	1984	Pacira Pharmaceuticals	[74]

API active pharmaceutical ingredient, *MTP-PEG* muramyl tripeptide phosphatidylethanolamine, *i.v.* intravenous, *i.m.* intramuscular

high-risk acute myeloid leukemia and has been approved in 2017 following successful phase III trials [107]. This lack of progress emphasizes the need for new approaches such as implementing patient selection to identify those most likely to respond to cancer nanotherapeutics, analogous to antibody drug patient selection already in clinical practice.

Ultimately the effectiveness of cancer nanotherapeutics can be limited due to the complex physiological and biological barriers in the body [108, 109]. Once administered into blood, an NP will interact with many components such as plasma proteins and immune cells that are intended by the body to seek and destroy foreign entities [110, 111]. Although our understanding and fine-tuning of NP biophysicochemical properties have improved over time, we are only now starting to appreciate to a much greater degree the contribution of blood components and the tumor microenvironment in limiting or enhancing cancer nanotherapeutic efficacy [33, 112, 113]. Furthermore, as NP therapies transition from animal models and into patients, it becomes increasingly important to consider the route of administration. Selecting the correct route of administration offers an opportunity to minimize the hindrance of biological barriers to NP circulation and delivery to target sites. In addition, the route of administration will determine the biological environment that the NP experiences and therefore should be taken into account in the design parameters of cancer nanotherapeutics [114]. Although the delivery of each nanomaterial and tumor type needs to be studied on an individual basis, general criteria that need to be understood and implemented as best as possible for the production of optimal cancer nanotherapeutics have emerged from investigations to date and will be discussed in this chapter.

8.2 Routes of Administration of Cancer Nanotherapeutics

The effectiveness of most chemotherapeutic drugs is dose-limited, and the maximum tolerated dose is set by systemic (i.e., off-target) toxicity [115, 116]. Alternative injectable routes of administration have been explored primarily to maximize the concentration of chemotherapies in the tumor relative to the rest of the body [117]. Administration by infusion remains the most common route for the delivery of chemotherapies, and therefore conventional chemotherapies are generally administered by intravenous (i.v.) or by intraperitoneal (i.p.) injections, depending on the cancer type and location [118, 119]. In the clinic, chemotherapies are less commonly administered by intramuscular, subcutaneous, intrathecal, intrapleural, intravesical, intra-arterial, or intra-tumor injection routes. Although i.v. and i.p. administration are the common routes of clinical administration of cancer nanotherapeutics, they can also be administered via the oral, nasal, retro-orbital, and intracranial routes in preclinical studies. Ultimately, the site and route of injection of cancer therapeutics will depend on the type and location of the tumor they are intended to treat. The contribution of route of administration to the overall efficacy endpoint of cancer therapies should be investigated in preclinical models. For example, it was shown that the efficacy of etoposide loaded tripalmitin NPs used for the treatment of

Dalton's lymphoma in tumor-bearing mice was highly dependent on the route of administration [120]. Subcutaneous injection led to a reduction of NP biodistribution to nontarget tissues and organs, and high accumulation in the tumor tissue, but not for i.p. injection. Intravenous distribution resulted in lower NP concentrations in organs of the mononuclear phagocytic system (MPS) compared to the free drug. High brain accumulation of the NPs was also found after i.p. injection. This study demonstrated that of the three routes investigated (subcutaneous, i.v., and i.p.), subcutaneous injection resulted in severalfold higher concentrations of NPs within tumors. In the following section, we discuss the key routes of administration of cancer nanotherapeutics utilized in the clinic.

8.2.1 Intraperitoneal Administration

The peritoneum is a membrane that lines the abdominal wall and wraps the organs surrounding the abdomen including the spleen, stomach, transverse colon, small intestine, and most of the liver [121]. The peritoneum is made up of two separate layers, the parietal layer that is attached to the abdominal wall and the visceral layer that is attached to the intraperitoneal organs (Fig. 8.3) [121]. The peritoneal cavity

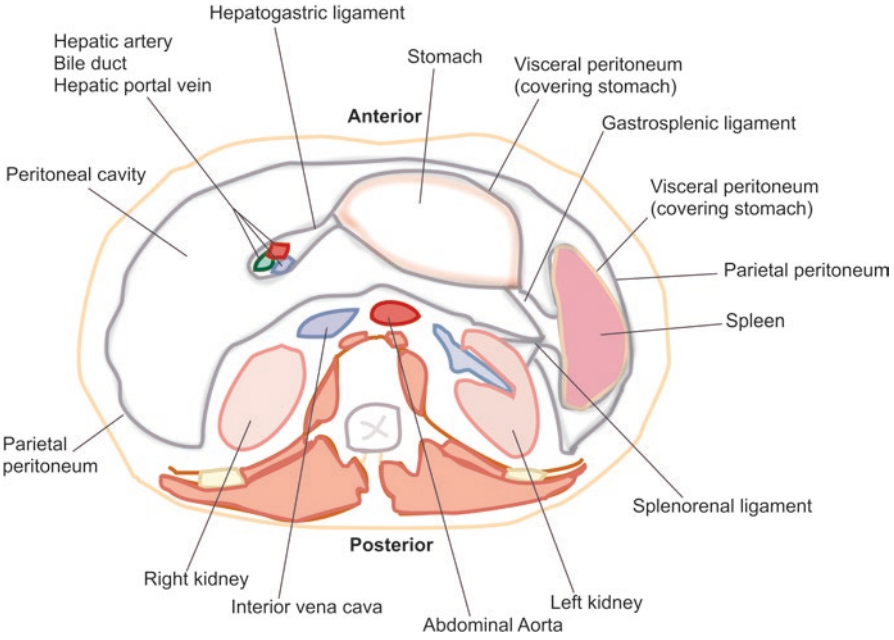

Fig. 8.3 Peritoneal cavity. The peritoneal cavity is the injection site for intraperitoneal injections. The peritoneal cavity is the space between the peritoneum around the abdominal wall (parietal peritoneum) and the peritoneum that surrounds the internal organs (visceral peritoneum)

refers to the space between these two layers. It is a thin layer filled with serous fluid that lubricates motion between the visceral and parietal layers, accommodating shifts in the intraperitoneal organs. The peritoneal cavity is connected across the abdominal cavity and forms a route through which tumor cells that have broken through the visceral membrane can metastasize [122]. The peritoneal cavity is therefore a site of metastasis for common cancers including ovarian, colon, pancreatic, gastric, and liver. As a result, i.p. delivery (i.e., injection into the peritoneal cavity) has become a well-established route of administration for these conditions. A well-recognized advantage of i.p. delivery is the capacity to achieve high local concentrations due to slow transport of drugs across the peritoneum to reach systemic circulation.

I.p. delivery has the advantage of using spatial proximity to expose tumors located in the peritoneal cavity to high drug concentrations, where 20–1000-fold higher drug concentrations have been achieved in cancer patients, compared to that measured after i.v. administration [123]. While few cancer nanotherapeutics formulated for i.p. have reached the clinic, preclinical evidence suggests that i.p. delivery of nanomedicines results in dosing to the tumor both from intraperitoneal release and, indirectly, from released drug and NPs that are transported into systemic circulation [121].

However, even where particles are transported out of the peritoneal cavity, differences in overall biodistribution have been noted. For example, less accumulation is seen in the lungs when compared to i.v. administration, which may be a consequence of the reduced "first-pass" effect in which i.v. administered NPs pass straight from the heart to the lung [124].

More recently a number of i.p. injected formulations and NPs have entered clinical trials. EGEN-001 is a formulation consisting of a plasmid encoding the cytokine IL-12 formulated with PEG-polyethyleneimine-cholesterol lipopolymer. Phase I trials have demonstrated the safety of EP-001 delivered i.p. in patients with recurring ovarian cancer, both alone [125] and in conjunction with i.v. chemotherapy [126]. Results for phase II trials of EGEN-001 in 22 patients were recently reported. The protocol administered EGEN-001 as a monotherapy at a dose of 24 mg/m^2 on a weekly basis [127]. Overall the trials demonstrated the practicality of i.p. delivery protocols, but limited efficacy has been shown. However, in light of preclinical understanding, results have been encouraging enough to proceed with a further phase I trial of EGEN-001 delivered i.p. in combination with liposomal doxorubicin delivered i.v. (ClinicalTrials.gov Identifier NCT01489371).

There have also been two recent trials of paclitaxel NP formulations delivered i.p. The first is Nanotax®, a Cremophor-free paclitaxel NP [128]. Nanotax® is produced using supercritical carbon dioxide resulting in 600–700 nm rod-shaped NPs which act as depots in vivo. Phase I trials involved i.p. delivery of Nanotax® to 21 patients with a variety of peritoneal malignancies using dose escalation every 4 weeks to a maximum of 275 mg/m^2. The results showed that 2 days following dosing, the concentration-time profile of peritoneal paclitaxel was elevated 450–2900 times above that of plasma. The formulation was well tolerated, with five patients surviving longer than 400 days.

The second i.p. delivered paclitaxel nanotherapy to reach phase I trials is Abraxane®, an albumin-bound formulation that has already been approved by the FDA for i.v. treatment of conditions including breast and pancreatic cancers [63]. A recent phase I trial enrolled 27 patients with peritoneal carcinomatosis (14 cases secondary to a gynecological malignancy, 12 cases secondary to a gastrointestinal malignancy, and 1 case of peritoneal mesothelioma). A 28-day dose escalation schedule was used with maximum dose of 170 mg/m^2 (ClinicalTrials.gov Identifier NCT00825201). Again the concentration-time profile of peritoneal paclitaxel was significantly elevated over plasma, by a minimum of 50-fold and a typical enhancement of 150-fold [129].

I.p. administration is not currently used as a main mode of NP delivery for targets outside of the peritoneum, the high interstitial fluid pressure poses a barrier to drug delivery via the peritoneal cavity, and if NPs are not able to cross the peritoneum, then prolonged local drug release can lead to peritoneal toxicity [123]. Therefore, i.p.-based cancer nanotherapeutics for drug delivery outside of the peritoneum should be designed to effectively traverse local barriers.

8.2.2 Intravenous Administration

Parenteral administration (injections or infusions) remains the most common route for chemotherapy as the entire administered dose reaches the circulation immediately and is a well-developed methodology (Fig. 8.4) [130]. The route of administration relies largely on the drug's pharmacological properties (e.g., absorption, metabolism, and half-life), and since most chemotherapies exhibit poor oral bioavailability, they are most commonly administered via intravenous injection. In patients, drugs are usually delivered into the subclavian vein. In the case of ongoing therapy, delivery is often through an implanted port [131, 132]. Alternative routes for intravenous administration are the cephalic vein in the arm or the femoral vein in the groin. The subclavian vein leads directly to the heart, resulting in rapid distribution of the drug through systemic circulation after a first pass through the lungs.

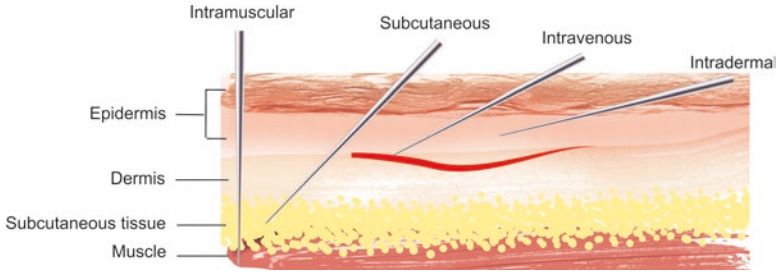

Fig. 8.4 Different routes of parenteral administration. Administration via the parenteral route can involve intramuscular, subcutaneous, intravenous, or intradermal injections, which are achieved with the appropriate sized needle and specific angle of injection

Delivery to the systemic circulation by intravenous administration has the advantage that it gives access to all respiring cells in the body, including any tumor cells. This makes it a strong general-purpose technique. However, this access is also a disadvantage in that it becomes harder to concentrate or target the therapy within the tumor, and in the case of NPs, they become prone to filtration by the liver and spleen. Since most chemotherapies are dose-limited by systemic toxicity, this is a major concern in clinical practice.

Following systemic administration, efforts are now focused to develop cancer nanotherapeutics that can reach targeted tissues at sufficiently high dosages for maximal clinical effects. This means that the NPs must be able to (1) circulate and remain for prolonged periods of time within the body, (2) cross the appropriate vessel walls to reach the tumors, (3) navigate the tumor space to reach the cancer cells, and (4) be internalized by these cancer cells. A number of biological barriers are faced by intravenously injected NPs, and preclinical investigations are currently attempting to address these. In this chapter, we focus on the barriers encountered by i.v. administration.

8.3 Biological Barriers

Intravenous administration into blood leads to NP clearance and accumulation in organs such as the liver, spleen, bone marrow, brain, and lungs [133]. Orally delivered NPs can be transported across the gut and to the liver via the first-pass mechanism through the hepatic portal vein [134]. NPs administered via inhalation can become entrapped within alveolar macrophages inside the alveoli [135, 136]. A final filtration barrier is the kidneys, where NPs can also be cleared based on their size and charge [137].

Healthy vessel walls allow the translocation of particles in the 5–12 nm size range [138]. In addition, by synthesizing and injecting rats with quantum dots (QDs) of varying sizes, Choi et al. showed that kidneys can rapidly filter NPs when their hydrodynamic diameter is <5.5 nm [139]. This suggests that NPs should be larger than this size in serum to avoid non-specific extravasation and renal clearance. NPs can also be cleared from circulation through the liver and spleen via the MPS. Using a range of polymeric micelle NPs, Cabral et al. found that particles larger than 100 nm were more likely to have a lower plasma half-life and accumulate in the liver when injected into murine models of colon adenocarcinoma [140]. Similarly, Moghimi et al. found that particles >200 nm were filtered more efficiently by the spleen [141]. Altogether, these older initial findings indicate that NPs should optimally fall within the 6–200 nm range for increased circulation times. In this section we discuss these aforementioned barriers as related to the journey of a NP once administered intravenously through blood and key organs.

8.3.1 Blood Circulation

Once administered into the body, NPs are subject to opsonization (absorption of plasma proteins to NP surfaces for recognition by phagocytes) [142] and come into contact with cells of the innate immune system, which include monocytes, macrophages, and dendritic cells that either circulate in blood or are resident in tissue, especially the lung, liver, lymph nodes, and spleen [143, 144]. These cells recognize the tagged NPs via the opsonins and pattern recognition receptors, and this interaction can lead to the rapid clearance and sequestration of NPs via phagocytosis [145]. This process, which is carried out by the MPS, can lead to the secretion of proinflammatory factors [108]. A variety of other plasma proteins also bind to NP surfaces, and this binding of serum proteins to NPs is now recognized as the "protein corona," which gives NPs an "in vivo identity," affecting their interactions at the plasma, organ, and subcellular levels, in addition to their clearance rate [112, 146–155]. The clearance rate of NPs in plasma can be tuned by considerations of their size, shape, charge, and surface chemistry. Up to now the main strategy used to circumvent or minimize unwanted recognition is PEGylation of the NP surface [19, 156, 157]. Other mostly preclinical attempts include using zwitterionic ligands such as cysteine or glutathione on the NP surface [158], and biomimetic strategies such as the use of self-recognizing peptides or proteins on the NP surface [159], or entrapping NPs within the cellular membranes of leukocytes, platelets, or red blood cells, which can mask the NP from recognition as nonself [160–162].

Strategies that can minimize the non-specific accumulation of NPs in organs and tissues can lead to higher doses reaching target sites and therefore lowered dose requirements. Another strategy to improve the circulation of NPs and facilitate endothelial contact and extravasation into tissues and tumors is to improve the margination of NPs by using non-spherical disclike NPs that are prone to lateral drift, with larger surface areas that can increase vessel wall contact with NPs [111, 163]. The thorough investigation of the effect of NP interactions with the MPS is very important as these therapeutics are mostly given to patients at highly advanced stages of cancer, who have already received chemotherapies with compromised immune systems.

8.3.1.1 Protein Corona Effects

The adsorption of plasma proteins provides the NPs with a biological identity that determines the physiological responses they elicit, ranging from cellular uptake and intracellular trafficking to pharmacokinetics (PK), biodistribution, and toxicity [154, 164]. Furthermore, it appears that the protein-type bound to the NP surface can influence its circulation lifetime, as recently binding of dysopsonin proteins (such as apolipoproteins and albumin) were shown to prolong NP presence in systemic circulation [151–153].

In relation to the protein corona, it was recently shown that PEGylated polystyrene NPs are highly prone to selective adsorption of clusterin (a 80 KDa chaperone protein), which led to reduced non-specific macrophage uptake of these NPs in vitro [165]. These findings and other results suggest that effects of PEGylation could be mediated by changes in the nature of the protein corona [165, 166]. Protein corona formation can also reduce the efficacy of the targeting ligands coupled to NPs [166] or, in some instances, promote organ-specific accumulation, as in the case of apolipoprotein E-mediated targeting of NPs to hepatocytes in vivo [167].

In clinical settings, NP-protein interactions were shown to trigger hypersensitivity reactions in patients via activation of the complement system [164]. Predicting such in vivo responses will require careful analytical techniques and protein fingerprinting to better understand how the NP corona influences pharmacokinetics, biodistribution, intra tissue, organ and tumor biodistribution, and therefore therapeutic efficacy of NPs [168–171].

8.3.2 Liver Clearance

It is estimated that on average less than 5% of injected NPs actually reach their intended target, and the liver can sequester and filter between 30% and 99% of administered NPs from the blood circulation [172]. NPs that are >5.5–10 nm are not cleared by the kidneys and therefore eventually become degraded by the hepatobiliary and MPS systems. Even though the liver is mostly composed of parenchymal hepatocytes, NPs are mostly prone to uptake by non-parenchymal cells such as Kupffer cells. Kupffer cells are macrophages in the liver that phagocytose NPs, and the uptake and retention of NPs within these cells are highly related to the NP surface charge, chemistry, and size [172]. In general, NPs that are highly charged (either cationic or anionic) are able to adsorb increased amounts of serum proteins, leading to aggregation and higher affinity to macrophage uptake [151]. Larger NPs (>100 nm) are also more prone to uptake by macrophages as they possess greater surface areas for interaction with these cells [151].

Physicochemical properties of NPs such as shape and surface chemistry can be optimized for reduced liver clearance and increased efficacy. For example, rod-shaped NPs were shown to be taken up significantly less than spherical NPs by the MPS [173–175]. Wormlike particles with high aspect ratios were hardly taken up by macrophages and had prolonged circulation times, perhaps due to lack of accessible binding surfaces by macrophages [173–175]. It has also been shown that rigid particles are more prone to uptake by macrophages and that NP elastic modulus can be tuned (decreased) to increase blood half-life circulation [176]. PEGylation and the use of zwitterionic surfaces, as mentioned, are also strategies to minimize macrophage uptake of NPs.

8.3.3 Kidney Clearance

Nanoparticle size has been widely explored in the design of effective nanomedicines. Most therapeutic NPs are within the 30–150 nm size range and are not subject to kidney filtration into the urine, unless they are degraded into particles <10 nm or if the glomerular filtration barrier is damaged by disease. Considering the influence of NP size on the biodistribution and clearance of NPs and size-dependent kidney transport barriers, it is therefore an important parameter for investigation.

Since the transport of NPs in circulation is influenced to a greater degree by the applied convective forces in blood than Brownian motion, the shape of NPs has an important effect on in vivo performance, biodistribution, and kidney clearance. A wide range of NP geometries have been manufactured using a variety of methods [177]. Interestingly, the kidneys are capable of filtering rigid NPs with high aspect ratios but with diameters smaller than the kidney filtration threshold. These NPs are predominantly single-walled carbon nanotubes (SWCNTs) with a rod length of 100–1000 nm and 0.8–1.2 nm diameter [178]. Even though SWCNTs have higher molecular weights (300–500 kDa) than plasma proteins (30–50 kDa), they were shown to be cleared by the kidney, implying that NP aspect ratio influences directional diffusion across endothelia [178]. One proposed underlying mechanism for this phenomenon is that hydrostatic forces orient carbon nanotubes perpendicularly to the kidney glomerular basement membrane and thereby facilitate the insertion of their <10 nm axis [178, 179].

Charge selectivity is also an important criterion for kidney filtration. This was demonstrated by investigating the kidney distribution of intravenously administered ultrasmall anionic NPs (3.7 nm QDs) as a model system, and accumulation was assessed using fluorescence imaging [180]. The majority of NPs were initially distributed within the peritubular capillaries or the glomerular arterioles, which then passed through the fenestrated glomerular endothelium and were gradually taken up by the mesangial cells (for up to 30 days) [180]. Only trace amounts of QDs could be detected in the urine, since the glomerular basement membrane is negatively charged, and filtration of anionic QDs was prevented due to repulsion, yet cationically charged QDs of similar size were found in much higher concentrations in the urine filtrates [180]. These studies provide a framework for understanding NP charge interactions across kidney cellular barriers, which mostly have negatively charged cell surfaces.

8.4 Tumor Microenvironment

The tumor microenvironment (TME) includes subpopulations of genetically diverse cancer cells, normal cells, vascular/endothelial cells, immune cells, and blood cells together with the tumor interstitium. All these components have a role in cancer progression [181]. Tumor-associated macrophages (TAMs), cancer-associated

fibroblasts, and endothelial cells have been shown to play a role in tumor progression. It is anticipated that reprogramming of the TME via non-cancerous cells can lead to tumor regression. For example, targeting stromal cells has gained attention recently [182, 183]. Stromal cells have a major role in tumor growth and maintenance—and their reprogramming can be a potential new avenue of cancer treatment approach [184]. Another striking example is the use of immune checkpoint blockade inhibitors to reprogram immunosuppressive TMEs. Nanomedicines offer solutions for the effective targeting of each of these components and pathways. In particular the combination delivery of multiple chemotherapeutics to tumor cells can be achieved using targeted NPs. Nano-enabled synergistic combination drug delivery to cancer cells can more effectively kill these cells and therefore minimize resistance [185].

Tumor vessels are irregularly spaced and structured relative to healthy vessels. Studies have shown that endothelial vessel gaps within the same tumor can have wide-ranging sizes, from 200 to 800 nm [186]. The vascular density within tumors is also nonuniform. For example, tumor cores tend to be less vascularized and to have compressed (and thus nonfunctioning) lymphatic vessels, which can result in necrotic areas [187]. This is attributed to varying levels of oxygenation within tumors, with clinical tests indicating that many tumors have regions of significant hypoxia [188]. Hypoxic tumors have been correlated with increased resistance to chemotherapy [189]. This correlation has been attributed to the increased difficulty of effectively targeting cancer cells within tumors due to poor and dysfunctional vascularization, as well as cancer cells' abilities to withstand nutritive deprivation and evolve to survive under hypoxic conditions, which may enhance treatment resistance and evasion [190]. Furthermore, there may be an increased potential for metastasis as the leaky and disordered tumor vasculature can lead to the infiltration of cancer cells into the normal circulatory system [191].

Even if NPs are able to cross into tumor interstitial spaces, they might not penetrate deeply into tumors due to pressure differentials, slow diffusion processes, and interactions with the tumor microenvironment. For instance, Stylianopoulos et al. observed that neutral particles tend to diffuse faster within tumors than charged particles [192]. They noted that collagen fibers in the tumor matrix have slightly positive charges, and this likely leads to slower diffusion of anionic particles within tumors. Similarly, Lieleg and colleagues studied the effect of geometric and electrostatic interactions between the tumor matrix and particles. They concluded that NP surface charge is a key factor affecting NP mobility, more so than the geometric and steric hindrances [193]. Transport of NPs within tumors is also affected by pressure differences between the microvascular and interstitial fluids, as well as the interactions between the tumor matrix and NPs. The EPR effect generally results in elevated tumor interstitial pressures as a result of fluid leakage from tumor vessels and inefficient drainage of fluids from the tumor core [191, 194].

The heterogeneity of cancer cell populations within tumors must also be considered, as different cells will react differently to nanotherapeutics. It is now known that cancer cells within the same tumors will exhibit varying characteristics, including morphologies, phenotypes, reaction to drugs, level of drug resistance, and

ability to metastasize. Denison and Bae suggest a tumor could be seen as a "new, independent organ acting within the host" [189]. Similar to organs that have complex structures and organizations with cells, each with specific functions and environments, they argue that recent evidence shows such complexities also exist within tumors [195]. For example, Caracas et al. propose that cancer stem cells, responsible for tumor formation, are found in their own niche microenvironment [196]. These contain cytokine sources (e.g., mesenchymal stem cells, endothelial cells, and lymphocytes) that contribute to the formation and growth of these cancer stem cells. Although multiple theories exist as to the cause of this heterogeneity between cancer cells, the connection between genetic, epigenetic, tumor microenvironment, and external factors remains to be elucidated.

8.4.1 Cancer Cell Uptake

Nanoparticles may be designed to act once they are within the tumor microenvironment where they can release their drug payload or intracellularly (i.e., inside cancer cells). In the former case, it should be sufficient to consider the tumor microenvironment characteristics previously discussed. In the latter, however, additional considerations must be kept in mind, including the mechanism of NP uptake into cancer cells and their transport. The mechanisms through which particles traverse the cell membrane can be categorized as (1) phagocytosis, (2) macropinocytosis, (3) clathrin-mediated endocytosis, (4) caveolae-mediated endocytosis, and (5) clathrin- and caveolae-independent pathways [197].

Clathrin-mediated endocytosis is the most studied receptor-mediated endocytic mechanism. It is the most commonly used endocytosis pathway in most cells, and it is normally involved in NP uptake by cancer cells. This process is hypothesized to be spontaneous, beginning with the binding of particles onto membrane receptors and the assembly of the AP2 protein complex [143]. A clathrin layer then starts forming, which eventually caves into the cell membrane and is excised, releasing clathrin-coated vesicles into the cell. These vesicles have been shown to range in size, from around 30 nm to over 1 μm in diameter [198, 199]. An understanding of the mechanism through which NPs traverse into cells is important as it determines the eventual fate of the particles and the cellular biological response. For instance, clathrin-mediated endocytosis has been used to penetrate lysosomal compartments, whereas caveolin-mediated endocytosis has been used to reach non-lysosomal compartments. As such, the ability to design nanotherapeutics that target specific uptake pathways will greatly impact their therapeutic efficacy.

Once NPs are taken up via endocytosis, endosomal escape is the next requirement. If NPs are delivering small hydrophobic drug molecules that are capable of permeating the endosomal lipid bilayer, then endosomal entrapment of NPs can still result in sufficient intracellular drug concentrations, and the drug is released over a period of time. However, endosomal release is required for the effective delivery of siRNA, miRNA, DNA, and other charged or hydrophilic small molecule drugs that

cannot permeate the endosomal membrane [200]. Furthermore, endosomal release should occur before the fusion process of endosomes with lysosomes as biomolecules can be degraded due to the lowered pH levels in lysosomes [201]. Endosomal escape mechanisms based on pH buffering and osmotic swelling have been investigated to facilitate endosome membrane destabilization [202–205].

Investigations on the effective cellular internalization of NPs have shown that NPs between 40 and 50 nm in size facilitate maximal uptake in in vitro studies [206]. Although it is worth noting that NP uptake can also be cell type-dependent [207]. Over the years, a major strategy to improve cellular uptake of NPs has been to include targeting ligands on the surface of NPs. These ligands could then specifically recognize overexpressed receptors on proliferating cancer cells. In the next section, we discuss active-targeting strategies.

8.4.1.1 Active Targeting

The bioconjugation of targeting moieties including antibodies, antibody fragments, peptides, aptamers, sugars, and small molecules to NP surfaces has led to the development of targeted nanotherapies and active-targeting approaches [26, 208–210]. The inclusion of such ligands can facilitate the direct binding of NPs and their subsequent uptake into cancer cells. These ligands bind targets specific to or overexpressed on the surface of proliferating cancer cells. Considerations to take into account when choosing an appropriate ligand include ligand size, affinity, immunogenicity, ease of scale-up, and manufacturing costs. For example, the use of antibody fragments is more ideal than whole antibodies as they lack an immunogenic component (the Fc-antibody region). The binding affinity of ligands (K_D) and receptor expression levels should also be investigated.

Active-targeting approaches can lead to therapeutic improvements when ligand-mediated cell internalization is achieved [211–214]. In addition to active-targeting approaches, effective tumor targeting also requires deeper tumor penetration and retention. For example, recently tumor-penetrating peptides such as iRGD with a R/KXXR/K C-terminal peptide motifs were used to facilitate neuropilin-1-mediated vascular permeability [215].

A number of actively targeted NPs administered i.v. are currently undergoing clinical trials, and these include the liposomal formulations MCC-465 (PEGylated doxorubicin liposome formulation with dimers of F(ab') fragments) [216], anti-EGFR ILs-dox (anti-EGFR (epidermal growth factor receptor) targeted) [217], SGT-53 and MBP-426 (transferrin receptor (TfR)-targeted liposomes) [218, 219], 2B3-101 (glutathione receptor-targeted liposome), and MM-302 (HER2-targeted PEGylated doxorubicin liposome) [75]. MM-302 was recently shown to improve the antitumor activity of oxaliplatin in HER-2-positive breast cancer, when administered after cyclophosphamide priming, and is currently the most advanced targeted NP in the clinic (undergoing phase III clinical trials) [220]. Since the overexpression of TfR, EGFR, and HER-2 receptors is observed in a range of cancers, these ligands are attractive active-targeting strategies [35, 221]. Other tar-

geted nanotherapies currently undergoing clinical trials are BIND-014 (polymeric NP targeted to prostate-specific membrane antigen (PSMA)) [222] and CALAA-01 (TfR-targeted NP) [24, 223].

The subcellular targeting of NPs can also be achieved using folic acid, low-density lipoprotein, cholera toxin B, mannose-6-phosphate, Tf, riboflavin, the tripeptide RGD, ICAM-1 antibody, or nicotinic acid-targeting strategies [224]. Cellular internalization with these ligands can occur via clathrin-dependent receptor-mediated endocytosis, caveolin-assisted endocytosis, lipid raft-associated endocytosis, or cell adhesion molecule (CAM)-directed cellular uptake [224–226].

Although various targeted nanotherapies are undergoing clinical trial investigations, no targeted NP has been approved as yet, and this lack of clinical translation can be due to multiple factors. These include our limited understanding of the nano-bio-interface with blood plasma components and their fate once targeted NPs have been systemically administered in vivo, unfavorable protein binding to the surface of NPs leading to rapid sequestration by the liver and spleen, inter- and intra-patient receptor expression level variation, lack of tumor penetration of targeted NPs, and inadequate affinities of the chosen ligands. Furthermore, targeting ligands add further complexity to the scale-up and manufacturing of NPs, which presents a further hurdle for their accelerated translation.

8.4.2 Chemoresistance

Chemoresistance remains a challenge for cancer nanotherapeutics and is therefore discussed here. In metastatic cancers, it is attributed with 90% of drug failures [227]. Chemoresistance can be intrinsic or acquired, and it may arise due to a process of natural selection: a subpopulation of cancer cells resistant to chemotherapy survives and grows. In particular, cancer stem cells are now believed to play key roles in evading chemotherapies and building chemoresistance through multiple mechanisms of action, including ABC transporter protein expression and enhanced DNA damage response [228]. Chow et al. have found that certain subsets of hepatic cancer stem cells are more resistant than others to cancer chemotherapeutics, and this is predicted by the presence of the multidrug resistance gene 1 (MDR1), which encodes P-glycoprotein (P-gp), an ABC transporter protein [229]. MDR1 has been shown to influence the efflux of various chemotherapeutics, and Chow's studies indicate that MDR1 leads to the ejection of doxorubicin and paclitaxel from primary hepatic tumor cells, increasing chemoresistance in these cell subsets. Inhibition of proteins involved in these mechanisms has been studied, but there has been little success as these studies have led to trial-ending adverse effects [230]. Moreover, different cancer cells express proteins with different and, at times, redundant functions, which suggests that administration of multiple therapeutics and inhibitors will be necessary. The co-delivery of multiple active pharmaceutical ingredients can facilitate synergistic cancer therapy and bypass mechanisms of drug release, and many such studies are currently underway and discussed elsewhere in detail

[231–236]. Drug delivery using targeted NPs capable of accumulating at high concentrations within cells can aid in potentially overcoming drug efflux. Studies in drug-resistant mouse models have shown an increased antitumor activity for targeted NPs such as folate-receptor-targeted polymeric micelles and transferrin-conjugated paclitaxel NPs, when compared to their nontargeted counterparts [231–236].

8.5 Emerging Therapies

The limits to cancer chemotherapy have driven the investigation of new treatment modalities. The most prominent of these are immunotherapies and genetic therapies such as those which make use of the RNAi pathway. Nano-formulations of these therapies have not yet been approved for clinical use, but in both cases, it is likely that nanotechnology will be a key enabler to an even greater extent than has been the case with more well-established chemotherapies. Below we review the lessons that are emerging from examples of these technologies that have reached clinical trials. It is likely that experience with these technologies will also be of most relevance to still emerging techniques such as delivery of mRNA molecules [237] or gene editing with CRISPR-based systems [238].

8.5.1 Genetic Therapies

RNA interference-based therapies show great promise in their potential for specificity and for targeting proteins thought "undruggable" by small molecules. However, the challenges faced by RNA delivery are at least as great as those faced by small molecules. The human body contains ubiquitous RNA degradation enzymes and immune recognition receptors that have specifically evolved to recognize RNA. Naked RNA molecules are small enough to be filtered by the kidney, resulting in rapid clearance [239].

Although RNAi is specific for a particular protein, activity in off-target healthy tissue can still result in toxicity. These factors put a premium on targeting of siRNA and miRNA payloads to diseased tissues. In addition, RNA faces the hurdle of entering the cytosol, which is required for its mode of action. DNA-based plasmid therapies must in addition reach the nucleus. While siRNA and miRNA molecules are generally smaller than mRNA or plasmids, they are still far larger (>12 kDa) than small molecule therapeutics and are negatively charged both of which make crossing the plasma membrane difficult.

While these factors can be addressed to some extent by chemical modification of RNA molecules [240], truly effective clinical translation will require that they are addressed through delivery systems and route of administration.

As can be seen from Table 8.2, trials are usually carried out using i.v. administration. The first exception is the hepatic intra-arterial administration of TKM 080301. In this case an angiogram was used to place a catheter at the time of each weekly dose for direct delivery of the nanoparticles. It is notable that even the liver, which is the easiest target for nanodelivery, has required investigation of such optimized administration. The second exception is the intra-tumoral injection of pbi-shRNA

Table 8.2 Gene or RNAi cancer therapies that have entered phase I or II trials

Name	Platform	API	Administration	Clinical trial identifier (clinicaltrials.gov)
CALAA-01	Cyclodextrin NP	siRNA	i.v., 30 min infusion	NCT00689065
PNT2258	Liposome	24-base ssDNA	i.v., 2 h infusion	NCT01191775, NCT01733238, NCT02226965, NCT02378038
TKM 080301	Lipid NP	siRNA	i.v., 30 min infusion	NCT02191878, NCT01262235
			Hepatic intra-arterial administration	NCT01437007
SNS01-T	Polyethylenimine NP	siRNA and plasmid	i.v.	NCT01435720
ALN-VSP	Liposome	Two siRNAs	i.v., 15 min infusion	NCT01158079, NCT00882180
Atu027	Liposome		i.v., 4 h infusion of drug diluted in 5% xylitol	NCT01808638
MRX34	Liposome	siRNA mimic	i.v., 2–4 h infusion	NCT01829971
DCR-MYC	Lipid NP	siRNA	i.v., 2 h infusion	NCT02314052
SGT53	Liposome	plasmid	i.v., 2 h infusion of drug diluted in 5% dextrose	NCT02340117, NCT02340156
miR-16 Mimic TargomiRs	Minicell	miRNA Mimic	i.v., 20 min infusion of drug in 20 mL saline	NCT02369198
siRNA-EPHA2-DOPC	Liposome	siRNA	i.v., 30 min infusion	NCT01591356
pbi-shRNA EWS/FLI1	Bilamellar invaginated vesicle	Plasmid	i.v.	NCT02736565
pbi-shRNA STMN1 LP	Bilamellar invaginated vesicle	Plasmid	Intra-tumoral injection	NCT01505153
DOTAP: Cholesterol-Fus1	Bilamellar invaginated vesicle	Plasmid	i.v., 30 min infusion of drug in 100 mL of 5% dextrose	NCT00059605
MTL-CEBPA	Liposome	dsRNAa	i.v.	NCT02716012

STMN1 LP. In this case although the drug was delivered intra-tumorally, the intention was to gather data relevant for i.v. delivery [241].

Overall the trials reveal that these agents are poorly tolerated without both oral and i.v. premedication, typically with dexamethasone, to reduce infusion-related reactions. For example, in the DOTAP: Cholesterol-Fus1, trial administration was attempted without premedication, but adverse reactions forced the adoption of premedication with dexamethasone and/or diphenhydramine. In the case of MRX34, fever, chills, rigors, and back pain at time of administration led to premedication with dexamethasone [242]. This was not sufficient to prevent the dropping of MRX34 from further development due to these adverse effects [243]. In the case of miR-16 Mimic TargomiRs designed against mesothelioma, poor toleration of infusion led to a low maximum tolerated dose of 1.5ug per week. This led the investigators to suggest that higher dosing could be achieved with intrapleural administration [244].

While immunogenicity is a problem for these formulations, in most cases trials have shown an acceptable MTD using premedication. An exception is for Atu027, which was tolerated in humans without premedication [93]. Based on preclinical data in animals including *rhesus* macaques, it has been suggested that siRNA-EPHA2-DOPC will not require premedication. The investigators suggest this may be due to the less immunogenic character of the neutral DOPC lipids in their formulation compared to more usual cationic formulations [245]. However, it should be noted that the Atu027 particles when loaded with RNA are strongly positively charged, having a zeta potential of +46mV [246].

The remaining hurdles are targeting (especially to organs other than the liver) and achieving efficacy. Both DCR-MYC [243] and PNT2258 [243] have recently been dropped from development for lack of efficacy prior to even entering phase III trials. It is likely that the efficacy of genetic therapies will be heavily influenced by the ability of the API to achieve endosomal escape [247, 248].

8.5.2 Cancer Nanoimmunotherapies and Consideration of Injection Route

There is currently a growing interest in developing immune-modulatory cancer nanotherapeutics. Nanotherapies can enhance immune cell activation or sensitize immune-resistant tumors to checkpoint blockade inhibitors and to facilitate in situ vaccine strategies. NPs have the advantage of protecting antigens from proteolytic degradation in vivo, and the possibility to optimize their delivery to dendritic cells, in addition to providing the ability to deliver multiple antigens, immunostimulatory agents, and also chemotherapies in combination.

Unlike traditional cytotoxic drugs, immunotherapeutic agents are aimed at modulating cells of the immune system. In some respects this makes immunotherapies stronger candidates than cytotoxic drugs for delivery of NPs [249]. Immunotherapies

therefore raise distinct considerations for targeting of NPs and for route of administration. Particles need not reach the tumor in order to stimulate a response. The examples of tecemotide and dHER2 ASCI discussed below show that the motivation for exploring subcutaneous (s.c.) and i.m. delivery for immunotherapies is already reaching the clinic.

A number of cancer nanoimmunotherapies that deliver antigens have entered clinical trials. The most advanced is tecemotide, a liposomal formulation of a peptide antigen derived from MUC1, a protein whose glycosylation and expression are aberrant in many epithelial cancers [250]. Tecemotide delivers peptide to antigen-presenting cells together with adjuvant derived from lipid A, resulting in a cytotoxic T cell response. Notably, a s.c. route has been chosen for clinical development, based on much earlier preclinical work in mouse models, which showed improved response in s.c. versus i.v. [251].

Tecemotide has been developed through numerous trials resulting in a phase III trial for stage III non-small-cell lung cancer [252]. The protocol consisted of administration of one dose of i.v. cyclophosphamide (300 mg/m^2, maximum dose 600 mg) before beginning immunotherapy [252]. The initial tecemotide therapy was eight consecutive weekly subcutaneous injections (806 μg lipopeptide). In the absence of progressive disease or toxicity, maintenance tecemotide was continued every 6 weeks until disease progression [252]. Disappointingly, the trial resulted in no significant improvement in overall survival [252]. However, the capacity to elicit a cytotoxic T cell response suggests that a combination therapy with immune checkpoint blockade inhibitors may be effective [253].

dHER2 ASCI is a liposomal formulation containing a peptide from the HER2 protein, which is commonly overexpressed in cancers, combined with AS15 adjuvant. In a phase I trial in patients with stage IV HER2 overexpressing cancers, 500 μg of truncated HER2 in the dHER2 ASCI formulation was administered intramuscularly every 2 weeks for six administrations. In addition, lapatinib was given orally. This was repeated for up to three cycles. Results showed that the formulation could induce an antibody response, raising the potential of raising patient antibodies with functionality similar to anti-HER2 antibodies such as trastuzumab [254].

Two further antigen-based cancer nanoimmunotherapies, both of which are administered i.v., have entered phase I trials, but limited information is publicly available. JVRS-100 is a cationic lipid-DNA complex that has entered trials for acute myelogenous leukemia (ClinicalTrials.gov Identifier NCT00860522). Lipovaxin-MM is a liposomal formulation of a melanoma antigen trial (ClinicalTrials.gov Identifier NCT01052142).

There are also two cancer nanoimmunotherapies that have entered clinical trials based only on their stimulatory or adjuvant effects, rather than antigen delivery. Mifamurtide (which is a liposomal muramyl tripeptide phosphatidylethanolamine) has been approved for marketing in the EU as an adjuvant for patients (aged 2–30 years) with nonmetastatic high-grade resectable osteosarcoma. Due to limited efficacy, it has not been approved in the USA [255]. The active component in mifamurtide is a lipo-tripeptide derived from a motif found in bacterial peptidoglycan. This is thought to stimulate antibacterial danger receptors in the innate immune response

resulting in an inflammatory response. Mifamurtide is given with combination chemotherapy consisting of methotrexate, doxorubicin, and cisplatin with optional ifosfamide. Mifamurtide is administered by i.v. infusion twice weekly at a dose of 2 mg/m^2, with dose escalation up to 2 mg/m^2 + 2 mg if no biological activity is observed after the first administration [255].

Finally, CYT-6091 is a novel PEGylated colloidal gold nanoparticle that is conjugated to recombinant human tumor necrosis factor alpha (TNF-α), which acts to stimulate immune response. In phase I trials in patients with solid organ cancers considered no longer responsive to conventional treatment, CYT-6091 reconstituted in sterile water was administered as a single i.v. push injection of no more than 20–30 s through a central catheter line. This was followed by flushing with an injection of 15–20 mL 0.9% sodium chloride solution. Doses were escalated from 50 μg/m^2 up to 600 μg/m^2. Results showed that the dose-limiting toxic hypotension induced by free drug was avoided, while the half-life of TNF-α was extended around fourfold over free drug [256]. As increased numbers of nanoimmunotherapies reach the clinic, the choice of administration route is a paramount factor that needs to be investigated early on in the preclinical stage of development, since immunotherapeutic efficacy is highly dependent on site of administration.

8.6 Regulatory Considerations

Prior to approval, nanomedicines are evaluated by, for example, the FDA and the European Medicines Agency (EMA) according to existing regulations governing standard pharmaceuticals and must satisfy the same safety requirements that apply to investigational new drugs (INDs) [257]. Therefore, in order to expedite their translation, nanomedicines must be rigorously characterized and optimized to good manufacturing practice (GMP) requirements. However, because nanomedicines' multicomponency and the added functionalities of targeting or imaging inherently complicate NP design, chemical steps should be minimized, rigorously tested, and optimized to ensure that NPs are amenable to scale-up. Stringent characterization of NP physicochemical properties under manufacturing, storage, and use conditions is necessary. The FDA pathway for the regulation of nanotechnology products has been discussed in detail elsewhere [258, 259]. Ultimately the rigorous and comprehensive characterization of nanomaterials and demonstration of reproducibility, safety, and efficacy are key concepts for the facile translation of new nanomedicines from the laboratory to the clinic [37, 260]. Furthermore, researchers working on the preclinical development of nanomedicines must ensure greater awareness of the added excipients and additives required for stabilizing and effective storage of clinical stage NPs. These components should be utilized earlier on in the preclinical development stage of NPs, so that any instability or adverse reactions can be minimized.

Chemistry, manufacturing and controls (CMC) and GMP requirements must continuously be met as NP technologies transition from preclinical to clinical

development and then to commercialization and throughout the market life of the product. Current pharmaceutical manufacturing unit operations can meet the demands of first-generation NPs (liposomes and polymeric NPs). However, the scale-up of complex nanomedicines, such as targeted NPs, multidrug combinations, and multistage, stimuli-responsive, or theranostic systems will require adaptation of current manufacturing unit operations.

As has been alluded to, the route of administration is also an important factor in the efficient translation of cancer nanotherapeutics and must be investigated on an individual basis for each NP type and payload. Possible toxic effects related to the route of administration should be investigated, where acute and/or repeat-dose toxicity studies with complete histological evaluation should be conducted using the designated clinical route of administration. For i.v. injections, NP compatibility with blood (e.g., in vitro hemolysis, protein flocculation, platelet activation) should be investigated. In addition to route of administration, rate of infusion should also be evaluated.

Investigations into in vivo animal models which can more accurately reflect late-stage cancer patient biology are also important. Some studies have demonstrated PK scaling across species (including humans) for different nanotherapeutics [29–31, 93]; however, discrepancies between preclinical and clinical models must be considered, and animal models are needed that more closely mimic the heterogeneity and vascularization of human tumors.

8.7 Outlook and Conclusion

Oncology is the area where nanomedicines are making a high impact. Research into NP transport to tumors and factors involved in their biodistribution and uptake within the TME needs to be expanded and will likely result in safer and more precise cancer nanotherapeutics. Investigating the challenges of controllable, reproducible, and scalable NP synthesis as well as large-scale NP screening and evaluation will facilitate more rapid clinical translation. The large-scale manufacturing and translation of targeted injectable cancer nanotherapeutics in sufficient quantities for clinical use is challenging. The cost-to-benefit ratio of new therapeutics is assessed by cost-effectiveness analysis (CEA) and is used to ascertain whether an NP product is more beneficial than standard therapy based on costs. In this context, for example, the average cost of Doxil (liposomal doxorubicin) per injection was estimated at $5,594 vs $62–162 for doxorubicin alone [261]. However, the reduced cardiotoxicity achieved for doxorubicin delivered in liposomes is certainly a beneficial factor, which justifies the use of this nanomedicine even at a higher cost. Therefore, it is important to consider the real cost-to-benefit ratio of cancer nanotherapeutics in today's healthcare market. Still, collaborative efforts between academia, small and large biotech, and pharma companies together with the initiation of a number of funding programs are encouraging the further development of nanomedicines.

As of August 2017, several first-generation, nontargeted nanomedicines that are not specifically tumor homing have received clinical approval. However, more than two decades have passed since the approval of the first nanotherapeutic Doxil in 1995. Second-generation targeted nanomedicines have not been approved yet, albeit several promising nanoparticle platforms have been tested in clinical studies. However, the full benefit of targeted nanotherapies has not yet been realized in the clinic; thus, more research and development are needed to ascertain the cost-to-benefit ratio of targeted cancer nanotherapeutics over the next decade.

Patient-to-patient variability and tumor heterogeneity within the same patient present a major biological challenge to the design and development of targeted nanomedicines. Increasing our understanding of tumor heterogeneity and the realization of effective EPR biomarkers can help identify "nanomedicine-responsive" patients and further improve their clinical outcomes. Simultaneously targeting multiple biomarkers may therefore be required for efficacy. The role of conjugation chemistries, linker lengths, flexibility, and ligand densities in optimizing the targeting efficiency of these second-generation nanomedicines is vital but often remains under-explored in preclinical studies, especially in relevant in vivo models of cancer. Further, for targeted nanomedicines to be successful, the cellular uptake and intracellular processing of the nanoparticle platform and the payload are vital. Most targeted nanoparticles use some form of receptor-mediated endocytosis as a mechanism for intracellular drug delivery and effects. Lysosomal degradation of the nanoparticles and the trapped payload following receptor-mediated endocytosis may be one of the reasons targeted nanomedicines have not shown the dramatic improvements in therapeutic indices that have been expected from these tumor-homing constructs. The development of novel targeting ligands (e.g., peptides and aptamers) that can selectively trigger endosomal escape of the nanoparticles prior to lysosomal degradation may prove to be a promising strategy toward improving the therapeutic indices of targeted nanomedicines. Another hurdle in the development of targeted nanomedicines has been the lack of relevant preclinical models for testing their targeting and therapeutic efficiencies. The development of more relevant in vivo models that can recapitulate the complexity of human disease could help in optimizing the design of targeted nanomedicines during their preclinical development and perhaps ensure a higher percentage of success during clinical trials. A modular approach of assembling targeted nanomedicines may not only help optimize the numerous key, design variables, but also simplify scale-up and the production of targeted nanomedicines.

Ultimately physicochemical parameters need to be optimized for successful development of targeted NPs. Optimization of these parameters will lead to minimizing toxicity, unwanted interactions with the immune system, rapid renal clearance, and minimal accumulation in MPS organs. The field is steadily progressing, and we will see targeted nanomedicines en route to becoming valuable therapeutics in oncology with greater impact in the near future. As evidenced by increasing numbers of preclinical and clinical publications, development of cancer nanotherapeutics is currently on a sharp rise. Our understanding of biological interactions of these therapies needs to also progress at a fast pace if we are to truly achieve the full

potential of nanomedicines in the clinic for oncology applications. Theoretical and mathematical models could be used as well to design better targeted nanomedicines prior to preclinical and clinical testing.

As our understanding of the "nano-bio"-interface between NPs and blood components, cells, tissues, and organs improves, this should facilitate better treatment outcomes and more successful clinical trials. Coupled with our greater understanding of tumor biology and inter-patient variability at the tumor genetic level, we should be able to design more improved and personalized nanomaterials for the delivery of chemotherapies. A multidisciplinary approach with collaborations between theoretical and experimental scientists, engineers, medical doctors, pharmaceutical and biotechnology industries, government and private funding agencies, and the regulatory agencies is therefore required to realize the true potential of targeted nanomedicines in the clinic. We anticipate that in the coming decades, the true potential of nanomedicines will be realized, on par with the significant progress observed from monoclonal antibodies, which only now are becoming more widely utilized in the clinic, more than 40 years since their discovery. There is still "plenty of room" for cancer nanotherapeutics.

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Chapter 9
Oral Nanotherapeutics for Cancer with Innovations in Lipid and Polymeric Nanoformulations

Alexander J. Donovan and Ying Liu

9.1 Oral Chemotherapeutic Drug Formulations: Overview of Current Challenges, Issues, and Opportunities for Oral Absorption

The guiding principle for formulation scientists, pharmaceutical directors, and clinicians alike is to prioritize pills and tablets over intravenous infusions. Certainly, these pronouncements are wholly justified because orally administered medicines have robust data to confirm their clinical efficacy: their dosing regimens are uncomplicated, not requiring a visit to the hospital or specially trained clinicians for administration. They are also minimally invasive and do not cause the patient excessive pain. Taken together, oral administration is the most facile and least invasive strategy for drug therapy [1, 2]. However, certain newly discovered antineoplastic agents represent a particular formulation challenge because of their physicochemical sensitivity, poor absorption, and significant side effects, virtually precluding the application of current design strategies employed in the pharmaceutical industry including stalwart processes like hot-melt extrusion [3].

Biologic therapies, pharmaceuticals that are manufactured in bioreactors using recombinant protein engineering, are poised to supersede conventional small molecule medicines as the first line of therapy for a number of conditions [4].

A. J. Donovan
Department of Chemical Engineering, University of Illinois at Chicago, Chicago, IL, USA
e-mail: adonov4@uic.edu

Y. Liu (✉)
Department of Chemical Engineering, University of Illinois at Chicago, Chicago, IL, USA

Department of Biopharmaceutical Sciences, University of Illinois at Chicago, Chicago, IL, USA
e-mail: liuying@uic.edu

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These medications present unique formulation challenges and must be given intravenously or subcutaneously to be properly absorbed [5].

Notwithstanding, significant capital investment in cancer research and development has enabled several oral chemotherapeutic medicines to be approved and marketed in the United States, Europe, and elsewhere for several years [6]. For example, imatinib (Gleevec®, Novartis) is an effective therapy against chronic myelogenous leukemia (CML) and countless other cancers; it nevertheless manifests a series of severe side effects traditionally encountered with other oncolytic medicines [7].

One of the complications in the design of an efficacious oral cancer therapy is the mode in which the gastrointestinal tract functions simultaneously as both a chemical and a physical barrier to drug absorption compared to parenteral administration routes (Fig. 9.1). The stomach churns with juices of high acidity that rapidly degrade pH-sensitive compounds [8]. A plethora of enzymes hydrolyze proteins and peptides, lipids, polysaccharides, and esters. More importantly, the epithelium acts as a semipermeable membrane, allowing the transport of only valuable nutrients, but preventing the absorption of large hydrophilic molecules and some hydrophobic compounds as well [9]. Moreover, it also secretes a layer of several micron-thick viscoelastic fluid filled with a cross-linked network of proteins that readily expels foreign particles out of the body [10].

As such, the first-pass metabolism of active pharmaceutical ingredients (APIs) given orally is frequently much more significant than after parenteral administration, which therefore requires oral dosages to be many folds higher to achieve the equivalent serum concentrations of the drug. For example, docetaxel's oral bioavailability is low because it is extensively degraded by gastric and hepatic cytochromes and prevented from permeating the intestinal epithelium by a glycoprotein transporter. Docetaxel is therefore intravenously formulated to mitigate this extensive first-pass metabolism. However, when dosed orally concomitantly with the antibiotic cyclosporine, which competes for cytochrome binding and inhibits the epithelial protein, serum docetaxel concentrations dramatically improve [11].

Additionally, simply reformulating parenteral chemotherapies for oral administration at higher dosages in order to attain commensurate blood levels may be impractical and raises significant toxicological issues. Upon transit of the gastric epithelium, all orally ingested drugs enter the circulation only to be transported directly to the liver [12]. Overloading the intestinal mucus barrier with a chemotherapeutic in order to trigger drug permeation may thus lead to potentially toxic hepatic accumulation, e.g., with oral tamoxifen citrate [13]. Mindful of such scenarios, researchers have primarily focused on oral chemotherapies for colon cancer (e.g., oral capecitabine) [14], where transmucosal permeability is not crucial for treatment efficacy. In addition, oral bioavailability of these drugs can be further improved by using small molecule receptor tyrosine kinase (RTK) inhibitors [15].

Despite the formidable obstacles facing oral administration of novel chemotherapeutic medicines, a number of approaches are currently being investigated to overcome these formulation challenges beyond the current conventional clinical practice. It was demonstrated in small and big animals that polymeric nanoparticles with controlled physicochemical properties can dramatically improve hydrophobic drug

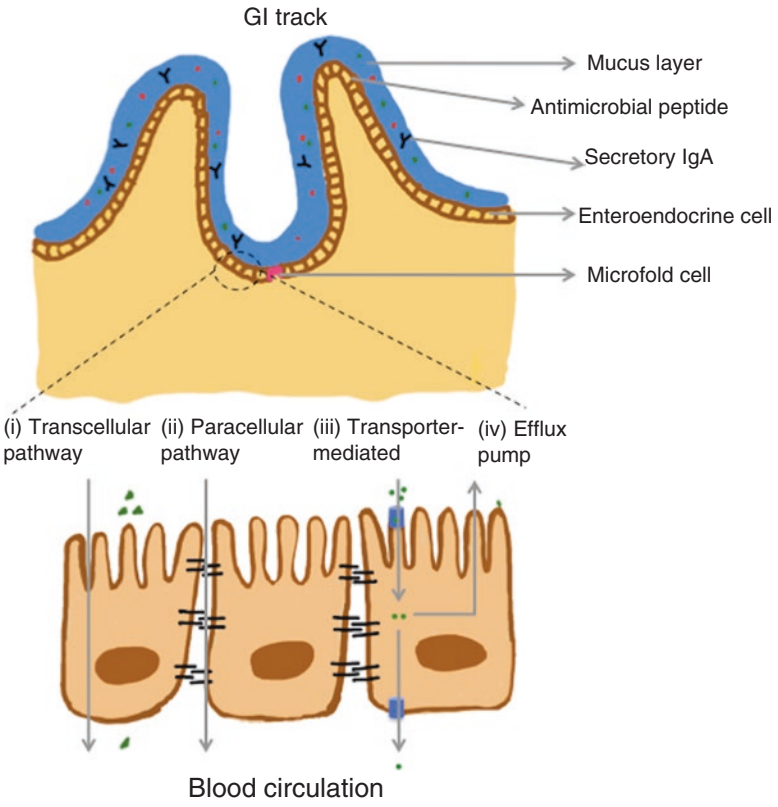

Fig. 9.1 Graphical description of the structural components of the mucosal barrier and drug transport mechanism across intestinal cells. The mucus layer acts simultaneously as both a physical and a chemical barrier, through the action of secretory immunoglobulins (secretory IgAs) and antimicrobial peptides. There are several possible mechanisms of drug transport through the intestinal mucosal barrier. The first pathway is the passive transcellular route. Through this pathway, drug permeates the cell passively by partitioning to cell membranes at both apical and basolateral sides. The second pathway is the paracellular route, where the drug transports through the tight intercellular junctions mediated by protein-protein interaction at different regions. The third pathway is the active transport route, where the drug is recognized by transporters and shuttled from the apical to the basolateral side. The fourth pathway is the mechanism of inhibiting drug permeation by efflux pumps. The efflux pumps expel the drug from cell membranes during the cell membrane partition process

oral availability [16–21]. O. M. Farokhzad et al. at MIT and Harvard Medical School have achieved a breakthrough in the oral delivery of biologic medicines by focusing on protein transporters distributed on the intestinal epithelium. By conjugating an antibody to a biodegradable polymeric nanoparticle containing the biologic medicine, the drug delivery system allows for the therapy to enter the bloodstream [22]. Chemical permeation enhancers (CPEs) such as surfactants and lipid compounds are another delivery strategy used to modify the morphology of the

gastrointestinal epithelium to enhance drug bioabsorption by orders of magnitude [23]. Perhaps even more promising for the pharmaceutical industry, nanotechnologies have enabled the invention of intelligent drug delivery vehicles employing natural lipid molecules and biocompatible synthetic polymers (e.g., platforms like phospholipid vesicles and PLGA nanoparticles, respectively) especially suited to transport drug cargoes to the targeting sites, promising enhanced efficacy and significantly reduced side effects [24]. Lipid- and polymer-based technology platforms for oral delivery of cancer chemotherapeutics will be discussed in the section below, with special emphasis placed on tailoring nanoparticle structure to modulate therapeutic function and in vitro assessments as a predictor of (and as a means to construct) in vivo pharmacological models.

9.2 The Big Picture: How Do Nanotherapeutic Systems Enable Oral Absorption?

Nanoscale drug delivery systems (DDSs) (with at least one dimension in the order of hundreds of nanometers or smaller) provide many advantages to current industry design strategies with regard to the formulation of orally administered cancer chemotherapies. Chemotherapeutic nanoparticles may actively or passively accumulate at the tumor site [25]. The APIs can be protected by immune assault and premature metabolism [26], which enhances the bioavailability of the APIs [27]. With these functional design elements, the lipid-based and polymeric nanotherapeutics explored throughout this chapter promise to outperform conventional oncolytic medicines. However, significant challenges remain in the development of easily accessible oral formulations.

Nanoscopic assemblies of lipids and polymers with hydrophobic constituents can be used to encapsulate poorly water-soluble drugs in their hydrophobic cores in order to minimize free energy. Rational design of these drug delivery systems can then ensure that the particles will be well dispersed in aqueous media and the drugs are absorbable either in the gastric mucosa or in the circulatory system, using structures with hydrophilic shells or other materials with amphipathic or surface active moieties for kinetic stability [28].

Free from the bulk solvent environment in the particle core, drug compounds are shielded from biochemical attack in the form of enzymatic hydrolysis or degradation, mitigating first-pass metabolism, for example, after oral ingestion. Chemically safeguarded in the particle interior, the compound's pharmacokinetics and pharmacodynamics are drastically altered, allowing for prolonged or enhanced therapeutic effect [29].

Decoration of the particle shell with polymers or biomolecules that favorably interact and entangle in the cross-linked mucin hydrogel comprising the intestinal mucous layer will promote drug release adjacent to the epithelial tight junctions to enhance absorption of poorly bioavailable compounds [30]. In instances where the

partitioning of the drug is so unfavorable, e.g., in the case of a very hydrophilic therapeutic peptide, then it is in practice and impossible for the compound to be transported across the epithelium even with the aid of a mucoadhesive carrier. In such scenarios the nanoscale drug delivery system (DDS) can be designed to penetrate completely through the GIT into the circulatory system [31]. Functionalization with polyethylene glycol (PEG) chains of certain molecular weights and at proper surface densities enables the DDS to penetrate certain vulnerable regions of the intestinal endothelium without becoming entangled in the mucus's proteinaceous web [32].

Equipped with an array of design strategies and precise control over the physical, chemical, and biological function of these nanoscale therapies, researchers and clinicians can successfully innovate efficacious lipid- and polymer-based DDSs for oral cancer chemotherapy. A review of the current state of the art and recognition of the challenges still remaining are discussed below.

9.3 Lipid Formulations and Technology Platforms

The application of lipid-based nanoformulations (Fig. 9.1) as drug delivery platforms overcomes several of the contemporary challenges encountered in the oral administration of oncolytic medicines: serving as a depot for hydrophobic and hydrophilic drugs alike, acting as a barrier to proteolytic and chemical degradation, and, perhaps most significantly, as a vehicle across tissues to target cells. Simultaneously, these nanotherapies offer a relatively benign and biocompatible alternative to other approaches employing synthetic polymers and chemicals as part of their formulary. In the following sections, nanoformulations incorporating both phospholipids and other related components are delineated. The most commonly studied type of lipid formulation is liposomes encapsulating hydrophobic or hydrophilic drug compounds.

Liposomes are spherical vesicles—hollow spheres consisting of one or more phospholipid bilayer shells ranging from tens of nanometers to microns in diameter. Resembling cells voided of their organelles, these vesicles naturally emerged as a primary drug delivery vehicle platform. With a facile synthetic route, they spontaneously self-assemble in aqueous conditions above their critical micelle concentrations (CMC). Due to their biomimetic structure—an aqueous core and selectively permeable bilayer membrane—liposomes could potentially encapsulate a diversity of drug compounds due to the vesicle's architecture.

Translation of liposomal formulations to clinical applications has achieved the most material progress to date in cancer chemotherapy [33]. Y Barenholz introduced Doxil® (nanoformulated doxorubicin), the first liposomal medication to gain regulatory approval by the Food and Drug Administration (FDA) in 1995 for Kaposi's sarcoma, a cancer manifested most frequently with AIDS patients [34]. The liposome shell consists of a very high melting temperature (T_m) of phosphocholine (HSPC) and a small amount of PEGylated phosphoethanolamine (DSPE-PEG_{2000})

to mitigate premature leakage of the drug and to confer it with "stealth" properties [26]. Despite the preconceived benefits of the liposomal formulation over the free compound, Doxil® has not been more universally adopted for treatment because of dose-dependent hypersensitivity reactions (HSRs) and other unforeseen side effects since its introduction more than two decades ago [35].

A myriad of other liposomal nanomedicines are in clinical development or have gained regulatory approval in North America and Europe (Table 9.1) [40, 45]. Amphotericin B, a potent antifungal agent which is poorly bioavailable and displays a severe number of side effects when administered intravenously, has been formulated into AmBisome® by Gilead, where the anti-infective compound is intercalated into the bilayer [36]. Epaxal is a liposomal vaccine, or "virosome," for hepatitis A. Inflexal V is a virosomal medication for influenza vaccination [40]. Myocet®, developed by Elan Pharmaceuticals and marketed by Cephalon in the European Union and Canada, is a non-pegylated formulation of doxorubicin for breast cancer treatment [43]. In addition, several other chemotherapeutic compounds have been successfully encapsulated as vesicle medicines. LipoCurc is a liposomal formulation of the poorly bioavailable anticancer compound curcumin and is currently in phase II trials for glioblastoma [40]. Daunorubicin (DaunoXome®, Galen Pharma) was approved for intravenous administration for Kaposi's sarcoma like its counterpart Doxil® [37]. The FDA approved a liposomal nanoformulation of vincristine (Marqibo®, CASI Pharmaceuticals) in 2012 for blood cancers [42]. Liposomal cytarabine (DepoCyt®, Pacira Pharmaceuticals) recently gained approval in 2010 for meningitis related to breast metastases [38]. PEGylated ("stealth") nanoformulations gaining entry to the market include nanoformulated cisplatin (Lipoplatin®, Regulon) for lung cancers [41], and a formulation of the topoisomerase I inhibitor Belotecan (S-CKD 602®, Chong Kun Dang) is in late-stage clinical trials [44]. However, none of the current liposome formulations is available for oral administration.

Beyond the inclusion of lipopolymers such as PEGylated lipids for prolonging circulation half-life, efforts to develop third-generation liposomal nanoformulations are focusing on platforms to develop therapies with additional or ancillary biochemical functionalities to home in on target histologies. Rather than relying on conventional passive methods such as enhanced permeation and retention (EPR) effects [25, 46], these next-generation smart medicines aim to exploit nonlinear, stimuli-driven, or threshold-switchable biochemical networks by responding to unique biochemical or environmental signals at their target organs. "Immunoliposomes" are phospholipid vesicles that have been chemically functionalized or physically decorated with immunoglobulins [47]. Chemically modified phospholipid head groups can easily be covalently linked to generic polypeptides via maleimide coupling chemistry under aqueous conditions if sulfhydryl groups are present [48]. Once decorated with antibodies, immunoliposomes can then home in on host cells that overexpress their target antigens [49]. However, developing oral available liposome-based therapy has to overcome the problem that liposomes are not stable under large fold dilution [51].

Table 9.1 Representative liposomal medicines at the clinical stage or on the market

Trade name	API[a]	Vehicle	Indication	Company	Clinical stage (year approved)	Approved country or region	References
AmBisome	Amphotericin B	Non-pegylated liposome	Antifungal	Gilead Sciences, Astellas Pharmaceuticals	1997	Australia, Canada, Europe, USA	[36]
DaunoXome	Daunorubicin	Non-pegylated liposome	Kaposi's sarcoma	Galen Pharma	1996	Brazil, Europe, USA	[37]
DepoCyt	Cytarabine	Non-pegylated liposome	Meningitis associated with breast cancer metastasis	Pacira Pharmaceuticals	2010	Canada, Europe, USA	[38]
Doxil	Doxorubicin	PEGylated liposome	Kaposi's sarcoma	Janssen Pharmaceuticals	1995	USA	[34, 35, 39]
Epaxal	Inactivated hepatitis A	Virosome	Hepatitis A	Crucell	1994	Asia, Europe, South America	[40]
Inflexal V	Inactivated influenza virus A/B	Virosome	Influenza A/B	Crucell	1999	Europe	[40]
LipoCurc	Curcumin	Non-pegylated liposome	Glioblastoma/cancer	SignPath Pharma	Phase II	Not yet approved	[40]
Lipoplatin	Cisplatin	PEGylated liposome	Lung cancers	Regulon	Passed Phase III	Not yet approved	[41]
Marqibo	Vincristine	Sphingomyelin/ cholesterol liposome	Blood cancers	CASI Pharmaceuticals	2012	Europe, USA	[42]
Myocet	Doxorubicin	Non-pegylated liposome	Breast cancer	Elan Pharmaceuticals	2000	Canada, Europe	[43]
Onivyde	Irinotecan	Non-pegylated liposome	Pancreatic cancer, breast cancer, pediatric sarcoma	Merrimack Pharmaceuticals, Baxalta	2015	USA	[40]
S-CKD 602	Belotecan	PEGylated liposome	Topo I inhibitor	Chong Kun Dang	Phase I	Not yet approved	[44]

[a]Active pharmaceutical ingredient

9.4 Polymeric Nanoparticle Formulations and Technology Platforms

Exceptional advances in the manipulation, design, and synthesis of biocompatible and biodegradable soft matter materials to nanometer length scales including functional block copolymers such as polyethylene glycol (PEG), poly(lactic-co-glycolic acid) (PLGA), polylactic acid (PLA), and polycaprolactone (PCL) have heralded a sea change in pharmaceutical research and development efforts, with significant financial and intellectual capital directed toward the invention and implementation of drug delivery platforms employing these materials [50]. Although >700 articles have been published on "polymeric nanoparticle cancer chemotherapy" according to the Web of Science to date, no platform has entered the US or European market (even though several are in early-stage human clinical trials) due to lingering questions about their possible presentation in humans [45]. Polymeric nanoparticles (Fig. 9.1) are advantageous for oral administration because they are structurally resilient [51] and kinetically stable (even under high dilution) [52], due to the super low CMC of the polymers. These nanoplatforms have the potential to be mucopenetrative [31] or actively transported [22] and can be made to be anti-immunogenic, biodegradable, and biocompatible [50].

9.5 Surface Design: Mucoadhesion and Mucopenetration

Although the encapsulation of therapeutic molecules in nanoscale lipid-based and polymeric drug delivery vehicles resolves many of the challenges encountered in the oral administration of chemotherapeutic drugs, including limited solubility, chemical degradation or proteolysis, pH sensitivity, etc., significant obstacles remain in transiting through the gastrointestinal barrier, so that the drug can be incorporated into the circulation and reach its target tissue. The viscous, gel-like secretions from the endothelium in the gastrointestinal tract consist of complex dispersions of biomolecules including lipids and glycoproteins (proteins posttranslationally modified with carbohydrates) which guard the human body against intrusion by malicious foreign actors like bacteria and virions while simultaneously allowing for the transit of valuable nutrients from food [9].

Mucin is the most abundant glycosylated protein produced by mucus-secreting cells and is primarily responsible for mucus's ability to modulate gastrointestinal transit of ingested matter, conferring mucus with rheological properties akin to a sticky and flexible spider's web. The mucin proteins act as monomers to assemble a cross-linked network of glycoproteins. Other constituents in the secreted hydrogel such as phospholipids, electrolytes, and other carbohydrates and proteins are relied upon to act in concert with the mucin web to adhere a wide diversity of molecular entities by exerting van der Waals, electrostatic, hydrophobic, steric, hydrodynamic, and mechanical forces [10]. Polymeric nanoparticles, for instance, which typically

have long polymer chains extending from their corona, can easily become entangled in the mucin mesh [53].

Once entrapped in the polymeric network, peristaltic forces act to expeditiously expel adhered foreign bodies [54]. Even if a nanoscale particle can release its drug payload once it is entangled, a sufficiently large burst release must occur to reach therapeutic concentrations in the blood stream [55]. However, this requires that the pharmaceutical compound possesses high permeability. Peptides and other small molecules which bear the biopharmaceutics classification class of II or IV will never reach the circulation at therapeutic concentrations after a robust burst release from mucoadherent nanoparticles [56]. Indeed, a large proportion of newly identified leads with oncolytic therapeutic potential are proteins such as immunoglobulins, hydrophilic peptides, and small molecules with extremely low bioavailability and permeability. In order to deliver these drugs as an oral formulation, mucopenetrating nanotechnologies are currently being sought and under development in the early preclinical stages [57].

Learning from nature's successes in circumventing this barrier will lead to a rationally designed drug delivery platform with mucus-penetrating properties. PEG is a hydrophilic polymer frequently utilized in parenteral drug delivery for its antiimmunogenic and biocompatible attributes. However, there was a lack of consensus in the scientific community regarding the interaction between mucus membranes and PEGylated micro- and nanoparticles. Justin Hanes and colleagues at Johns Hopkins University systematically investigated the effects of PEG on particle transit through the GIT using PEG molecular weight and surface density as variables. Using robust and sophisticated tracking instrumentation and analysis to calculate the effective diffusivity of the particles, it was concluded that low molecular weight (2000 Da) polymers decorated on particle surfaces at maximum numbers possess the ability to transit the mucus layer quite effectively. However, longer PEG chains (~10,000 Da) may become entrapped in the mucin network, whereas nanoparticles of lower PEG surface coverage interact electrostatically with the mucus layer, promoting elimination [32, 58].

9.6 In Vitro and In Vivo Assessments of the Orally Administrated Nanotherapeutics

9.6.1 Assessment of Release of Therapeutics and Transport of Nanocarriers Using In Vitro Models

Development of experimental platforms that predict quantitatively the in vivo drug release and response behaviors of nanoparticle drug delivery systems from in vitro measurements is a formidable task, but it is paramount in implementing and characterizing nanotherapies that will be efficacious in the clinical setting [59]. Numerous techniques have already been devised to quantify release rates including dialysis,

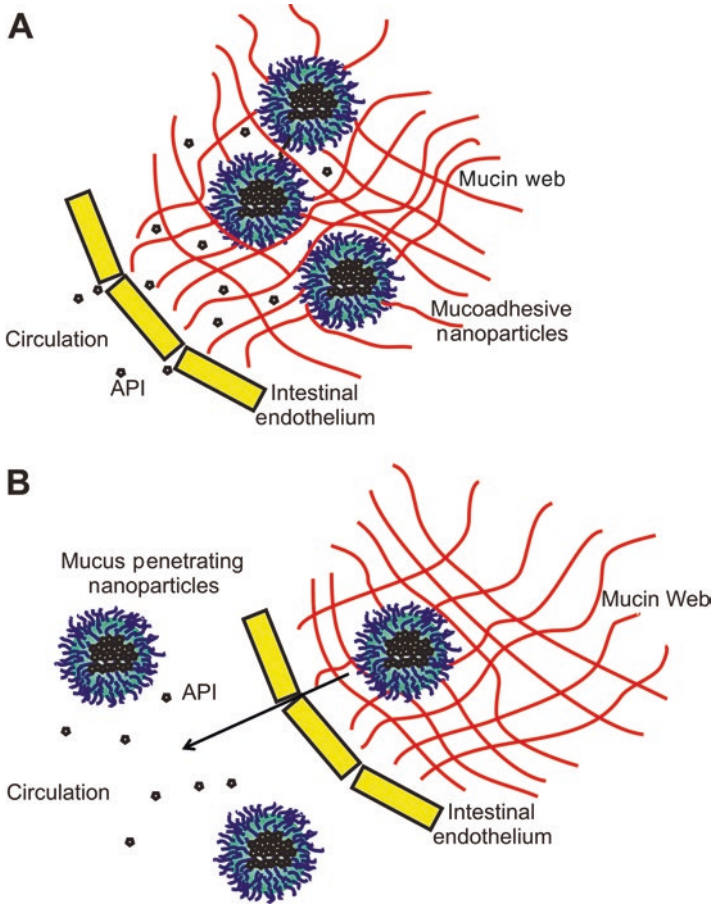

Fig. 9.2 (**a**) Mucoadhesive polymeric nanoparticles are entangled in the mucin mesh near the intestinal epithelium. The active pharmaceutical ingredient (API) is released in a burst and transits the gastrointestinal barrier into the circulation. (**b**) Mucus-penetrating nanoparticles are able to gain entry into the circulation and may provide efficacious vectors for peptides such as insulin and biologics for oral administration

two-phase systems with artificial membranes, and more exotic analytical tools such as artificial organs employing microfluidic architectures. These in vitro methods for characterizing nanoparticle drug delivery systems for oral administration will be discussed more in detail below after an introduction to two important processes involved in these assessments—transport and transmucosal permeability.

Mass transport of therapeutic compounds from the particle interior into the circulation involves manifold steps (Fig. 9.2). For a nanoparticle that is not mucous penetrating (Fig. 9.2a), the drug first must diffuse out from the nanoparticle. The magnitude of this transport can vary wildly as well depending on the hydrophilicity or ionizability of its functional groups. Having arrived in the bulk medium, the therapy must now cross the intestinal epithelium to reach the blood circulation

and ultimately its target [60]. In this case, the main goal is to maintain the sustained release of the drug from the nanoparticle and drug delivery across the intestinal epithelium without a reduction in the drug concentration originally encapsulated in the nanoparticle.

Frequently, the most significant bottleneck in drug compound transport materializes when the molecule is transiting the mucus layers lining the epithelium. In instances of oral administration of nanomedicines, drug compounds must be delivered through the intestinal epithelium via the thick mucus lining to gain entry to the vasculature. Transmucosal permeability describes the propensity of a chemical compound to transit through the protective mucus layers surrounding epithelial tissue. The fraction of therapeutic medicine able to permeate the mucus lining can vary by several orders of magnitude depending on the drug's physicochemical properties and biological metabolism [61]. Both transport and transmucosal permeability are therefore aspects of the intestinal epithelium that are modeled to gain a greater understanding of how these processes can be manipulated to improve oral administration and delivery of nanotherapeutics.

9.6.1.1 In Vitro Intestinal Co-culture Models

Anne des Rieux and colleagues devised a paradigm to study nanoparticle release and transport through specialized areas of the intestinal epithelium into regions particularly vulnerable to transit of foreign bodies such as Peyer's patches. The epithelium protecting these tissues is more permeable to nanoparticulate entry and is called the follicle-associated epithelium (FAE). Co-culturing of two different cell lines, Caco-2 and Raji, precipitated a transformation of some Caco-2 into M cells, a histology resembling the FAE, as confirmed by differential expression of β_1-integrin measured via immunofluorescence. Flow cytometry was then employed to interrogate the rate of transport of nanoparticles decorated with various moieties. It was determined that negatively charged particles (such as carboxylated particles) more facilely crossed the epithelial barrier compared to positively charged particles with amine functionalizations [62]. Sizeable improvements can be realized after upturning the Caco-2 culture before introducing Raji cells. This single modification significantly increases the conversion of Caco-2 into M cells and leads to more robust and reproducible NP transport results [63]. Other researchers have attempted to construct more advanced analogues of the intestinal mucous layer by including other cell types. Filipa Antunes and colleagues developed a co-culture with Caco-2, Raji, and mucous-secreting cells and investigated the transport of peptide drug delivery systems [64].

9.6.1.2 Semipermeable Artificial Membranes

Measuring in vitro drug release kinetics using semipermeable membranes is a well-established and robust technique for nanoparticulate drug delivery platforms, which can be either a diffusion-controlled one-phase system (dialysis) or a two-phase

setup with sink conditions. In dialysis, a colloidal nanotherapy suspension is separated by a semipermeable membrane from another chamber containing only solvent. A concentration gradient is utilized to transport the drug compound from the particle core to the bulk solvent and across the semipermeable membrane into the second compartment. Drug release kinetics are characterized by measuring the drug concentration in the second compartment as a function of time [65]. Dialysis methods have been employed extensively for nanoparticle chemotherapies in particular. P.K. Gupta et al. in 1987 employed dynamic dialysis to measure the rates of doxorubicin release from protein microparticles synthesized from bovine serum albumin (BSA) [66]. Eliana Leo and coworkers investigated doxorubicin release from gelatin nanoparticles utilizing the same technique in the presence of an intestinal protease trypsin which helps to hydrolyze proteins in the human digestive system [67]. However, release kinetics measurement by using these dialysis methods is often limited by drug solubility.

Quantification of drug release kinetics from nanoparticles encapsulating hydrophobic compounds can be interrogated by exploiting both diffusive transport and the molecule's partition coefficient simultaneously. In two-phase sink conditions, the nanocarrier is dispersed in an aqueous suspension which is segregated by a semipermeable, artificial membrane from an organic phase miscible with the drug. The compound's low water solubility acts as a driving force for it to partition into the sink compartment containing the organic phase [21]. Drug release kinetics are subsequently determined in a manner equivalent to conventional single-phase dialysis techniques [68].

9.6.1.3 Microfluidic Artificial Organs

Dialysis and two-phase sink systems crudely simulate the permeation of pharmaceutical ingredients through the gastrointestinal mucosa and their release kinetics in the body. However, even the most complex intestinal co-culture models fail to closely approximate the in vivo pharmacokinetics and pharmacodynamics because the cellular surroundings are so foreign to its physiological environment. In these experimental systems, epithelial and immune cell histologies such as Caco-2 and Raji cells are grown in culture dishes as single layers lacking the three-dimensional architecture and morphological complexity of the intestinal villi from which they are derived.

In the past decade, scientists and researchers have initiated an effort to construct environments which more closely mimic human physiology using novel fabrication and machining techniques such as microfluidic technology to construct miniature artificial organs to study the feasibility of newly invented drug delivery platforms before they enter the marketplace or begin human clinical trials (Fig. 9.3) [69]. A successful example has been realized in the HuMiX platform, a cellular co-culture system exploiting a modular microfluidic technology (Fig. 9.3) [69]. Some research groups have even fabricated devices with multiple major organ mimetics such as the liver, spleen, brain, and lungs in an effort to create a micro-human in silico [70].

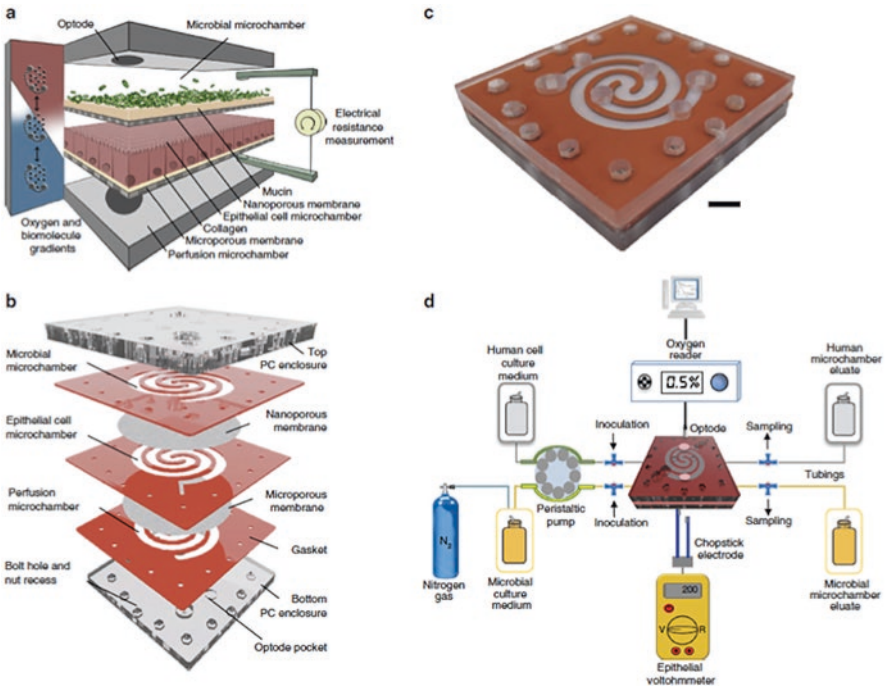

Fig. 9.3 The HuMiX platform, a modular microfluidics-based co-culture device [69]. (**a**) Schematic illustration of the key features of the HuMiX platform; (**b**) expanded view of the HuMiX device; (**c**) image of the assembled HuMiX device with the scale bar equivalent to 1 cm; (**d**) diagram of the experimental setup

9.6.2 In Vivo Assessments of Efficacy

Once lipid-based and polymeric nanotherapeutic platforms have been systematically evaluated using robust and predictive in vitro drug release and permeability models like dialysis and the intestinal co-culture model, research may progress into the succeeding preclinical stages with animal models of cancer. Below, an outline of the current endeavors and the accompanying analytical methods to assess oral bioavailability, pharmacokinetics/pharmacokinetics (PK/PD), and drug toxicity is discussed.

Typical approaches to evaluating in vivo efficacy entail grafting tumors on model animals such as nude mice, orally administering a nanoparticle suspension by gavage and sacrificing the animals at various time intervals. Overall efficacy is evaluated by the reduction in tumor volume versus a suitable control [75–77]. The blood and major organs (e.g., liver, lungs, spleen) are harvested, and the drug and its key metabolites are measured to determine crucial pharmacokinetic and pharmacodynamic parameters, including those to verify that the drug is within the therapeutic

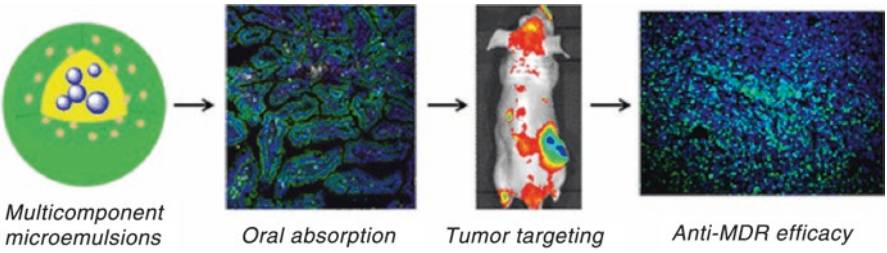

Fig. 9.4 In vivo evaluation of multicomponent microemulsions administered orally for tumor targeting and anti-multidrug-resistant (anti-MDR) breast cancer treatment [75]. The potent chemotherapeutic etoposide was co-formulated in the microemulsions with coix seed oil and ginsenoside Rh2 to promote synergic antineoplastic activity against aggressive, drug-resistant breast cancer. The emulsion system successfully targeted anti-MDR breast cancer in a nude mouse model

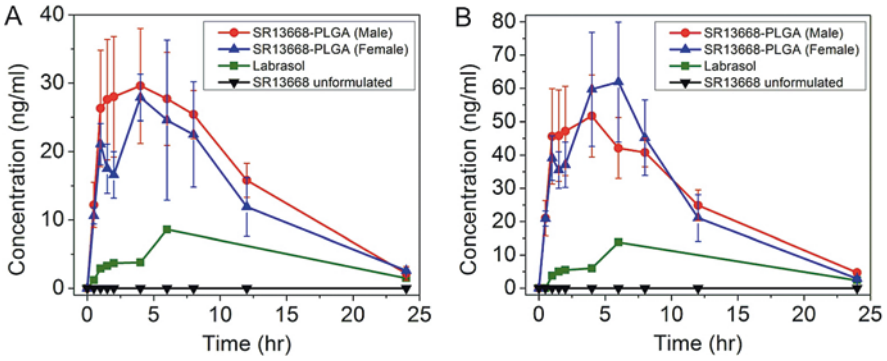

Fig. 9.5 SR13668 mean levels in beagle dog (**a**) plasma and (**b**) blood. Dogs were orally dosed at drug level of 2.8 mg/kg. Comparisons were between PLGA nanoformulation, Labrasol®, and drug in 0.5% methylcellulose (as the unformulated neat compound) [20]

concentration, and determine where the nanoparticles/drugs accumulate (Fig. 9.4). Chromatographic methods, such as high-performance liquid chromatography (HPLC) and mass spectrum protocols, are often relied upon heavily for these PK/PD and toxicological assessments. Although a majority of the market-available anticancer drugs are delivered via intravenous administration, several nanotechnologies have shown promising results from oral administration. The Liu group at the University of Illinois at Chicago used a scalable process to generate high-drug-loaded PLGA nanoparticles to orally deliver SR13668, a cancer-preventive compound. Compared with Labrasol®, the nanoformulations helped to achieve two orders of higher oral bioavailability of SR13668 in mice and one order of higher bioavailability in beagle dogs [20, 21](Fig. 9.5). S. Bisht et al. demonstrated satisfactory efficacy of rapamycin-loaded polymeric nanoparticles using a mouse model of pancreatic cancer. In vivo pharmacokinetic studies demonstrate that their nanorapamycin formulation had superior or equivalent serum rapamycin levels over a 24-h

period compared with conventional oral rapamycin [74]. Golla et al. encapsulated doxorubicin into lactoferrin (polypeptide) nanoparticles and evaluated the preclinical outcomes in rats grafted with a hepatic tumor. The measured outcomes were greatly improved in comparison to conventional doxorubicin therapy, as the cancer was specifically targeted by the nanoparticles [71]. O.M. Farokhzad of MIT and Harvard Medical School is a leader in oral delivery of nanoparticle-encapsulated biologic medicines, employing active transmucosal transport. Nanoparticles encapsulating insulin transported across the intestinal epithelium by the FcRn receptor had permeations in an order of magnitude greater than passively loaded nanoparticles [22]. Vong and colleagues disregard transmucosal permeability and target colon cancer with their redox nanoparticles. Of course, colon cancer is a less formidable target for oral chemotherapy than other malignant neoplasms, as the drug does not need to exit the gastrointestinal tract to reach the tumor location. However, the drug must still retain its integrity in the low pH conditions of the stomach and sustained released in the colon. The RNP^{O}s effectively neutralized oxygen radicals in neoplastic tissue in the large intestine, leading to positive outcomes in mice. Endoscopy was used to evaluate experimental outcomes [73]. Recently, the clever design of nanocapsules by Benita and colleagues, that were administered orally and used the lymphatic system to improve systemic circulation, led to a significant increase in docetaxel oral bioavailability in rats and an increase lung cancer treatment efficacy [78]. A similar strategy is to activate the immune response by using bacteria [79]. Several notable oral nanotechnologies in preclinical development for cancer and other indications are listed in Table 9.2.

9.7 Decision Crossroads and Best Practices

Exponential advances in information technology and automation have engendered a new paradigm in drug discovery efforts, enabling high-throughput analysis of hundreds of thousands of compounds per day to identify leads [80]. This increase in productivity in the initial screening stages however does not entirely translate into an increased rate of efficacious drug products entering the marketplace [81]. The subsequent phase requires modification of the lead compound's physicochemical properties in an effort to balance its solubility, bioavailability, and toxicity, without comprising its pharmacological activity, and often remains a formidable challenge to medicinal chemists, especially in the case of highly hydrophobic drugs. Further formulation of these poorly soluble molecules into oral dosage forms is thus prohibitive with current industrial processing technologies [82]. A number of currently available antineoplastic agents, as well as many of the next cohorts of leads identified in screening efforts, fall into this category and thus are not economical to formulate for oral administration with current industry practices [83]. Moreover, safety concerns relating to hepatotoxicity of orally administered chemotherapeutics have yet to be addressed with these conventional oral dosage formulations.

Table 9.2 Some notable oral nanotechnologies in preclinical development for cancer and other indications

API[a]	Vehicle	Vehicle type	Indication	Research group	Reference
Curcumin	PLGA [2] NP [3]	Polymeric	Opioid tolerance	Y. Liu et al.	[18, 19]
Doxorubicin	Lactoferrin NPs	Proteinaceous	Liver cancer	A.K. Kondapi et al.	[71]
Insulin	Fc-conjugated PEG-PLA [4] NPs	Immunopolymeric	Diabetes mellitus	O.M. Farokhzad et al.	[22]
Paclitaxel	Polyelectrolyte-stabilized MLVs [5]	Polymer-lipid hybrid	Breast cancer	K. Thanki et al.	[6, 72, 73]
Rapamycin	NMA622 NPs	Polymeric	Pancreatic cancer	A. Maitra et al.	[74]
RNP^{O}	RNP^{O6} NPs	Polymeric	Colorectal cancer associated with colitis	Y. Nagasaki et al.	[73]

[a]Active pharmaceutical ingredient [2];poly(lactic-co-glycolic) acid [3];nanoparticle [4];poly(ethylene glycol)-poly(lactic acid) [5];multilamellar vesicles [6];redox nanoparticle

Lipid-based and polymeric drug delivery systems are arguably the next tools in the oral cancer chemotherapy arsenal, with the capability to circumvent the current obstacles in drug formulation and development [24]. These nanotechnologies facilitate oral absorption by functioning as a depot for highly hydrophobic drugs, effectively enhancing their solubility, delaying their metabolism, and altering their release kinetics. Oral nanoformulations would therefore mitigate toxicological issues, as smaller dosages would be required to achieve equivalent serum concentrations and minimize impact on the liver. Passive and active targeting to the tumor location mitigates cytotoxicity without initiating undesired responses in other organs throughout the body. Finally, enhanced efficacy is gained through controlled release characteristics accomplished by intelligently tailored particle design.

Lipid nanocarriers such as liposomes, liquid crystalline nanoparticles, and solid lipid nanoparticles (SLNs) are at the forefront in cancer chemotherapy, predating other next-generation delivery systems with entrance to the market [84]. Lipid-based delivery platforms offer several advantages over other systems requiring synthetic materials with their inherent biocompatibility, facile synthetic schemes, and potential stealth properties [33]. Nonetheless, lipid-based nanocarriers often lack structural integrity and stability after high dilution in the bloodstream to deliver therapeutic concentrations of oncolytic medicines to their targets effectively [51]. Further, the majority of lipid-based nanotherapeutics approved by the FDA are approved for parenteral administration (Table 9.1) due to their hydrophobicity, with only solid lipid nanoparticles (SLNs) having the potential to be orally formulated [85].

Polymer-based nanovehicles have garnered considerable attention as a feasible alternative to lipid-based systems, enabled by the design of polymers and copolymers that have received generally recognized as safe (GRAS) status from major regulatory bodies [86] and the development of sustainable processes that do not require the use of toxic organic solvents. Polymer drug delivery platforms such as micelles, polymersomes, and nanoparticles are kinetically and structurally stable, even under high shear or strain and extreme concentration dilution below their critical micelle concentration, therefore being more naturally suited for oral administration [52]. However, considerable uncertainty about the clinical manifestations that may present in human patients is hindering the approval of these platforms, with no such treatment approved to date. Promisingly, however, several polymeric nanoparticulate therapies are undergoing human clinical trials at the time of this writing [45].

Lipid-based and polymeric nanoparticle delivery platforms may appear transformative in their ability to treat human disease, yet questions still remain about whether these newly engineered technology platforms could feasibly supersede conventional oral chemotherapies in the future. Human clinical trials may only be successful for parentally administered nanoparticle formulations, as gastrointestinal transit may prove an insurmountable challenge. Further, the unique biology of cancer compared to other chronic diseases states may limit the usefulness of nanoparticulate drug delivery platforms as simply alternative prophylactic regimens. Moreover, reproducibility and scalability of nanoparticle synthesis are essential to ensure the translation of fundamental research to clinical applications. However, the discussion in this chapter provides substantial evidence to the contrary that researchers have reached a tipping point in the development of a portfolio of oral nanoparticle-based medicines. The tools currently exist to effectively transit the gastrointestinal tract, identify and target neoplastic tissue, and characterize its pharmacokinetics and pharmacodynamics. The conditions that must be fulfilled to realize adoption of oral nanotherapeutics for widespread clinical use are becoming more apparent every day: (1) scale-up and manufacturing of platforms with appropriate economies of scale and quality expectations are coming more into view; and (2) additional evaluations relating to the safety of nanotherapeutics and the ramifications of the use of nanotechnologies, more broadly speaking, must still be addressed by appropriate regulatory bodies.

With the virtues of an abundance of nanotechnologies currently being reported in the literature, the path to choose the most suitable platform for specific oral cancer chemotherapies may seem challenging at first pass. Nonetheless, lipid-based and polymeric drug delivery technology platforms have evolved past the proof-of-principle stage into clinical practice in recent years, with complementary attributes available to the formulation of scientist and clinician alike to ensure both proper medication design and effective clinical outcomes for a diverse array of antineoplastic compounds with varying physicochemical properties and pharmacological activity. Antiangiogenic peptides could be encapsulated in the hydrophobic core of a mucous-penetrating block copolymer nanoparticle to directly transit into the blood stream. Amphipathic small molecules could be incorporated into the

transmembrane compartment of a solid lipid nanoparticle or the phospholipid bilayer of a stealth liposome. These laboratory techniques function together as the next toolkit in fighting cancer.

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Chapter 10
Topical and Transdermal Nanomedicines for Cancer Therapy

Yanqi Ye, Jinqiang Wang, Wujin Sun, Hunter N. Bomba, and Zhen Gu

10.1 Introduction

Recent advances in transdermal nanomedicine for anticancer therapy have shown the impressive progress that has been made toward enhancing the bioavailability of therapeutics and their treatment efficacy [1–3]. One commercially available example of transdermal delivery is the nicotine patch, which can be self-administrated by people experiencing smoking cessations [4]. This delivery platform is able to transport the drug molecules through the epidermal and dermal tissue of the skin while enabling a fraction of the drug to enter the systemic blood circulation in a

Y. Ye · J. Wang · W. Sun
Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, Raleigh, NC, USA

Division of Pharmacoengineering and Molecular Pharmaceutics and Center for Nanotechnology in Drug Delivery, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
e-mail: yye4@ncsu.edu; jwang65@ncsu.edu; wsun9@ncsu.edu

H. N. Bomba
Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, Raleigh, NC, USA
e-mail: hnbomba@ncsu.edu

Z. Gu (✉)
Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, Raleigh, NC, USA

Division of Pharmacoengineering and Molecular Pharmaceutics and Center for Nanotechnology in Drug Delivery, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
e-mail: zgu@email.unc.edu

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controllable manner [5]. Topical and transdermal delivery of therapeutic agents offers several advantages over conventional oral and intravenous drug delivery systems. For instance, topical treatment of cutaneous melanoma locally targets the site of disease, thereby reducing the drug exposure to the body and maximizing organ preservation. It is also capable of inducing a substantial immune response with the abundance of dermal dendritic cells comparable to traditional intramuscular route. Important aspects of topical and transdermal administration in comparison to systemic therapy include prevention of agent loss from hepatic first-pass metabolism, minimization of enzymatic degradation, enhancement of localized pharmacodynamics, ease of turning the dose "on/off," and maintenance of plasma concentration [6–8]. However, from a biological point of view, the skin acts as a protective interface between the interior of the body and the external environment to reduce the invasion of biological agents, radiation, chemicals, physical damage, and dehydration [8]. The barrier property of the skin is attributed to the outermost epidermis layer, resembling a composite of cornified cells and lipid bilayers through which therapeutic molecules migrate via diffusion [9]. As a result, molecular transportation through the epidermis layers and the systemic delivery of drugs across the skin are highly impeded. This process is limited by the hydrophilicity and size properties of the drug molecules [10]. Compounds with molecular weights larger than a few hundred Dalton or highly polar and hydrophilic compounds cannot efficiently pass the skin by passive diffusion, highlighting the need for the delivery vehicles.

In this chapter, we have discussed the recent developments in the field of transdermal technology, including the use of chemical, electrical, ultrasonic, structural, and photoacoustic systems for enhancing efficacy of the delivery. We have also summarized the use of nanocarriers in transdermal applications for targeted drug delivery. These nanoparticles include liposomes, micelles, dendrimers, nanoparticles, polymer conjugates, and inorganic materials with a focus on their use in transdermal delivery for anticancer applications.

10.2 Methodology of Anticancer Drug Delivery

From understanding the physiological properties of the skin barrier to manipulating reversible skin disruption for therapy, transdermal drug delivery systems have made great progress in the past two decades. To further enable delivery with controlled release and absorption kinetics, various methods have been developed to enhance delivery efficacy of a variety of therapeutics (Fig. 10.1).

10.2.1 Chemical Enhancers

Over 350 types of penetration enhancers have been identified to enhance skin permeability for facilitated transdermal absorption of drug molecules [11]. It has been identified that three independent mechanisms are responsible for enhancing

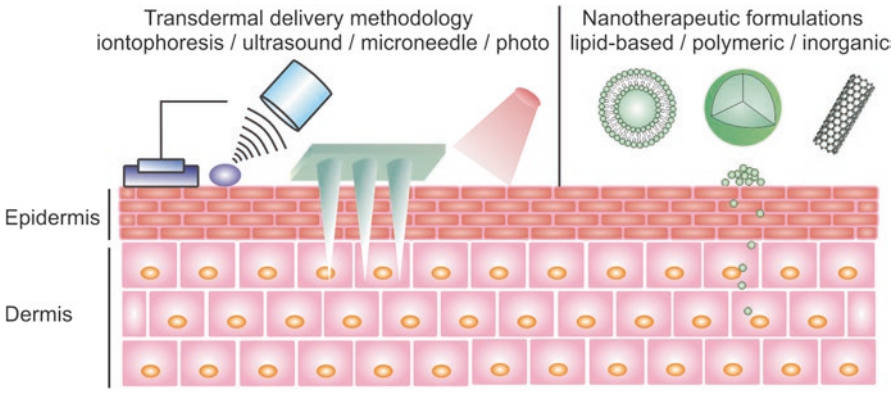

Fig. 10.1 Schematic of recent developments in transdermal nanomedicine for anticancer application, including active transport methodology to increase skin permeability and the formulation of delivered nanotherapeutics

membrane penetration via skin barrier perturbation, fluidization, and extraction of lipid bilayers [12]. However, chemical enhancers have been employed largely for skin-permeable compounds, while efficient delivery of large skin-impermeable drugs such as protein and nucleic acids remains challenging. Chen et al. reported that the short synthetic peptide, ACSSSPSKHCG, enabled macromolecular drugs to cross the skin barrier and reach systemic circulation. In vivo phage display and time-lapse studies demonstrated that a transient cavity in the skin barrier was created by the peptide to facilitate the absorption of transdermal protein drug into intact skin [13]. In addition to the penetration pathways of small hydrophobic molecules, Hsu et al. identified and utilized a skin penetrating and cell entering (SPACE) peptide for delivering siRNA and macromolecules across cellular- and tissue-level barriers [14, 15]. Shastri's group found a family of naturally occurring glycosylated triterpenes, avicins, which exhibited high skin permeability [16]. However, side effects regarding the health of the skin raise safety concerns for the use of chemical enhancers in the clinic. For example, the irritation response and the conformation changes of stratum corneum when exposed to high concentration of chemicals or prolonged exposure make them unsuitable for routine use. The implementation of standardized procedures relies on techniques like high-throughput screening studies to gain insight into the mechanisms of the chemical penetration enhancers [12, 17].

10.2.2 Iontophoresis

Iontophoresis is a noninvasive technique utilizing electric current as a driving force for increasing the penetration of drugs by electrically charging biological membranes, in this case the skin [18]. A continuous low-intensity electric current, usually less than 1 mA, is applied to generate repulsion of the drug from the reservoir

chamber into the skin with an electrode of opposite charge. The electroporation allows the transient disruption of the lipid bilayer structure of biological membranes in a programmed three-step process: (1) electrophoretic movement, (2) passive diffusion, and (3) electroosmosis. The process promotes the passive diffusion of molecules and potentiates skin permeabilization, which may last hours after the application of the energy pulse [19, 20].

Depending on the dose and the intensity of the energy applied, the iontophoresis system is applicable either for local cutaneous or systemic therapeutic effects following the anticancer drug entry into the superficial dermal capillaries [21]. To evaluate the clinical application of iontophoretic devices as potential anticancer therapies, DeSimone's research group tested the devices in a group of different orthotopic mouse and canine models, including locally advanced and nonmetastatic pancreatic cancer models [22]. It was found that these devices delivered high levels of cytotoxic drugs, gemcitabine and cisplatin, to the mice tumor, enhancing the therapeutic index of the drugs, reducing systemic side effects of the drugs, and offering new modalities to prevent local tumor invasion. The combination iontophoretic delivery of two drugs, 5-fluorouracil and leucovorin, into the buccal tissue of a pig animal model also showed potential in the treatment of head and neck cancer (Fig. 10.2) [22]. In another example, electrochemotherapy was applied, which consisted of the administration of a non-permeable bleomycin after the localized treatment of high-voltage pulses. This permeabilized the local tissue exposed to the electrical field for treating subcutaneous tumor [23].

The SynCon vaccine is a representative example that has been approved by the US Food and Drug Administration (FDA), which is used for the therapy and prevention of prostate cancer, lung cancer, breast cancer, hepatitis B, hepatitis C, and leukemia [24]. Although an iontophoresis delivery system has the potential to provide personalized delivery rates for ionic drugs, it still requires an electric field across the

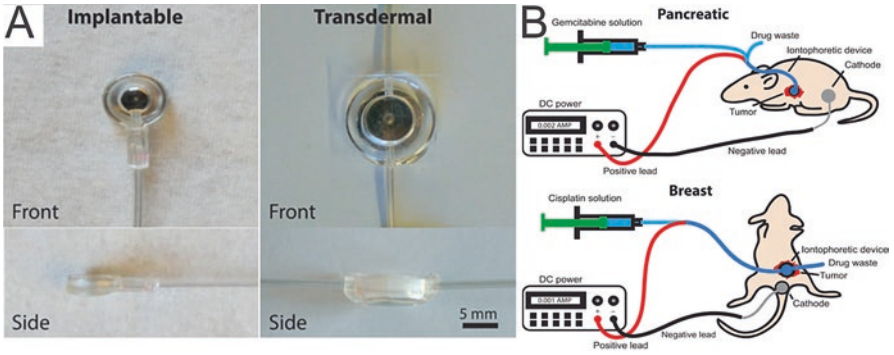

Fig. 10.2 Iontophoretic devices used for the delivery of cytotoxic agents to solid tumors. (**a**) Front and side images of the implantable and transdermal devices. (**b**) Device treatment setups in the pancreatic (implanted device) and breast (transdermal device) cancer models where the drug is supplied to the device, using a syringe pump and electrical current via a DC power supply. Positive and negative leads connect to the device and counter electrode, respectively. (Reproduced with permission from [22] (Science Translational Medicine))

skin, which requires high-power input and can cause serious side effects like skin irritation [25]. Further exploration of this technique is imperative for mass human application and translational utility.

10.2.3 Sonophoresis

The application of ultrasound, also called sonophoresis, is a technique that increases the transient permeation of therapeutics across biological membranes [26]. Dermal exposure to ultrasound may cause phenomena such as cavitation, convective transport, thermal effects, and mechanical effects [27, 28]. Between the ultrasound transducer and the skin surface, the cavitation bubbles are readily produced, imploding and oscillating in the dermal interstitial fluids. During this process, submicroscopic defects and micro-vibrations of the epidermis were caused by the application of ultrasonic waves, increasing the kinetic energy of molecules in the skin [29]. Mitragotri and colleagues showed that cavitation induced microjets and shock waves during the treatment with low-frequency ultrasound. It opened transport pathways for NPs into the skin's sebaceous glands and enabled the subsequent thermolysis which was activated by the NIR light [30]. Rubio et al. also discovered the most favorable cationic surface charge for nanoparticles to penetrate a Franz diffusion cell after the application of ultrasound [31]. The synergistic effect of low-frequency ultrasound together with the surfactant sodium lauryl sulfate has been approved by the FDA to enhance the local uptake of anaesthetics as skin pretreatment [26]. The SonoPrep, approved in 2004 by the FDA, employs 53–56 KHz low-frequency ultrasound at an average time of 15 s, allowing a 100fold increase in reversible skin permeability.

10.2.4 Microneedle

Microneedles (MN) were designed for insertion across the stratum corneum painlessly and therefore create transport pathways for drug diffusion through viable epidermis to capillary networks [32, 33]. Silicon [34], metal [35, 36], and polymer [37–39] needles of micrometer dimensions and tailored geometries were developed to meet different functionalities, such as increased skin permeability, liquid microinjection, and stimuli responsiveness [40–42].

In clinical trials, the first-generation solid MNs have been widely used to punch microscopic holes into the skin layer before the application of drug-loaded patches. Recently, Gu and coworkers developed a physiologically self-degradable MN patch for localized and advanced delivery of anti-programmed cell death protein 1 (aPD1) antibody toward melanoma. The needles pierced into the immune-cell-rich epidermis and delivered aPD1 to regional lymph and capillary vessels, promoting the immune cells to attack the tumor [43]. Gu's group also integrated combination

nanotherapeutics into the polymeric MN system to transport 1-methyl-*dl*-tryptophan (1-MT) and aPD1 across the stratum corneum to the local tumor. This delivery platform improved the interaction with tumor-infiltrating lymphocytes, increased the local retention of the drug, and reduced the toxicity due to systemic circulation (Fig. 10.3) [44]. Walsh et al. demonstrated for the first time that integrating the MN array with their nanotopography technology dramatically loosened tight junctions in squamous epithelium and enhanced transdermal delivery of drugs [45]. Robust antigen-specific immune response with a lower dosage of vaccine is associated with MN-mediated delivery compared to the conventional administration routes, which is largely due to the highly immunosuppressive environment in the dermal region compared to the muscle site. Impressive progress has been made in the fabrication of polymeric MNs for the transdermal delivery of small molecular drugs, therapeutic proteins, and vaccine compounds, but few studies have reported gene delivery applications. Additionally, current MN platforms for transdermal delivery have been suggested to be more effective on animal species rather than human patients. It appears that there are several contributing factors to the limited number of human trials conducted so far: skin irritation, local inflammation, and the maximum amount of drug that can be loaded in the MN structure. Barrier disruption of human skin requires different needle geometries such as an elongated MN path length and personalized patch size, which should be systematically studied for the translation of preclinical studies into clinical trials.

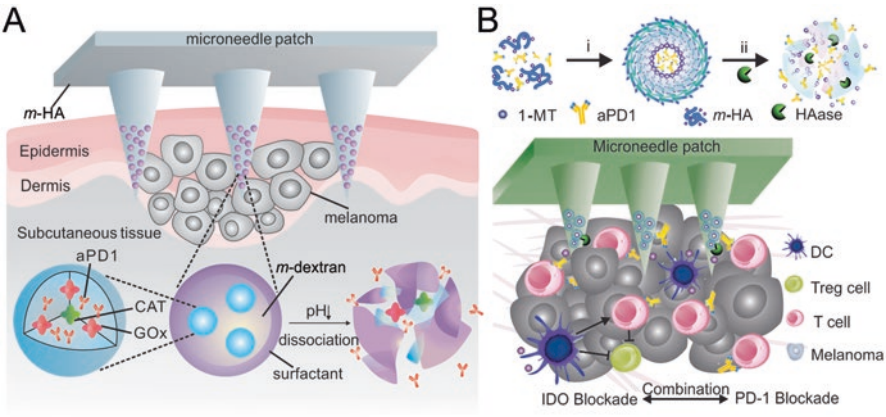

Fig. 10.3 Schematics of microneedle-based transcutaneous delivery approach for the treatment of melanoma. (**a**) An innovative self-degradable MN patch for the sustained delivery of aPD1. The MN is composed of biocompatible hyaluronic acid integrated with pH-sensitive dextran NPs. (Reproduced with permission from [43]. Copyright (2016) (Nano Letters)). (**b**) A synergistic immunotherapy strategy that locally targets the immunoinhibitory receptor PD1 and immunosuppressive enzyme IDO for the treatment of melanoma through a MN-based delivery system. (Reproduced with permission from [44]. Copyright (2016) (ACS Nano))

10.2.5 Phototherapy

Lasers can be used to create micropores at a specific width, depth, and density [46–48]. Therefore, this method enables diffusion of topically applied therapeutic agents in a rate- and spatiotemporally controllable manner [49]. Due to the relatively short exposure time, there is low risk for the microsecond- to millisecond-pulse-generated heat to propagate into deep tissue [50–53]. The short laser pulses were able to minimize the pain and generate photoacoustic waves that permeabilized the stratum corneum for large molecules [54].

Jung et al. reported a nanographene oxide-hyaluronic acid (HA) conjugate transdermal patch for melanoma skin cancer treatment using a near-infrared (NIR) laser. The HA conjugate promoted the transdermal delivery of drugs into tumor sites through the abnormal cancerous skin barrier. After photoablation therapy with NIR irradiation, no recurrence of tumorigenesis was found compared to continuous tumor growth for control groups (Fig. 10.4) [55]. Chen et al. developed a combination of chemotherapy and photothermal therapy for treating superficial tumors. When irradiated with NIR light, the encapsulated lanthanum hexaboride (LaB6) nanoparticle in the poly(vinyl alcohol)/polyvinylpyrrolidone transdermal patch absorbed the laser energy and converted it into heat, triggering doxorubicin (DOX) release from the MNs to the tumor [42]. During this process, the transdermal barrier is disrupted, and micron-scale holes are generated within the stratum corneum and epidermis, which can lead to inflammation.

10.3 Nanoformulations of Therapeutics

To enhance the delivery efficiency to targeted skin sites and address the shortcomings associated with therapeutic modalities, drugs have been preformulated into nanomedicine prior to incorporation into transdermal devices. Nanotherapeutic systems that harness the material chemistry and systematic pharmaceutics generate a variety of nanoformulations for topical and transdermal delivery of active therapeutics [2, 56–58]. Nanocarriers such as lipid nanovesicles, polymeric nanoparticles, and inorganic nanocarriers have been studied for the spatial- and/or temporal-controlled delivery to the diseased sites [59–63] (Table 10.1). At the same time, with the difference in nanoparticle materials and physicochemical properties (e.g., size, shape, zeta potential, stiffness, surface modification, and targeting ligand), the corresponding biological processes can be tuned for various therapeutic outcomes. Rationally designed nanocarrier systems are expected to improve the absorption of drugs, potentiating their penetration through the stratum corneum and improving pharmacokinetic and targeted response of the active compound.

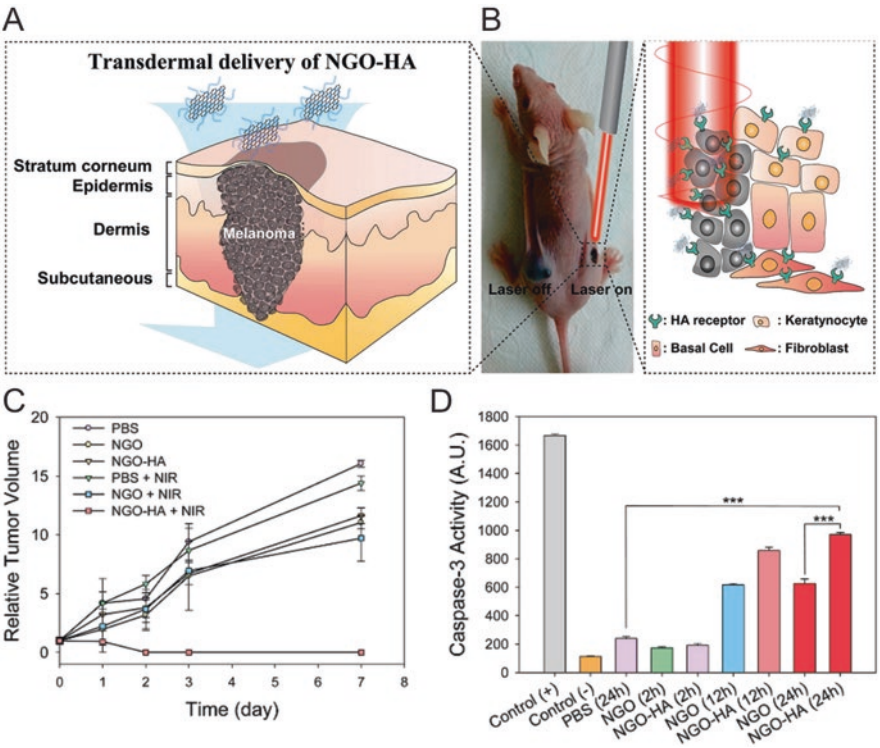

Fig. 10.4 Schematic illustration of transdermal delivery. (**a**) Topical delivery into melanoma skin cancer cells and (**b**) the following photothermal ablation therapy using a near-infrared laser. (**c**) Relative tumor volume with increasing time. Treatment groups are combinations of nanographene oxide (NGO), hyaluronic acid (HA), nanographene oxide-hyaluronic acid (NGO-HA) conjugates, and near-infrared (NIR) laser indicated in the graph. (D) Caspase-3 activity in tumor tissues by ELISA for detection of apoptosis-mediated cell death (***$P < 0.0001$). (Reproduced with permission from [55]. Copyright (2014) (ACS Nano))

10.3.1 Lipid Nanovesicles

Lipid-based nanovesicles are optimized to permeate through the epidermis lipid lamellar regions by creating transient hydrophilic opening through the lipid bilayers [64, 81]. This hydration pathway is affected by nanovesicle adaptability, which is attributed to deformation to match the size of an opening in a skin barrier [10, 65, 66]. Therefore, lipid bilayer vesicles with a relatively high membrane elasticity are comparably more adaptable than the lipid-coated nanodroplets [59, 68, 82–85]. Less deformable or more rigid nanocarriers need higher energy input to transport them through the skin barrier. In contrast, with increasing lipid bilayer elasticity, the energy that is needed for vesicular opening decreases. Lipid nanovesicles that confer high flexibility and elasticity are readily favorable to squeeze through the stratum corneum through water activity and osmotic force. Another prerequisite for

Table 10.1 Examples of nanoformulated therapeutics

Nanoformulations	Material	Drug	Size (nm)	Zeta potential (mV)	Advantages	Ref.
Lipid nanovesicles						
Cationic liposome	PEG-PEI complex	Tamoxifen	270	40	Skin penetration and drug accumulation	[64]
Unilamellar vesicles	EggPC, Pad–PC–Pad	Fluorescence	100	−5.46 ± 0.4281	Shear stress sensitive for targeted drug delivery	[65]
Unilamellar liposome	DPPC, DSPC	Neomycin	–	–	Enhanced local release of drugs by hyperthermia	[66]
Stratum corneum liposomes	Ceramide, cholesterol, palmitic acid, cholesteryl sulfate	5-Aminolevulinic acid	500	–	Skin permeation and retention	[67]
Core-multishell nanotransporters	Polyglycerolamine	Lipophilic dye nile red	20–30	–	Cutaneous uptake	[59]
Solid lipid nanoparticles	Solid lipid ATO	Lipophilic dye nile red	150–170	–	Skin penetration, epidermal targeting	[59]
Nucleic acid nanoparticle conjugates	Lipid-based transfection agent	EGFR siRNA	13	–	Personalized, topically delivered gene therapy	[68]
Transfersomes	Lecithin, HA	DOX	251.4 ± 10.4	–	Transdermal lymphatic drug delivery	[69]
Polymeric nanoparticle						
Realgar nanoparticles	Polyoxyl (40) stearate	Realgar	150	–	Low toxicity	[70]
Nanogels	Chitin	Curcumin	70–80	49.34	Effective transdermal penetration	[71]
Nanogel/peptide	DEAMA, EGDMA, methoxy-PEG	Antigen peptide hgp-100	67.8 ± 15.1	15.2 ± 7.5	Anticancer vaccination	[72]
Polymeric nanoparticles	PVA, PLGA	Nile red	48.0 ± 5.6	–	Skin surface retention	[73]

(continued)

Table 10.1 (continued)

Nanoformulations	Material	Drug	Size (nm)	Zeta potential (mV)	Advantages	Ref.
Polymeric nanoparticles	PLGA	OVA	357.5 ± 45.7	−20.3 ± 3.5	Selective targeting of antigen to skin DC, skin immunization	[40, 74]
Nanoparticle	Chitosan tripolyphosphate	Plasmid DNA	287.25 ± 14.12	41.45 ± 0.43	Transfection potential for topical gene delivery	[75]
Unimer polyion complex	PEG-PLL	21mer/21mer siRNA	50	−24.7 ± 0.4	Systemic siRNA delivery	[76]
Inorganic nanocarriers						
HA-AuNR nanocomplex	Hyaluronate-gold nanorod	Death receptor 5 antibody	15.69 and 91.28 nm	−4.20 ± 1.20 mV	Theranostic platform for noninvasive treatment	[77]
Layer-by-layer polymer-coated AuNP	Polyelectrolyte	Imatinib mesylate	98.5 ± 4.3	32.3 ± 1.3	Drug loading capability	[78]
Carbon nanotube membranes	Crystalline CNTs	Nicotine	7		Programmable long-wear patch device	[79]
Bioconjugated quantum dot probe	Quantum dots, PEG	Tumor-targeting ligand	10–15		Diagnostic and therapeutic tool	[80]

transporting the lipid nanovesicles through the skin barrier is the transient and local perturbation-induced opening of the biological gaps between the adjacent cells. Usually, the hydrophilic passage within the skin is initiated through mechanical or chemical energy and could be widened by the hydrophilic surface of lipid vesicles [10]. The uptake of lipid nanovesicles (<180 nm) into skin keratinocytes has also been studied, which suggests that they could easily traverse the cellular membrane, permeabilize throughout the cytosol, and reach the nucleus [86]. Apart from the flexibility, elasticity, hydrophobicity, and size, the delivery systems can also improve the permeation of substances through the epidermis layers, for instance, surface properties, surfactant concentration, targeting property, controlled release, as well as biological degradation. Doxorubicin loaded into a transfersome by lipid hydration was investigated for its lymphatic delivery efficacy through the transdermal route. It was found that the permeation was enhanced, and this was attributed to the altered crystal conformation of lipid in the skin, the improved fluidity and deformability, as well as the hydrophilic surface properties [69]. In turn, stable drug efflux could be achieved through the formulation of solid lipid nanoparticles. The experimental paclitaxel-loaded lipid nanoparticles were subjected to histopathological study. The treatment of skin cancer was accompanied by the sustained permeability coefficient, enhancement ratio, and minimum dosage-related side effects [87]. Silva et al. formulated bullfrog oil microemulsion showing significant skin permeation for treatment of B16F10 melanoma [88]. Bhatia et al. evaluated the effect of topically applied tamoxifen-loaded liposome on inhibition of skin carcinogenesis. The liposome preparations increased the cytotoxicity toward skin HaCaT melanoma versus free drug in the liquid form, along with enhanced drug retention and better skin permeability [89].

10.3.2 Polymeric Nanoparticles

From the standpoint of material design, biocompatible polymers are useful for preparing smart transdermal devices capable of responding to the physiological environment. This includes natural polymers like dextran, alginate, hyaluronic acid, carrageenan, or synthetic polymers such as poly(*D,L*-lactide-co-glycolide) (PLGA) [24, 90–97]. Drugs that are enclosed by polymeric networks could be transported through the skin barrier and triggered to release through diffusion, competitive dissociation, or degradation in a controlled manner [70, 71, 98, 99]. Compared to the lipid nanocarriers, polymeric nanoparticles have a tendency to accumulate on the skin surface with lower flux rates but higher retention times. Polymer hydrophobicity, particle size, and surfactant type used during preparation also influence their skin interaction performance [73].

Toyoda et al. utilized the electric interaction between biocompatible nanocapsules and the skin epidermis layer and showed how biological molecules could be transported through the stratum corneum. Amine-functionalized PLGA nanocapsules were formulated by using in situ precipitation together with encapsulation

procedure. The increased skin penetration was attributed to the electric interaction generated by the positive charges and nanoscopic sizes of the molecules [72]. Zhao et al. showed that the topical cream of a traditional Chinese drug with tetraarsenic tetrasulfides as the active ingredient could successfully treat melanoma in vivo. Compared to the intraperitoneal route as the control, nanoparticles that were dermally delivered to tumor-bearing C57BL/6 mice displayed notable survival rates and tumor regression coupled with low systematic toxicity [99]. Zaric et al. delivered chicken ovalbumin (OVA) to cutaneous draining lymph nodes by biocompatible PLGA-NPs integrated with MN arrays. PLGA-NPs exerted prevention of the OVA antigen from proteolytic degradation and facilitated uptake by skin antigen-presenting cells (APCs). Compared to intravenous injection, the authors claimed that this platform restricted the entry of the encapsulated nanotherapeutics into the systemic circulation. In murine models of melanoma, the NPs were delivered to the afferent lymphatics in a targeted and sustained manner, which subsequently induced the accumulation and presentation of antigen-specific effector CD4+ cells and improved anticancer efficacy [40, 74].

10.3.3 Inorganic Nanocarriers

Inorganic materials such as gold nanorods, iron oxide nanoparticles, carbon nanotubes, and mesoporous silica nanoconstructs have also been used for the preparation of therapeutic nanoparticles [100–103]. Among the inorganic nanocarriers, gold has shown potential in a variety of biomedical applications such as drug delivery and bioimaging [76, 104–107]. Gold particles possess advantages including well-defined surface chemistry, which enables tailored functionalization with biological ligands [108–111]. The unique properties of numerous morphologies, such as spheres, rods, cubes, shells, disks, and prisms, have also received extensive attention in the last decade. Lee et al. developed a hyaluronate-gold nanorod/death receptor 5 antibody (HA-AuNR/DR5 Ab) complex to serve as a transdermal nanocarrier for noninvasive theranostics of skin cancer. AuNR was harnessed as a photoacoustic imaging agent and light-to-heat transducer upon absorbing NIR. However, the predominant surface conjugation approach involves complex chemistry and compromised stability in the ionic medium [77]. To overcome these restrictions, Labala et al. developed a layer-by-layer polyelectrolyte-coated AuNP (LbL-AuNP) integrated with anodal iontophoresis for transdermal delivery of an anticancer agent, imatinib mesylate. Compared to chemical conjugation techniques, the multiple, layered structure possessed greater drug loading capability and surface charge property that could be important for transdermal transportation [78]. In the study by Labouta et al., they showed that size influences molecular penetration behavior, of which 6-nm AuNP showed a substantial degree of penetration compare to AuNP with a size of 15 nm. The accessibility through the skin is primarily attributed to their physicochemical features such as the surface hydrophobicity, size, shape, and surface chemistry (Fig. 10.5).

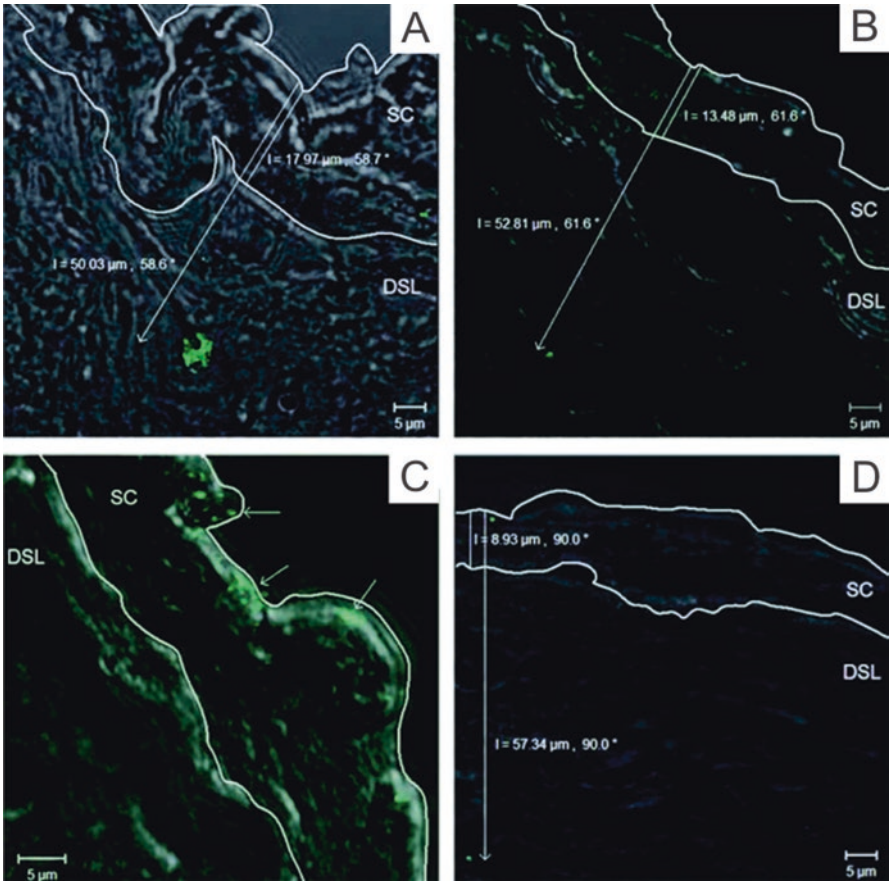

Fig. 10.5 Fluorescence images of penetration of different surface coatings and sizes of gold nanoparticles (GNPs) in the human skin stratum corneum (SC) and deeper skin layer (DSL). (**a**) 6-nm dodecanethiol-coated GNPs in toluene, (**b**) 6-nm lecithin-coated GNPs in water, (**c**) 15-nm citrate-coated GNPs in water, and (**d**) 15-nm cetrimide-coated GNPs in toluene with gold nanoparticles indicated in green. (Reproduced with permission from [10]. Copyright (2011) (Nanoscale))

Son et al. described a multifunctional, wearable-on-the-skin system for diagnosis and therapy via integration of gold nanoparticle-assembled nanomembranes. This transdermal patch mainly consisted of three parts: data storage modules, diagnostic tools, and therapeutic actuating elements [112]. Carbon nanotube (CNT) membranes have been known to have atomically smooth graphitic cores, functional surface chemistry, and efficient electrochemical functionalization. These membranes were incorporated into a switchable transdermal patch on human skin to pump out nicotine electrophoretically at low power, with 12 days of battery life [79]. Therapy utilizing spherical nucleic acids has the expectations in fighting genetic diseases with success in its first human trial [113]. In this study, three major components including spherical nucleic acid nanoparticle conjugates (SNA-NCs)

with densely coated oligonucleotides, targeting epidermal growth factor receptor (EGFR), and Aquaphor were topically applied to the skin of C57BL/6 J mice. The 10-day treatment period inhibited EGFR mRNA expression and EGFR protein with a knockdown rate of 52% and 75%, respectively, compared to nonsense SNA-NC. It provided compelling evidence for RNA-mediated gene suppression to treat skin tumors, irritation, inflammation, and other absence-of-gene skin disorders [68, 114].

10.4 Conclusion

In recent years, we have witnessed exciting collaborations from academia, the pharmaceutical industry, and the public for the clinical translations of transdermal drug delivery systems for cancer treatment [115]. Several challenges remain that include broadening the range of active drugs amenable to the transdermal route, optimizing the physicochemical properties of the therapeutic formulations, and addressing the safety concerns associated with materials involved in transdermal delivery.

First, translation criteria rely profoundly on a thorough understanding of how transdermal devices interact with the physiological skin environment, such as furrows, follicles, and eccrine ducts. In addition to the general evaluation methods, more detailed research should be carried out to investigate the permeability behavior of the skin, the delivery of the nanomedicine passing through the skin barrier, the drug retention effect in the local tissue environment, the biodistribution, and the clearance mechanisms. In addition, the stimuli-responsive release of nanotherapeutics can facilitate improvement of transdermal drug delivery. The targeting and release effects in response to physiological signals such as pH, redox, enzymes, glucose, and ATP gradient should be fundamentally investigated [91, 116, 117]. However, considering the timescale of pharmaceutic regulation, it is not surprising that more time is required to overcome translational challenges in the field of transdermal nanomedicine.

Second, given the importance of uniformity and safety in clinical trials, the rational design of the nanotherapeutics with transdermal device should follow the standard synthesis procedures, including the capability to fabricate transdermal devices with specific physicochemical properties and reproduce ability. It requires reducing variability in formulation/device morphology, surface properties, optimal therapeutic doses, and pharmacokinetics and stability while decreasing their development costs. The success of commercially available products like the nicotine patches (NicoDerm®) should be used as a stepping stone for the continued development of transdermal technology incorporating nanomedicines for cancer applications.

Last but not least, new techniques for noninvasive drug delivery and new evaluation methods for skin permeation should be explored for the development and translation of transdermal devices. Systems with significant therapeutic efficacy but without highly complex design and characterization would be ideal candidates for clinical translation. One promising direction for the design of new and efficient formulations could be inspired from nature by mimicking the structures and compositions of biological objects, such as bacteria and viruses.

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Chapter 11
Cancer Nanotherapeutics Administered by Non-conventional Routes

Kyle C. Roche, Yusra Betul Medik, Zach Rodgers, Sam Warner, and Andrew Z. Wang

11.1 Introduction

Nanomedicine has recently emerged as a powerful tool for improving the efficacy of cancer therapeutics. These improvements are attributable to the advantageous properties that nanoparticle drug formulations (NDFs), nanomaterials combined with drugs, display including enhanced solubility of hydrophobic drugs, relatively high drug-carrying capacity, and prolonged drug release profiles [1, 2]. Conventionally, NDFs are administered intravenously and passively target cancerous lesions through the enhanced permeability and retention (EPR) effect [2, 3]. The EPR effect is a process in which systemically circulating nanoparticles penetrate through the "leaky" tumor vasculature where they accumulate due to poor lymphatic drainage [2, 3]. While intravenous administration of nanoparticles has proven successful, there are notable limitations associated with this administration strategy. Firstly, intravenous nanoparticle administration results in off-target

K. C. Roche · Y. B. Medik · S. Warner · A. Z. Wang (✉)
Laboratory of Nano- and Translational Medicine, Carolina Center for Cancer Nanotechnology Excellence, Carolina Institute of Nanomedicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Department of Radiation Oncology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
e-mail: kyleroche@gwmail.gwu.edu; yusra@email.unc.edu; swarne14@jhmi.edu; zawang@med.unc.edu

Z. Rodgers
Department of Chemistry, School of Physical and Mathematical Sciences, Westminster College, New Wilmington, PA, USA
e-mail: rodgerzl@westminster.edu

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accumulation and toxicity in normal organs including the kidneys, liver, and spleen. The liver and spleen are particularly susceptible to off-target toxicity due to the presence of phagocytic macrophages that sequester nanoparticles from the vasculature [2, 3]. Secondly, the utility of intravenously administered nanoparticles is limited to vascularized tumors. Consequently, efforts have been made to investigate whether the delivery of NDFs directly to anatomical locations with tumor burden has the potential to increase therapeutic efficacy while avoiding the off-target toxicities associated with conventional intravenous nanotherapeutic administration [2, 3].

A number of non-conventional administration techniques have been developed that are capable of delivering NDFs in an anatomically targeted fashion. Importantly, the tissue-specific environment being targeted and the device being used for delivery must be accounted for when fabricating NDFs. For example, NDFs being delivered to lung cancers must be able to maintain their integrity following aerosolization and avoid cellular clearance by resident immune cells [4]. To date, NDFs delivered via non-conventional routes have been used to treat several cancers including pulmonary cancer, intraocular melanomas, colorectal cancer, bladder cancer, and cervical cancer [2, 3]. Non-conventional nanotherapeutic routes of administration that will be discussed in this chapter include inhalation, ocular injection, rectal administration, intravesicular administration, and vaginal administration (Fig. 11.1). Obstacles/considerations will be discussed for each of these methods in addition to the underlying rationale of these anatomical targeting approaches as they apply to

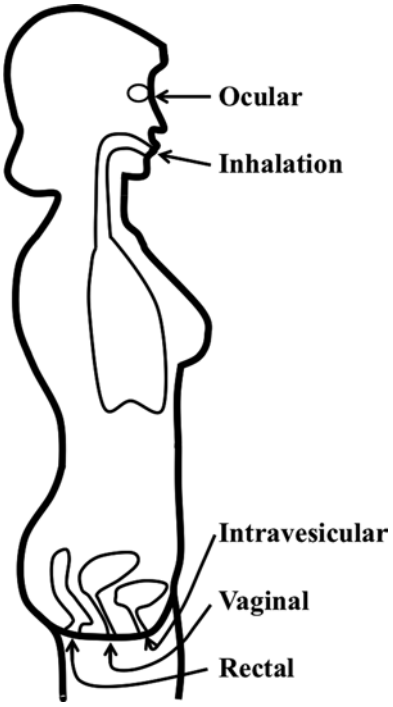

Fig. 11.1 Non-conventional sites of nanoparticle drug formulation administration

cancer therapy. While non-conventional NDF delivery has yet to be adopted in clinical practice, the results from clinical trials and preclinical studies are promising. Aerosol NDF delivery to the lung is the most extensively studied non-conventional NDF delivery approach and will likely be adopted in the clinical setting in the next few years [2–4].

11.2 Aerosol Nanotherapeutic Delivery

Lung cancer is responsible for over 30% of cancer-related deaths. The majority of lung cancer patients are ineligible for surgical intervention and require chemoradiotherapy [5]. The standard treatment for advanced lung cancer consists of administering gemcitabine and cisplatin intravenously followed by radiotherapy. Problematically, patients undergoing this treatment regimen often experience pain, nerve damage, and allergic skin reactions, highlighting the need for the development of targeted chemotherapeutic administration strategies [5]. A number of studies have investigated whether direct drug delivery to the lung using aerosolized NDFs can improve cancer therapy [4]. Aerosolization is a process in which nongaseous substances are broken down into particles small enough to be carried by a gaseous medium [6, 7]. Aerosolized nanoparticle administration to the lung has the potential to increase the therapeutic index of current lung cancer treatment regimens by increasing chemotherapeutic concentrations in tumors arising in pulmonary tissues while minimizing systemic exposure [8]. However, there are a number of obstacles that must be overcome to produce effective aerosolized NDFs. These formulations must be able to endure aerosolization, deposit on the pulmonary tissues containing cancerous lesions, and avoid mucociliary and immune clearance mechanisms [7].

Aerosolization is a relatively harsh process. There are a number of devices that can be employed to achieve nanoparticle aerosolization including nebulizers, dry powder inhalers, soft mist inhalers, and pressurized metered-dose inhalers. Nebulization is by far the most commonly utilized aerosolization method for pulmonary nanoparticle administration [4, 7]. Nebulizers use force generated from either compressed air (jet nebulizers), rapidly vibrating piezoelectric crystals (ultrasonic nebulizers), or vibrating mesh (mesh nebulizers) to produce a mist out of nanoparticle-containing solutions [6]. NDFs administered via jet nebulizers must be able to withstand shear forces and extensive exposure to liquid-air interfaces. NDFs administered using ultrasonic nebulizers must be designed in such a way as to avoid undergoing heat-induced degradation. For example, the use of liposomal nanoparticles composed of high phase transition temperature lipids and cholesterol for increased rigidity, effectively reduces NDF degradation during ultrasonic nebulization [9]. Note that successful nanoparticle administration via nebulization is not limited to liposomes [4, 7]. Dry powder inhalers use force generated from either compressed air or the negative pressure produced by inhalation to disperse powdered NDFs into the air [6]. Dried NDFs designed to be delivered using this device

must demonstrate minimal inter-particulate adhesive/cohesive forces as effective aerosolization depends on their ability to undergo airflow-induced disaggregation [4, 7]. Previous efforts have shown that the addition of disaggregation enhancers or carrier particles such as lactose to dried NDFs can improve aerosolization and deposition dynamics [10, 11]. Additionally, dry powder inhalers containing NDFs must be stored in cool dry areas, as high humidity environments can result in poor drug disaggregation and delivery [7]. Other methods of facilitating pulmonary nanoparticle delivery include the use of pressurized dose inhalers and soft mist inhalers, although they have not been widely implemented to date. Pressurized metered-dose inhalers use force generated from pressurized containers containing liquefied gas propellants, such as hydrofluoroalkanes, to eject aerosolized drug formulations [7]. The use of this delivery system for NDF administration is less favorable due to limited dose administration per actuation and high drug deposition on oral tissues [6]. Soft mist inhalers use force generated from a compressed spring to move liquid through a nozzle system, aerosolizing the nanoparticle-containing solutions. This system is easy to use, and aerosolized droplets created using this system have demonstrated relatively high lung depositions when compared to pressurized metered-dose inhalers due to slower aerosolized particle velocity [12, 13].

In addition to the choice of delivery system, another important consideration to be taken into account when designing aerosolized NDFs is the intended pulmonary deposition site within the lung. Particles are deposited onto pulmonary tissue by either impaction or sedimentation. Particles with diameters over 10 microns generally deposit in the oropharyngeal region via impaction, a process in which the trajectory of the particles fails to conform to the curves of the respiratory tract resulting in early deposition. Particles with diameters ranging between 5 and 10 microns are deposited in the central airways, while particles ranging from 1 to 5 microns are deposited in small airways through sedimentation, a process in which the force of gravity overcomes the kinetic force of the particles as they slow down. Particles less than 1 micron either deposit in the alveoli or remain suspended in the air and subsequently exhaled without deposition (Fig. 11.2) [13]. Recently, the development of hygroscopic aerosolized particles that increase in size as they interact with and adsorb water from the highly humid environment present in the lungs has been developed. This NDF avoids oropharyngeal deposition and while depositing at relatively high efficacy within the central and small airways of the lung [14, 15].

Another design consideration for aerosolized NDFs is the physiologic barriers they encounter following deposition. Nanoparticles deposited in the upper airways must be able to penetrate the mucus layer, whereas nanoparticles deposited in the lower airways must avoid being cleared by resident macrophages [4]. The upper airways of the lung are coated with a layer of mucus that is constantly renewed via mucociliary clearance, a process in which ciliated cells push mucus secreted by goblet cells out of the lung and into the esophagus via coordinated ciliary beating (Fig. 11.3) [7]. Highly glycosylated mucin fibers containing inter-dispersed hydrophobic domains interact with nanoparticles through hydrogen bonding and hydrophobic and electrostatic interactions [4]. Consequently, this mechanical defense mechanism impedes successful deposition of nanoparticle formulations. A number

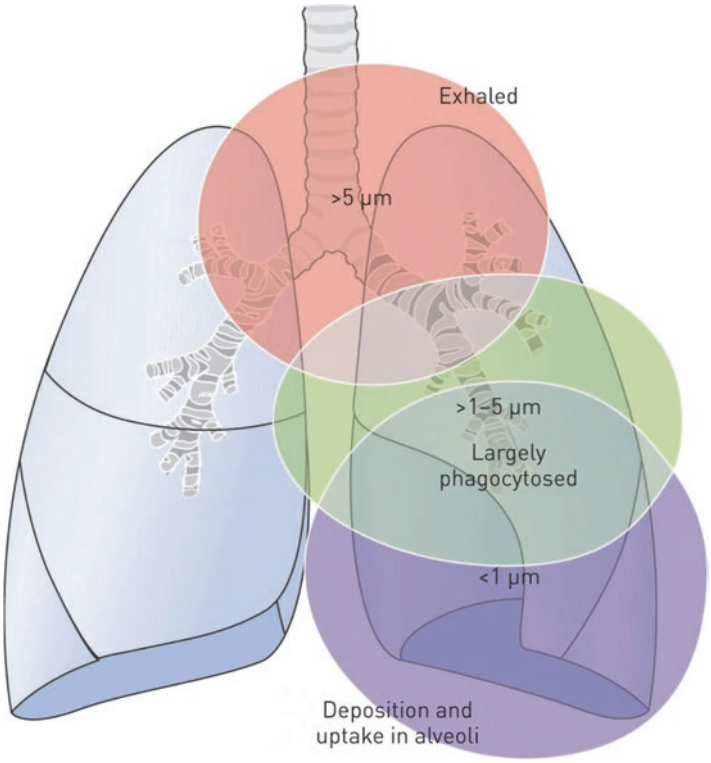

Fig. 11.2 The pulmonary deposition of aerosolized nanoparticle-containing droplets is size-dependent. Larger droplets (>5 μm) deposit in the upper airways and are lost to mucociliary clearance or fail to deposit and are lost to exhalation. Smaller droplets (1–5 μm) deposit in the smaller airways of the lung and can be phagocytized by pulmonary macrophages. The smallest droplets (<1 μm) deposit in the alveoli of the lung

of strategies can be applied to nanoparticle formulations to promote mucus penetration [16, 17]. One widely applied strategy that has proven successful is to coat nanoparticles with low molecular weight uncharged hydrophilic polymers, such as PEG. Alternatively, the co-administration of mucolytics can also promote penetration [18]. The efficacy of NDFs delivered to the lower airways is in part dependent on their ability to evade phagocytosis by resident alveolar macrophages. Nanoparticle size, shape, and charge can be altered to reduce uptake by alveolar-residing macrophages. The optimal nanoparticle size for avoiding macrophage-mediated clearance is unclear. A few studies have determined that particles with greater diameters (~1–6 μm) are most readily phagocytized by macrophages, while smaller particles (~0.2) are able to avoid uptake. In addition to size, shape also plays an important role in immune evasion [19]. One study found that rod-shaped nanoparticles were more effective than spherical-shaped particles at evading phagocytosis by macrophages in vitro [3, 20]. Finally, neutrally charged nanoparticles are preferable for avoiding immune detection, and many studies have shown macrophage uptake and

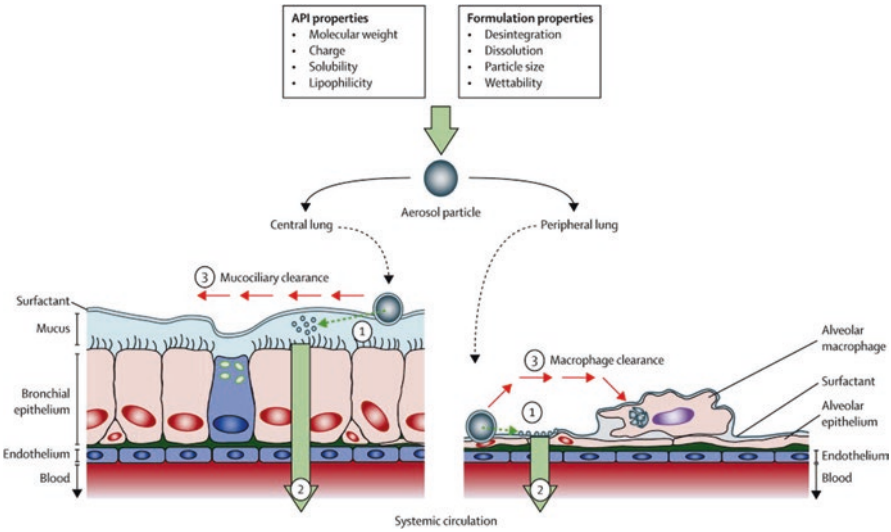

Fig. 11.3 Physiologic mechanisms of nanoparticle drug formulation (NDF) clearance from the lung. NDFs that deposit in the upper airways are subject to mucociliary clearance, whereas NDFs that deposit in the lower airways are cleared by pulmonary macrophages

clearance of various nanoparticle formulations increase with charge, both positive and negative. However, neutrally charged nanoparticles typically exhibit poor storage stability in solution and may require lyophilization with cryoprotectants so that they can be stored in powder form [19].

A number of clinical trials have evaluated the efficacy of aerosolized NDFs. Results from clinical trials assessing the efficacy of aerosolized liposomal formulations loaded with IL-2, 9-nitro-20(s)-camptothecin (9NC), cisplatin, 5-FU, and doxorubicin are promising [21]. In a phase I clinical trial, patients with pulmonary metastases were given liposomes containing IL-2 using a jet nebulizer [21]. Impressively, no significant toxicity was observed. Further clinical investigation is necessary to determine whether this treatment strategy represents an effective anticancer therapy. In another phase I clinical trial, lung cancer patients were treated with aerosolized liposomes loaded with 9NC. Liposomes were formed with dilauroylphosphatidylcholine (DLPC). The aerosol droplet size used for pulmonary delivery of the 9NC liposomal complex ranged between 1 and 3 μm [22]. Treatment-induced side effects were generally mild, the most common of which were nausea and vomiting. Of the 25 patients treated, 2 underwent partial remission and an additional 3 experienced stable disease. Finally, two separate phase I trials have evaluated the therapeutic efficacy of liposomes loaded with cisplatin for the treatment of pulmonary carcinomas. In a dose escalation study, 17 patients with lung carcinomas were treated with aerosolized liposomal cisplatin. Liposomes were fabricated with dipalmitoyl phosphatidylcholine and cholesterol. Note that the mean aerosolized droplet diameter was 3.7 μm as measured by cascade impaction [23]. Side effects

were generally mild including fatigue, dyspnea, nausea, vomiting, and hoarseness. Notably, pulmonary function was largely unaffected following the administration of the first round of treatment, and over 70% of patients enrolled in the study experienced stable disease [23]. An additional phase I trial assessed the efficacy of aerosolized liposomal cisplatin treatment in the setting of metastatic pulmonary disease. Nineteen patients were given liposomal cisplatin via a nebulizer. Of the 19 patients treated, 13 experienced significant respiratory side effects, and 1 demonstrated sustained partial response [24]. These results are promising and warrant further clinical study of aerosolized NDFs for lung cancer treatment.

In addition to the clinically approved NDFs mentioned above, a number of NDFs are currently being examined in the preclinical setting. A major focus in aerosolized NDF design is the development of NDFs that actively target cancerous lesions via either surface modifications or external magnetic field gradients [4, 7, 14]. Coating nanoparticles with molecules that target proteins on the surface of cancerous cells is a commonly implemented approach to improve the efficacy and specificity of NDF delivery. For example, aerosolized delivery of NDFs coated with luteinizing hormone-releasing hormone (LHRH) effectively reduced cancer burden in a murine lung cancer model. LHRH-targeted NDFs were prepared by conjugating DSPE-PEG-NH2 and DSPE-PEG-COOH nanoparticles loaded with paclitaxel with LHRH peptide. This LHRH-targeted NDF measures 110 ± 20 nm in diameter with a polydispersity index of 0.4 and a zeta potential of 60.3 mV [25]. Importantly, targeting moieties can also double as therapeutic agents. For example, functionalization of NDFs with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) increased the therapeutic efficacy of the NDF by targeting death receptors present on cancerous cells. Porous PLGA microparticles were loaded with doxorubicin, and subsequently their surface was conjugated with TRAIL. The mean diameter of resultant nanoparticles was 11.5 ± 0.4 μm [26]. Targeting these death receptors not only promoted NDF accumulation in tumors but also increased the therapeutic benefit of the NDF by working synergistically with doxorubicin to promote proapoptotic signaling in cancerous cells (Fig. 11.4).

An alternative targeting approach that is being investigated in preclinical studies is the use of external magnetic field gradients to control the site of aerosolized magnetic NDF deposition within pulmonary tissues. Note magnetic nanoparticles are commonly referred to as superparamagnetic iron oxide nanoparticles (SPIONs) [27]. SPIONs have a nanocrystalline magnetite core (Fe_3O_4) and a biocompatible coating with functional groups for conjugation of therapeutics. Different polymer shells such as chitosan, PEI, and PEG have been developed. As proof of principle, one study evaluated aerosolized SPION-mediated delivery of model chemotherapeutics and biotherapeutics to pulmonary tissues with and without external magnetic gradients. Impressively, the use of external magnetic field gradients increased the localization and deposition of SPION to target tissue by two- to threefold. In addition to enhancing localization, magnetic fields applied to SPIONs can also destroy cancerous lesions via hyperthermia [27]. Researchers assessed the efficacy of PEG-coated iron oxide nanocubes in mild tumor magnetic hyperthermia treatment. Specifically, when SPIONs are exposed to a rapidly altering magnetic field,

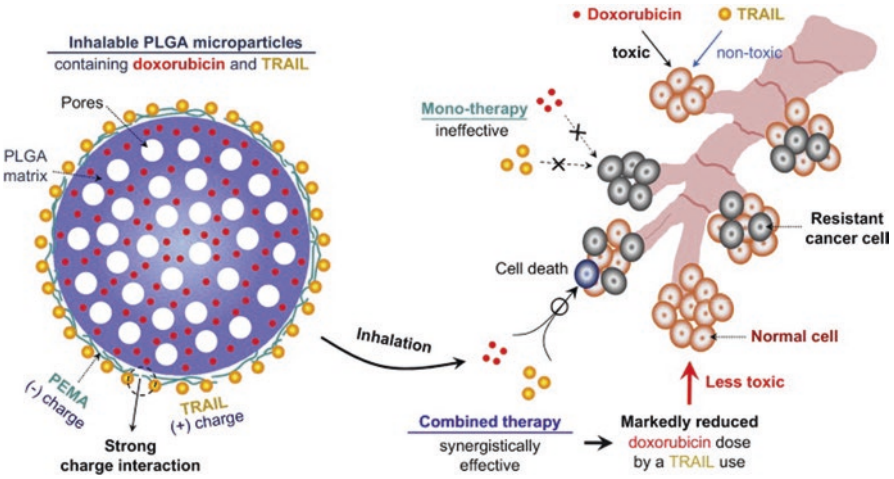

Fig. 11.4 Coating aerosolized nanoparticle drug formulations containing doxorubicin with TRAIL increases their cancer treatment efficacy. Conjugated TRAIL acts as both a targeting moiety and a direct therapeutic agent that works synergistically with doxorubicin

they rapidly heat due to hysteresis loss, the induction of eddy currents, and Neel relaxation. This heat is transferred to surrounding tissue via conduction resulting in cancer cell necrosis. Note that to date, no aerosolized SPION formulations have been used to treat tumors via hyperthermia. Nonetheless, this possibility will likely be explored in the preclinical setting in the near future.

11.3 Ocular Nanotherapeutic Delivery

The detection and treatment of ocular cancers is challenging. Uveal melanoma is the most common ocular cancer. These cancers arise from melanocytes present in uveal tissues including the iris, ciliary body, and choroid [28, 29]. Unfortunately half of all patients treated for primary uveal melanomas die within 15 years due to metastatic spread, highlighting the need for better detection and therapeutic treatment strategies. Commonly implemented treatment methods for ocular cancers are enucleation, external beam radiotherapy, local resection, cryotherapy, and brachytherapy [28, 29]. Previous efforts to treat ocular cancers using chemotherapeutics have proven unsuccessful (success rate of single-agent treatment regiments <10%), in part due to an inability to deliver sufficient quantities of chemotherapeutics to the ocular tumors [28, 29]. While the utilization of intra-arterial injection of chemotherapeutics into the ophthalmic artery has increased the efficacy of chemotherapy, in many cases this approach still fails to deliver sufficient quantities of chemotherapeutics to ocular tumors to achieve pathologic complete response [30]. Alternative delivery approaches, including intratumoral injection and implantation, have been

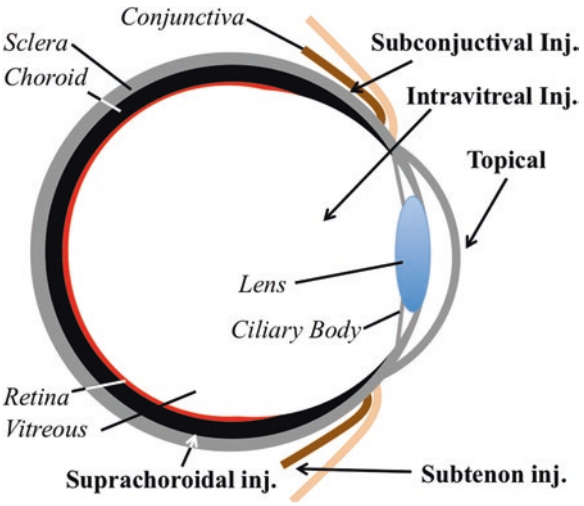

Fig. 11.5 Anatomical targeting sites of the eye used for direct ocular injection of nanoparticle drug formulations. (Inj, injection)

investigated; however these approaches necessitate invasive surgery and carry the inherent risk of visual impairment. Additionally, surgical intervention may promote tumor extension and orbital dissemination [28, 29]. Consequently, there has been an immense amount of interest in developing noninvasive chemotherapeutic delivery techniques. The topical administration or injection of NDFs represents a relatively less invasive approach for delivering chemotherapeutics to ocular cancers in a prolonged manner.

As with direct pulmonary administration, the delivery method used to administer NDFs to ocular cancers represents a major consideration. Currently, there are four delivery methods that are suitable for nanotherapeutic administration including topical eye drops, intravitreal injection, injection into the suprachoroidal space, and periocular injection (Fig. 11.5) [31, 32]. The use of topical eye drops for nanotherapeutic administration is attractive due to its noninvasive nature and ease of administration. The efficiency of this delivery method, however, is relatively low. NDFs deposited on the ocular surface elicit reflex tearing and must be able to avoid enzymatic degradation as well as mechanical clearance due to blinking and nasolacrimal drainage [33]. In the event of successful deposition, NDFs must then traverse the corneal epithelium and lens barrier and diffuse across the vitreous humor [31, 32]. In preclinical studies, this method has been used to successfully treat cancers residing in the anterior segments of the eye, such as the cornea and iris, but not in cancers residing in the posterior eye tissues, likely due to poor drug permeation. In the future, this delivery method may be suitable for treating posterior eye cancers, as many studies have investigated ways of enhancing the ocular tissue penetration capacity of NDFs [34]. For example, a number of studies have concluded that positively charged liposomes ranging from 105 to 125 nm in diameter are more capable of interacting with the negatively charged mucins on the ocular surface reaching posterior ocular tissues [35].

In contrast to topical eye drop administration, intravitreal injections surpass the corneal barrier resulting in increased chemotherapeutic concentrations in posterior eye tissues. Importantly, this procedure is more invasive than topical eye drops and carries significant risks including visual impairment, endophthalmitis, retinal detachment, vitreous hemorrhage, and cataract development. Therefore, the number of injections given should be limited as much as possible [33–35]. To this end, a major consideration when designing NDFs to be administered via intravitreal injection should be their stability and ability to support prolonged drug release. Notably, the inherent risk of tumor dissemination following intravitreal penetration remains a significant concern and has limited clinical adoption of this practice.

Additional routes of ocular administration include injection into suprachoroidal space and periocular tissues (Fig. 11.5) [31, 32]. A relatively new procedure, suprachoroidal space injections using microneedles, represents an effective method for delivering drugs to the choroid and ciliary body [35]. Specifically, NDFs are injected into tissue pockets between the sclera and choroid. In a recent study, it was found that the post-injection diffusion of fluorescently labeled microparticles can be controlled by altering their surface chemistry. For example, the addition of hyaluronic acid to the surface of NDFs promotes particle diffusion [36]. Periocular injection is less invasive than intravitreal injection and more effective at delivering drugs to intraocular tissues than topical eye drop delivery. Periocular delivery involves injecting nanoparticles into the subconjunctival, suprachoroidal, or subtenon tissues. Commonly incurred side effects that accompany this delivery strategy include increased intraocular pressure, corneal endothelial dystrophy, hyphema, and strabismus [33, 35]. Clinically, periocular injection of free chemotherapeutics, such as carboplatin, represents an effective adjunct therapy for the treatment of retinoblastoma, and periocular injection of nanoparticles loaded with carboplatin has been clinically investigated (discussed below).

The chosen method of nanoparticle delivery to ocular cancers plays an important role in nanoparticle design, and we have just begun determining the optimal nanoformulations for each delivery method. Like many NDFs, NDFs designed to target cancerous lesions within the eye can be coated with a variety of targeting molecules such as EpCAM, folate, and CD44 [33, 35]. Ocular tissues are relatively sensitive, and therefore, an additional criterion that must be considered during nanoparticle design is local toxicity. A number of particle formulations have already been shown to cause ocular damage. Formulations consisting of dendrimers, polystyrene, carbon nanotubes, and titanium dioxide can elicit adverse effects in ocular tissues including direct tissue damage, inflammation, and hypersensitivity [37]. In contrast, liposomes and silicon-based quantum dots are relatively well-tolerated.

There are currently no clinically applied nanomedicine-based treatment regimens for ocular cancers. While preclinical and clinical studies have confirmed that intraocular NDF administration improves drug delivery, in vivo data assessing the feasibility of this treatment strategy is limited due to a lack of ocular cancer animal models [33]. One preclinical study showed that the intraocular drug concentrations were significantly greater in animals intraocularly injected with polymethylmethacrylate nanoparticles loaded with carboplatin when compared to animals injected

with free drug. Polymethylmethacrylate nanoparticles were prepared by free radical emulsion polymerization in a carboplatin containing aqueous solution. The average diameter of nanoparticles was 110 ± 10 nm with a negative zeta potential [38]. Consistent with this study, a phase I clinical trial performed using the same NDF on patients scheduled to undergo uniocular enucleation found that intravitreal injection of this carboplatin containing NDF was able to support sustained drug release into retinal tissues for at least 72 h in human subjects [39]. Importantly, no carboplatin was detected in the systemic vasculature of any patients undergoing treatment. The therapeutic efficacy of this treatment strategy will have to be assessed in future studies. In addition to improving drug delivery, intraocular delivery of NDFs has also been shown to improve therapeutic response of eye cancers in preclinical studies. A recent preclinical trial demonstrated that dendrimeric nanoparticles containing carboplatin periocularly injected into the subconjunctival space of transgenic mice containing retinoblastomas reduced tumor burden [40].

In addition to NDFs containing chemotherapeutics, NDFs containing biotherapeutics have also been assessed in preclinical trials. As proof of concept, NDFs containing a nucleic acid construct encoding green fluorescent protein (GFP) were administered to mice via subretinal or intravitreal injection. Excitingly, both injection strategies resulted in robust GFP expression in target tissues [41]. More recently, a preclinical study assessed the feasibility of treating intraocular melanoma by intravitreal injecting vectosomes, a light-sensitive NDF containing antisense oligonucleotides (ODN). Vectosomes were composed of oligonucleotides bound to the C-terminal amino acids of herpes simplex virus structural proteins. The diameter of the resultant NDFs ranged from 0.3 to 1 μm. This study found that intravitreal injection of vectosomes, followed by light stimulation, resulted in efficient ODN release in retinal and retinal pigment epithelial cells [42].

An alternative approach that is currently being developed in vitro is the use of NDFs to combat cancer via photodynamic therapy. Photodynamic therapy is a treatment approach that relies on the excitation of photosensitive compounds using light to produce reactive oxygen species, thereby killing cancerous cells [33]. Two recent in vitro studies have verified the validity of this approach in vitro by exposing retinal blastula cells to NDFs containing photosensitizing agents [43, 44]. These NDFs demonstrated efficient cellular uptake and elicited phototoxicity within cancer cells following exposure to light. The use of photodynamic therapy to treat cancerous lesions in vivo will likely be explored in the near future, as this approach allows for a high degree of spatial targeting capacity.

11.4 Vaginal Nanotherapeutic Delivery

Approximately a quarter of a million women succumb to cervical cancer each year with another half a million new cases arising annually. Contemporarily, surgical intervention performed in concert with radiotherapy cures 80–95% of women presenting with early-stage cervical cancer [45]. Unfortunately, many of these patients

experience negative side effects including impaired sexual function and infertility. Systemic administration of chemotherapeutics to patients with cervical cancer is commonly employed as a last line of treatment due to a low efficiency of drug delivery to the cervical epithelium and detrimental off-target effects, emphasizing the need for more targeted therapeutic approaches [45]. As with previously discussed anatomically targeted delivery methods, vaginal administration results in higher drug concentrations and reduced systemic toxicity.

The physiologic characteristics of vaginal tissue necessitate consideration when designing NDFs for vaginal delivery. The cervicovaginal mucus lining is a formidable physical barrier. As in the lung, coating nanoparticle surfaces with nonadhesive PEG allows nanoparticles to penetrate through the mucus layer and access the underlying tissue. Nanoparticle size should also be tailored to promote mucus penetration. The cervicovaginal mucus lining is a tightly knit structure composed predominantly of glycoprotein lipids with pores ranging from 270 to 410 nm [45]. Nanoparticles exceeding 500 nm, therefore, are unlikely to achieve mucus penetration. An alternative approach to developing mucus-penetrating NDFs is to develop NDFs that adhere to the mucus lining. In doing so, NDFs can transiently release drugs topically over the course of approximately 24 h, being cleared passively as the mucus layer is renewed.

Additional characteristics of cervicovaginal physiology that must be accounted for are the resident microbiota and pH of the vaginal canal. The squamous epithelium of the vaginal wall is constantly renewed as cells are sloughed off into the lumen of the vagina, acting as a food source for resident lactobacilli. Importantly, these bacteria secrete lactic acid and effectively lower the pH of the lumen to approximately 4, thereby hindering the ability of various pathogenic agents to colonize and grow. When lactobacilli populations are destroyed, the pH rises, thereby, increasing the susceptibility of the vagina to pathogenic colonization (bacterial vaginosis) and the risk of inflammatory disease [45]. Therefore, NDFs to be delivered through the vaginal canal should be stable in acidic conditions and ideally minimally impact resident lactobacilli populations.

There are currently no clinical trials underway investigating the use of vaginally delivered nanotherapeutics for either vaginal or cervical cancer treatment. However, promising preclinical data suggests that there is merit in this treatment strategy. One study demonstrated that cancer initiation and establishment could be prevented by prophylactic treatment with camptothecin (CPT)-loaded PLGA nanoparticles [46]. CPT-loaded PLGA nanoparticles were prepared using a single-emulsion oil-in-water method and demonstrated a mean diameter of 158 ± 62 nm. Specifically, this study showed that CPT-loaded nanoparticles delivered via vaginal lavage were able to prevent the formation of tumors from vaginal epithelium with activated oncogenic (Kras) and inactivated tumor suppressor (Pten) genes. Preclinical studies have also shown that vaginally delivered nanotherapeutics can treat established cancers. In one study, PLGA nanoparticles coated with PEG and loaded with paclitaxel were effective in treating tumors derived from implanting TC-1 cancer cells to the vaginal wall [47]. Paclitaxel-loaded, mucus-penetrating PLGA particles were fabricated using nanoprecipitation. The average diameter of nanoparticles was 239 nm with a

zeta potential of −7 mV. Interestingly, particles lacking the PEG coating were ineffective, presumably due to decreased mucus penetration and subsequent clearance. Future studies will likely evaluate the in vivo treatment efficacy of NDFs containing multiple therapeutic agents, including biotherapeutics. A recent study evaluated that efficacy of a NDF containing paclitaxel and a Bcl-2 siRNA. Solid lipid particles were prepared using solvent emulsification/evaporation. This NDF had a mean diameter of 167.09 ± 9.67 nm and zeta potential of 23.24 mV. They found that solid lipid NDFs containing paclitaxel and Bcl-2 siRNA were more effective in eliciting tumor cell death in vitro than NDFs containing only one of these therapeutic agents [48]. The effectiveness of this NDF will likely be assessed in tumor-bearing animal models in the near future.

11.5 Rectal Nanotherapeutic Delivery

Colon and rectal cancers are the third most common cancers diagnosed each year and the second leading cause of cancer-related deaths in the United States. Conventional treatment methods for rectal cancer include surgical resection and intravenous administration of 5-FU [49]. However, due to its relatively short half-life, poor stability (enzyme dihydropyrimidine dehydrogenase-induced degradation), and the broad off-target effects, increasing efforts have been focused on developing novel drug delivery routes of 5-FU to the colon including direct rectal administration and oral colon-targeted and rectal drug administration [50]. It is important to note that the applicability of direct administration of NDFs to the rectum is not limited to treating colonic disease in situ. In fact, the colonic epithelium is very permissive to drug uptake and may represent an ideal route of delivery for systemic drug delivery. In this section, we will discuss the use of oral and rectal NDF delivery methods for treatment of colorectal cancer (CRC). Specifically, we will discuss the limitations and considerations that must be taken into account designing NDFs to be delivered via these methods and the current state of their clinical use.

There are a number of complications associated with oral nanotherapeutic delivery that are avoided in rectal delivery. Oral delivery of NDFs to the colon necessitates traveling through the gastrointestinal tract. Once swallowed, nanoparticles must endure the low pH environment in the stomach and enzymatic degradation in both the stomach and small intestine while avoiding drug deposition and absorption before reaching the colon [51]. To this end, strategies have been developed to avoid nanotherapeutic deterioration and deposition in the upper GI while increasing drug release in the colon. Many pH-dependent drug release systems have already been developed that prevent drug deposition in the upper GI by encapsulating drugs in pH-dependent polymers [50, 52]. For example, the inclusion of an enteric coating on 5-FU containing nanoparticles prevents pH-dependent nanotherapeutic degradation in a simulated gastric environment [53, 54]. Problematically, these systems tend to deposit high doses of drugs in the small intestine after exiting the stomach

due to a rapid pH change. Efforts have also been made to develop drug formulations that demonstrate time-dependent release. Unfortunately, the variability in time it takes for the stomach to empty its contents has hindered the clinical adoption of this approach [50]. Finally, efforts have also been made to take advantage of the relatively high microbial concentrations in the colon by designing drug formulations that require enzymatic activation by bacteria residing in the colonic lumen [50]. While none of these strategies have proven successful on their own, combinations of these techniques have been successfully applied to drug delivery strategies resulting in significant free drug deposition specifically in the colon. In the future these methods may be applied to NDF design.

Rectal nanotherapeutic administration avoids many of the complications that limit the efficacy of oral nanotherapeutic delivery. However, both oral and rectally administered nanoparticles must be designed with the physiology of the colon in mind. Two major considerations include the presence of mucus layer atop the colonic epithelium and a diverse and abundant microbiota. As previously discussed, the addition of a PEG coating can increase mucus penetration; however, it has also been shown to reduce enzymatic degradation by resident bacteria [17, 50]. Another significant limitation in rectal administration is the physical drug delivery. The human colon is about 5 feet in length; therefore, physically reaching the proximal colon is not contemporarily feasible.

Currently, there are no clinically approved colorectal cancer treatment strategies that utilize rectal administration of NDFs. However, preclinical studies have demonstrated that direct rectal delivery of NDFs via suppository or enema may be effective. A number of NDFs carrying chemotherapeutics designed for direct administration into the rectum have been developed. One study found that negatively charged, lipidoid nanoparticles delivered via enema were able to accumulate and deliver siRNAs to the epithelium of the large intestine through a lipid raft endocytosis, demonstrating a predilection for uptake in CRC epithelial tissues. Negative lipoid nanoparticles were synthesized by conjugating lipidoid 98 N12-5 with PEG and cholesterol in an aqueous solution containing 35% ethanol. siRNA-loaded lipidoid nanoparticles had a mean diameter of 90 nm and a zeta potential of -8 mV [55]. A commonly employed approach to reduce NDF clearance from the lumen of the large intestine is suspending NDFs in hydrogels and mucoadhesive polymers [50]. For example, DOX-loaded liquid nanotubes suspended in a hydrophilic gel are able to support durable pH-dependent drug release profiles that were able to elicit cancer cell death in vitro. Nanotubes were formulated using Aqua, a molecule that is redox-active and pH sensitive. Ribbonlike nanotube aggregates demonstrated a width of 0.2–0.6 nm and a length of 4–10 nm with a zeta potential of 23.73 mV [56]. As mentioned previously, rectal nanotherapeutic delivery strategies represent a promising strategy for treating a multitude of cancers given that NDF delivered via the rectum can support durable systemic drug delivery. To this end, one study found one CRC nanotherapeutic formulation carrying irinotecan was found to prolong systemic drug bioavailability more effectively than oral or intravenous drug administration [57]. A more recent study found that a double-reverse thermosensitive nanocarrier system (DRTN) can promote durable systemic delivery of irinotecan

[58]. This system is comprised of solid lipid nanoparticles homogenously suspended in a thermopolymerizing hydrogel. This system avoids the initial rapid increase of drug concentrations within the vasculature as seen following intravenous administration and supports sustained drug release. Importantly, this favorable pharmacokinetic profile translated to enhanced therapeutic response in a subcutaneous xenograft tumor model, and this delivery system may be assessed in the future.

11.6 Intravesical Nanotherapeutic Delivery

Bladder cancer is the fourth most commonly occurring cancer in men, and over 70% are diagnosed as nonmyoinvasive, superficial lesions [59]. In cases of nonmyoinvasive bladder cancer, the standard treatment regimen consists of transurethral resection followed by immunotherapy and/or chemotherapy. Problematically, systemic treatment strategies are largely ineffective, because the urothelial layer of the bladder is not well vascularized [59, 60]. Consequently, standard treatment involves intravesicular administration of either the immunotherapeutic Bacillus Calmette-Guerin (BCG) or chemotherapeutics such as valrubicin, mitomycin C, adriamycin, or thiotepa. The addition of postoperative immunotherapy has been shown to prolong disease survival and reduce tumor recurrence; however, recurrence rates remain high (up to 60% at 2 years following treatment) [60]. Additionally, the side effects of intravesicular BCG administration can be problematic, including BCG infection, sepsis, and death [60]. Therefore, the development of effective nanotherapeutics for bladder cancer treatment remains an active area of research.

There are a few significant limitations and considerations that need to be accounted for when designing nanotherapeutics for intravesicular administration. First, the large majority of drug is lost following urination. Patients undergo catheterization and treatment every 4–6 weeks, predisposing them to local irritation, infection, and bladder fibrosis [60]. The development of NDFs that are able to avoid clearance and support prolonged drug release profiles may reduce the number of catheterizations required for treatment. Multiple studies have found that cationic nanoparticles are able to avoid clearance supporting drug release for at least 6 hours post administration. One recent preclinical study in particular demonstrated that nanoparticles made solely from FDA-approved materials (mPEG-PLA and DOTAP) were able to support sustained release of DOX, resulting in a prominent reduction in tumor growth rates in vivo [61]. Another important consideration is how nanotherapeutics interact with urine following intravesicular delivery. The chemical composition of the urine can vary dramatically, particularly with regard to pH (the normal pH range for urine is 4.5–8.0), which can strongly influence the stability and drug release rates of nanotherapeutics. For example, MMC is stable at neutral pH, but readily deteriorates in acidic or basic conditions. Importantly, measures have been developed to reduce the effects of residual urine on the efficacy of nanotherapeutic deposition and drug release, such as ultrasound-guided removal of residual urine and neutralization of urine using oral sodium bicarbonate.

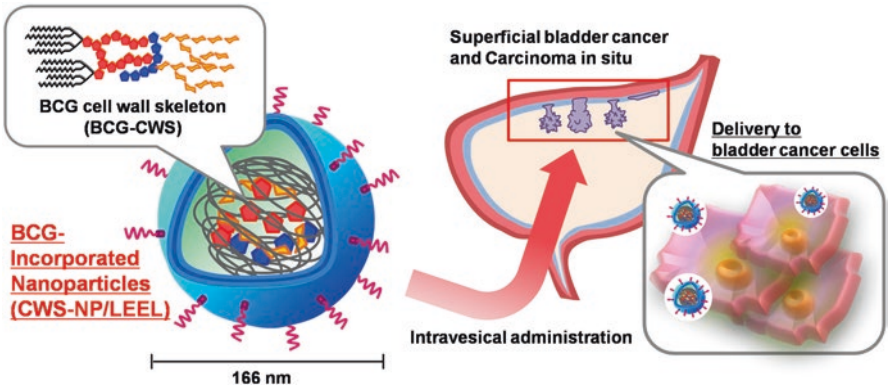

Fig. 11.6 Intravesicular administration of nanoparticle drug formulations containing Bacillus Calmette-Guerin cell wall skeletons represents an effective method for treating superficial bladder cancer

Many preclinical studies have been conducted evaluating the efficacy of intravesically delivered nanotherapeutics. Cumulatively, these studies demonstrate that this treatment strategy is safe and more effective than free drugs at combating bladder cancer in most cases. Again, standard treatment for nonmyoinvasive bladder cancer consists of surgical intervention followed by treatment with BCG, which carries some inherent risks including systemic infection. One preclinical study took a nanomedicine-based approach to mitigate these risks by encapsulating the bacterial wall skeleton of *Mycobacterium bovis* BCG (BCG-CWS) in liposomes (Fig. 11.6) [62]. This liposomal formulation consisted of R8 (octaarginine)-modified liposomes encapsulating BCG cell wall skeletal components. The mean diameter of this NDF was 166 ± 2 nm with a zeta potential of 31 mV. This NDF was effectively taken up by mouse bladder tumor cells (MBT-2) in vitro and impeded tumor development in mice bearing MBT-2 tumors. This approach was also effective in treating rats with naturally occurring bladder cancers. Preclinical studies have also demonstrated that a variety of polysaccharide-based nanoformulations loaded with MMC have promising drug release profiles when compared to free drug administration. Chitosan–polycaprolactone nanoparticles were prepared by the W/O/W double emulsion method. The average diameter of nanoparticles was 318 nm with a polydispersity index of 0.18 and a zeta potential of 10.5 mV. In addition to immunotherapeutics, the delivery of chemotherapeutics with NDFs continues to be explored in the preclinical setting [63]. One study found that NDF-mediated delivery of DOX improves drug retention, penetration, and cellular uptake resulting in a more robust therapeutic response for the treatment of bladder cancer in vivo [64]. As with NDF delivery to the lumen of the large intestine, NDFs were suspended in bioadhesive gels. Bioadhesive nanoparticles were prepared by ionotropic gelation of thiolated chitosan (chitosan–thioglycolic acid conjugate) in bioadhesive chitosan gel or in sodium tripolyphosphate. The average diameter of the resultant NDF was 174.5 ± 3.76 nm with a zeta potential of 32.1 mV. A recent study found

2% chitosan gels maintain their integrity in artificial urine and therefore have the potential to improve the residence NDF delivery [64].

Recent clinical trials have demonstrated that intravesicular delivery of taxane carrying NDFs may be safe and improve the efficacy of bladder cancer treatment. The safety of taxane-loaded nanotherapeutics has been assessed in two phase I trials. In one trial, 60% of bladder cancer patients treated with paclitaxel-hyaluronic acid bioconjugates demonstrated complete treatment response with no systemic drug exposure [65]. In a separate trial, 56% of patients given albumin-bound paclitaxel experienced mild side effects, mainly dysuria. Furthermore, 28% of patients demonstrated no evidence of disease posttreatment. Note that albumin-bound paclitaxel (Abraxane®) is an FDA-approved NDF [66]. The efficacy of this nanotherapeutic was also addressed in a phase II follow-up study [67]. Of the 28 patients enrolled in the study, 32% of the patients experienced mild adverse treatment effects. Notably, 36% demonstrated a complete treatment response, which remained durable for 1 year.

11.7 Concluding Remarks

The majority of cancer treatment strategies that rely on NDFs utilize intravenous administration. NDFs delivered via this route passively target cancerous lesions by the EPR effect, a process by which NDFs extravasate the leaky tumor vasculature and accumulate due to poor lymphatic clearance. In contrast, non-conventional routes of NDF administration actively target anatomical locations to increase local drug bioavailability in a sustained manner while reducing systemic side effects. A major limitation of intravenous NDF administration is the ability of the immune system to rapidly clear NDF from circulation. Many studies have demonstrated that coating nanoparticles in PEG can significantly enhance the circulation time of NDFs by limiting the immune detection and elimination. This NDF modification has also been investigated in the context of non-conventional NDF delivery. Specifically, studies have shown that coating aerosolized NDFs in PEG reduces pulmonary clearance by resident macrophages and increased mucus penetration in pulmonary, vaginal, and large intestinal tissues.

A promising approach for increasing the ability of NDFs to home to cancerous cells is modifying their surface with targeting molecules. In the context of intravenous NDF, this design strategy has been moderately successful. The limited success achieved by this approach is thought to be due to increased immune recognition and clearance of NDFs that contain these modifications. Importantly, NDFs delivered via non-conventional routes are not subject to the relatively high levels of immune surveillance present in the vasculature. Consequently, NDFs coated with targeting molecules may prove more effective when delivered via non-conventional routes. Future studies focusing on targeted non-conventionally delivered NDF design should evaluate the impact that these targeting moieties have on cancer targeting, mucus penetration, and immune clearance. Of note, a major challenge in intrave-

sicular NDF administration is the development of NDFs that are able to escape physical clearance during urination. In this case, the development of a NDF that is able to adhere to epithelial bladder tissue may be sufficient to improve therapeutic efficacy.

Non-conventional NDF treatment strategies are gaining traction due to the intuitive nature of the hypothesis underlying their efficacy, that targeted administration results in increased therapeutic availability. The clinical adoption of these treatment strategies has the potential to improve the therapeutic index of a variety of cancers, especially those that are poorly vascularized. Furthermore, the use of non-conventional NDF delivery routes has the potential to greatly reduce treatment costs by reducing total dose of drug required during treatment. Of the non-conventional routes of administration discussed, aerosolized NDF delivery is the only method that has undergone significant assessment in phase I and II clinical trials, the results of which warrant further assessment in phase III clinical trials. Notably, the effectiveness of this treatment strategy may be further enhanced by the development of more effective aerosolization devices and physical targeting strategies including the use of magnetic NDFs administered under the influence of a magnetic field gradient.

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Part III
Regulatory Considerations for Nanomaterial Drug Products

Chapter 12
Regulatory Considerations for Cancer Drug Products Containing Nanomaterials

Mamta Kapoor, Kathryn Hughes, and Katherine M. Tyner

12.1 Introduction

The inclusion of nanomaterials in drug products has increased in recent years, and the United States (US) Food and Drug Administration (FDA or the Agency)[1] has received several hundred applications from companies seeking to move these products to market [1]. These products and applications are often complex, and the ways this technology is used are myriad [1]. Among all of these nanotechnology-related submissions, the most commonly stated indication is for treatment of cancer. As described in previous chapters, nanomaterials may improve cancer treatments due to enhanced drug dissolution, drug distribution, and targeted delivery mechanisms (passive/active), which can significantly improve drug accumulation at the cancer site while reducing adverse effects [2–4]. Reflecting both the promise of these materials and the high-risk tolerance for novel treatments among clinicians and cancer patients [5, 6], FDA saw an 8% increase in submissions for cancer therapeutics containing nanomaterials between 2011 and 2016 [7].

[1] The US Food and Drug Administration is a government agency responsible for (1) protecting the public health by assuring the safety, effectiveness, quality, and security of human and veterinary drugs, vaccines, and other biological products and medical devices; (2) ensuring safety and security of most of our nation's food supply, all cosmetics, dietary supplements, and products that give off radiation; (3) regulating tobacco products; and (4) advancing the public health by supporting innovations that facilitate more effective, safer, and affordable medicines [1]. In the USA, all companies wishing to sell or market a new pharmaceutical must first submit an application for review to the FDA.

M. Kapoor · K. Hughes · K. M. Tyner (✉)
Center for Drug Evaluation and Research, US Food and Drug Administration, Silver Spring, MD, USA
e-mail: Mamta.kapoor@fda.hhs.gov; Kathryn.hughes@fda.hhs.gov; Katherine.tyner@fda.hhs.gov

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Applications for new pharmaceuticals in the USA are reviewed by the FDA's Center for Drug Evaluation and Research (FDA/CDER), which evaluates the safety and efficacy of both drug substances[2] and drug products.[3] The degree of innovation and variety in the drug products containing nanomaterials is broad, and FDA has developed conceptual and analytical frameworks to capture consistently the risks introduced by new therapeutic approaches. When reviewing drug product applications, the Agency considers the entire product, from the data demonstrating clinical efficacy to the chemistry, production, storage, and delivery method(s). These last four areas are collectively evaluated as the quality attributes of a drug, and FDA determined that these have particular importance for drug products containing nanomaterials. The relevant quality attributes are discussed in detail in this chapter following a brief overview of the mechanisms of action of nanomaterials within products designed to treat cancer and a description of the regulatory structure in which applications for cancer therapeutics are reviewed.

12.1.1 Nanomaterials in Anticancer Drug Substances and Products

The Centers for Disease Control and Prevention notes that cancer is at present the second most common cause of death in the USA [8], and as a class of diseases, it is a focus of government-funded research initiatives [9] . The progression of the disease and its potential for causing loss of life make it a good target for the development of innovative drug substances (and drug products) [5, 6]. However, many anticancer drug substances suffer from poor water solubility and toxicity issues [10], which reduce the overall efficacy and safety of the compounds.

Using nanomaterials within the drug product is one potential method for resolving these issues because a material's physicochemical properties can change with particle size. For example, by reducing the particle size of a drug substance to the nanoscale, the effective surface area can be increased severalfold to modify surface-related characteristics such as apparent rate of dissolution [11, 12]. Alternatively, the properties of nanoscale drug carriers can be "borrowed" to improve the bioavailability of a less-soluble or less-tolerated drug substance by facilitating longer circulation in vivo and targeted delivery (passive/active) [3, 13]. Thus, nanomaterials are used by drug developers to significantly improve drug accumulation at the cancer site while reducing adverse effects seen in the use of conventional formulations [2, 4, 14].

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[2] "an active ingredient that is intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease or to affect the structure or any function of the human body, but does not include intermediates use in the synthesis of such ingredient" 21 CFR 314.3.

[3] "a finished dosage form, for example, tablet, capsule, or solution, that contains a drug substance, generally, but not necessarily, in association with one or more other ingredients" 21 CFR 314.3.

12.1.1.1 Nanotechnology-Based Platform Technologies

Several nanotechnology-based platform technologies such as liposomes, nanoparticles, micelles, and drug conjugates have been developed to take advantage of the properties of nanomaterials to deliver drug substances to cancerous tissue. They have been applied to support the reformulation of approved cancer drugs as well as to deliver new medicines (Tables 12.1 and 12.3) in order to reduce the size of tumors or related cancer events without damaging healthy tissues. These materials take a number of forms and may be lipidic, metallic, polymeric, or proteinic in nature. They can be used to facilitate drug solubilization and enzymatic stability and/or enhance cellular uptake via either complexation or covalent conjugation with the drug.

In many cases, these materials are tailored to target the tissues passively (e.g., DaunoXome®, Taxotere®, Marqibo®, Genexol, Doxil® [see Box 12.1]) or actively (e.g., Tf-LPN-G3139, MCC-465) by taking advantage of the particular characteristics of a tumor's microenvironment (i.e., the enhanced permeability and retention effect) or the surface binding features of cancerous cells [13, 15–25].

Box 12.1 Doxil®: Passive Targeting of Tumors with Liposomes
Adriamycin®, a doxorubicin hydrochloride injection, was approved in 1993 for treatment of various types of cancer. Even though it was an effective anticancer therapy, Adriamycin® caused severe cardiotoxicity, among other side effects. To overcome this issue, a PEGylated liposomal formulation of doxorubicin hydrochloride (Doxil®) was developed by Janssen Pharmaceuticals (FDA approved in 1995). Doxil® was designed to be able to passively target to tumor regions, thereby minimizing cardiotoxicity effect [26].

Emerging approaches for nanotechnology-based cancer treatment include the use of two anticancer drugs incorporated into a single product (e.g., ALN-VSP, CPX-351, and CPX-1) and the use of a two-stage system requiring the use of external stimuli to activate the product. The second category of drugs, using so-called "*SMART*" delivery systems, are designed to become active upon exposure to heat, ultrasound, radiofrequency, or some other energy-based trigger. The product may then change state (e.g., Thermodox®[4]) or enable the particle to directly affect the cell by, for example, locally increasing the temperature within a cell to modify permeability or to cause direct damage to the cell (e.g., Auroshell, NanoTherm™) [13, 27–29].[5]

[4] Thermodox® is a thermally sensitive liposomal doxorubicin formulation developed by Celsion Corporation (http://celsion.com/thermodox/). When targeted to the tumor site and exposed to temperature of 40 °C–45 °C (via radiofrequency thermal ablation, high-intensity focused ultrasound, etc.), the heat-sensitive liposomes release the encapsulated doxorubicin into and around the targeted tumor.

[5] Many of these products are considered "combination products" and would be handled by the FDA Office of Combination Products, http://www.fda.gov/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/OfficeofScienceandHealthCoordination/ucm2018184.htm.

Table 12.1 Examples of cancer therapeutics using nanotechnology in the US market[a]

Drug product	Description	Active ingredient	Nanotechnology platform[b]	Type of cancer	Route	Approval year
Oncaspar®	Pegaspargase injection: PEGylated L-asparaginase	L-asparaginase	Polymer-drug conjugate	Acute lymphoblastic leukemia	IV, IM	1994
Doxil®	PEGylated doxorubicin HCl liposomes for injection	Doxorubicin HCl	Liposome	Ovarian cancer, AIDS-related Kaposi's sarcoma, multiple myeloma	IV	1995[c]
DaunoXome®	Daunorubicin citrate liposomes for injection	Daunorubicin citrate	Liposome	Advanced HIV-related Kaposi's sarcoma (relapse)	IV	1996
Taxotere®	Docetaxel for injection	Docetaxel	Micelle	Breast, prostrate, gastric adenocarcinoma, head and neck cancer, non-small cell lung cancer	IV	1996[d]
Eligard®	Polymeric matrix formulation of leuprolide acetate, sustained release	Leuprolide acetate	Nanoparticle	Palliative treatment of advanced prostate cancer	SC	2004
Abraxane®	Paclitaxel protein-bound particles for injectable suspension, albumin-bound	Paclitaxel	Nanoparticle	Pancreatic, lung and breast cancer	IV	2005
Marqibo®	Vincristine sulfate liposomes for injection	Vincristine sulfate	Liposome	Philadelphia (Ph) chromosome negative (−) acute lymphoblastic leukemia	IV	2012
Onivyde®	Irinotecan HCl liposomes for injection	Irinotecan HCl	Liposome	Advanced pancreatic cancer	IV	2015

[a]The list excludes antibody drug conjugates and products with particle size greater than 1000 nm
IV intravenous, *IM* intramuscular, *SC* subcutaneous, *PEGylated* contains polyethylene glycol, *HCl* hydrochloride
[b]The nanomaterial structure terminologies do not represent CDER labeling or naming conventions and are used only to describe/interpret the type of structure of the nanomaterial in identified drug products for the purpose of this chapter
[c]First ANDA approved in 2013
[d]First ANDA approved in 2014

12.2 Regulatory Guidance for Drug Products Containing Nanomaterials

The drug application review process at the FDA is the same irrespective of whether the product involves the use of nanotechnology, and the Agency has not adopted a regulatory definition of nanotechnology [30]. However, to help industry identify the use of nanotechnology in their products, the Agency has issued a final guidance document on whether FDA-regulated products involve the use of nanotechnology [31]. Guidance documents are a mechanism by which the Agency communicates to industry and to the public, and they represent FDA's current thinking on a topic. They do not create or confer any rights for or on any person and do not operate to bind FDA or the public [32]. Per the nanotechnology guidance [31], FDA and sponsors may evaluate submitted applications for a drug product to determine:

1. Whether a material or end product is engineered to have at least one external dimension, or an internal or surface structure, in the nanoscale range (approximately 1 nm to 100 nm).
2. Whether a material or end product is engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects that are attributable to its dimension(s), even if these dimensions fall outside the nanoscale range, up to one micrometer (1000 nm).

In addition to the overarching nanotechnology guidance, there are several drug product-specific guidances (e.g., product-specific bioequivalence[6] guidances) and guidances for classes of products (e.g., liposome guidance) (Table 12.2). For

Table 12.2 Guidances on drug products involving nanotechnology (partial list) [63]

Title	Status	Date
General guidances		
Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology	Final	June 2014
Liposomal Drug Products: Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation	Final	April 2018
Product-specific guidances for cancer therapeutics		
Bioequivalence Guidance on Megestrol acetate	Draft	February 2010
Bioequivalence Guidance on Paclitaxel	Draft	September 2012
Bioequivalence Guidance on Verteporfin	Draft	April 2014
Bioequivalence Guidance on Daunorubicin Citrate	Draft	July 2014
Bioequivalence Guidance on Lanreotide Acetate	Draft	July 2014
Bioequivalence Guidance on Doxorubicin Hydrochloride	Draft	September 2018

[6] "Bioequivalence is defined as the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study" (Code of Federal Regulations 21CFR 320.1).

Table 12.3 Examples of cancer therapeutics using nanotechnology in clinical trials

Drug product	Description	Active ingredient	Type of cancer	Phase	Status	Ref.
Liposomes						
Lipocurc	Liposomal curcumin	Curcumin	Advanced cancers	I/II	Ongoing	[83]
MRX34	Liposomal injection containing microRNA miR-34, a naturally occurring tumor suppressor	miR-34 microRNA	Multiple cancers	I	Terminated	[74]
siRNA-EphA2-DOPC	EphA2 gene targeting using neutral liposomal small interfering RNA delivery	Anti-EphA2 siRNA	Advanced cancers	I	Recruiting	[76]
SGT-53	Transferrin-targeted liposome loaded with the p53 gene	p53 gene	Children with refractory or recurrent solid tumors	I	Recruiting	[90]
MCC-465	PEGylated liposomal doxorubicin tagged with monoclonal antibody GAH	Doxorubicin	Colorectal cancer	I	Complete	[21]
MBP-426	Liposomal oxaliplatin	Oxaliplatin	Gastric, gastroesophageal, or esophageal adenocarcinoma	I/II	NA	[81]
Atragen	All-trans retinoic acid liposomes	All-trans retinoic acid	Advanced renal cell carcinoma	II	NA	[69]
Liposome annamycin	Liposomal annamycin	Annamycin	Acute lymphocytic leukemia	I/II	NA	[85]
Atu-027	Liposomal formulation of siRNA against protein kinase N3 the vascular endothelium	Anti-protein kinase N3 siRNA	Advanced or metastatic pancreatic cancer	I/II	Complete	[82]
LEP-ETU	Liposome-entrapped paclitaxel easy-to-use formulation	Paclitaxel	Metastatic breast cancer	II	Complete	[88]
Endo TAG-1	Paclitaxel-loaded cationic liposomes	Paclitaxel	Breast cancer	II	Complete	[67]
CPX-1	Irinotecan HCL; floxuridine liposomes	Irinotecan HCL and floxuridine	Advanced colorectal cancer	II	Complete	[68]

CPX-351 (Vyxeos™)	Cytarabine; daunorubicin liposomes	Cytarabine and daunorubicin	Acute myeloid leukemia	III	Complete	[59]
ThermoDox® (LTSDEL)	Thermositive and PEGylated liposomes encapsulating doxorubicin hydrochloride	Doxorubicin	Hepatocellular carcinoma	III	Recruiting	[86]
L-BLP25	Liposomal formulation of L-BLP25, a peptide vaccine	L-BLP25, a peptide vaccine	Non-small cell lung cancer	III	Complete	[70]
Lipoplatin	Cisplatin-loaded long circulating liposomes	Cisplatin	Nonmetastatic pancreatic cancer	III	Complete	[91]
Myocet®	Non-PEGylated liposomal doxorubicin	Doxorubicin	Metastatic breast cancer	III	Recruiting	[78]
Micelles						
NK012	Polymeric micelle anticancer drug encapsulating 7-ethyl-10-hydroxycamptothecin (SN-38), an active metabolite of Irinotecan	7-ethyl-10-hydroxycamptothecin (SN-38), an active metabolite of Irinotecan	Small cell lung cancer	II	Complete	[79]
Cynviloq™ (Genexol-PM)	Micellar diblock copolymeric paclitaxel formulation. Paclitaxel is in the micellar core	Paclitaxel	Non-small cell lung cancer	II	Complete	[75]
Nanoparticles						
Tf-LPN-G3139	Polyethylenimine-containing and transferrin-conjugated lipid nanoparticle system for antisense oligonucleotide (G3139, anti-Bcl2) delivery	G3139 antisense oligonucleotide	Acute myeloid leukemia	Preclinical	–	[20]
ALN-VSP	Lipid nanoparticle formulation of 2 siRNAs - anti-KSP and anti-VEGF	Anti-KSP siRNA and anti-VEGF siRNA	Solid tumors with liver involvement	I	Complete	[84]
Nanosomal docetaxel lipid suspension	Nanosomal docetaxel lipid suspension	Docetaxel	Advanced or metastatic breast cancer	II	Complete	[89]

(continued)

Table 12.3 (continued)

Drug product	Description	Active ingredient	Type of cancer	Phase	Status	Ref.
NanoTherm™	Aminosilane-coated superparamagnetic iron oxide delivered locally	Superparamagnetic iron oxide	Recurrent glioblastoma	I	Active	[29]
ABI-009	Nanoparticle albumin-bound rapamycin	Rapamycin	Advanced malignant perivascular epithelioid cell tumors	II	Recruiting	[73]
BIND-014	Docetaxel nanoparticles for injectable suspension	Docetaxel	KRAS mutation positive or squamous cell non-small cell lung cancer	II	Complete	[66]
Nanoparticle-drug conjugates						
CRLX-101	Covalently conjugating camptothecin to a linear, cyclodextrin-polyethylene glycol (CD-PEG) copolymer that self-assembles into nanoparticles	Camptothecin	Advanced non-small cell lung cancer	II	Complete	[87]
Cyt-6091 (Aurimune)	Tumor necrosis factor (TNF) bound to PEGylated colloidal gold nanoparticles	Tumor necrosis factor (TNF)	Advances solid tumor	I	Complete	[77]
Polymer-drug conjugates						
NKTR-105	PEGylated-docetaxel	Docetaxel	Refractory solid tumors	I	Active	[71]
Auroshell	PEGylated gold-silica nanoshells	Gold-silica	Primary and/or metastatic lung tumors	I	Recruiting	[72]
Etirinotecan pegol (NKTR-102)	PEGylated-irinotecan	Irinotecan	Locally recurrent or metastatic breast cancer	III	Ongoing	[80]
Opaxio® (paclitaxel poliglumex)	Paclitaxel conjugated to a biodegradable, water-soluble polyglutamate polymer	Paclitaxel	Ovarian cancer	III	Ongoing	[65]

Route of administration is intravenous unless otherwise indicated. This is a partial list as this does not include ALL products
DOPC 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine, *KSP* kinesin spindle protein, *VEGF* vascular endothelial growth factor, *siRNA* short-interfering RNA, *HCl* hydrochloride, *NA* Not available

nanoparticle technology, an example of product-specific guidance is the bioequivalence guidance on lanreotide acetate [33] (a polymer-based depot injection [34]). As the FDA receives more applications related to active targeting and other (advanced) nanotechnologies as discussed above, additional relevant guidance(s) may be drafted to help streamline the application submission and review process. It should be noted that a comprehensive review of the submission for drug products containing nanomaterials was conducted by FDA. Within the review, it was noted that approval rates for drug products containing nanomaterials were comparable to both small molecule and biologics [7].

12.2.1 The Application Review Process

Figure 12.1 depicts the drug development process and steps where drug applications are submitted to the FDA by a sponsor (usually the manufacturer or potential marketer). Once a drug application is received by FDA/CDER (see Box 12.2), it is assigned to the appropriate division for review based on the product's indication (for new drugs) or its dosage form (for generic drugs[7]).

Box 12.2 The Role of CDER within FDA
Within the FDA, the Office of Medical Products and Tobacco houses the Center for Drug Evaluation and Research (CDER). The mission of CDER is "to protect and promote public health by helping to ensure that human drugs are safe and effective for their intended use, that they meet established quality standards, and that they are available to patients." This is in part achieved by overseeing research, development, manufacturing, premarketing, and postmarketing activities pertaining to drugs (prescription, generic, and over the counter) [35]. As per the US FDA, a "drug" may be defined as "a substance recognized by an official pharmacopoeia or formulary; a substance intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease; a substance (other than food) intended to affect the structure or any function of the body; a substance intended for use as a component of a medicine but not a device or a component, part or accessory of a device; biological products are included within this definition and are generally covered by the same laws and regulations, but differences exist regarding their manufacturing processes (chemical process versus biological process)" [36].

[7] As per the US FDA, "a generic drug is a medication created to be the same as an existing approved brand-name drug in dosage form, safety, strength, route of administration, quality, and performance characteristics."

For new drugs (containing new drug substances), after successful preclinical testing in animals, studies are conducted by (or via) the sponsor to determine whether the product is safe for initial use in human subjects and if the testing in human would demonstrate benefits that outweigh the potential risks and that the product will not expose humans to unreasonable risks when used in early phases of clinical trials. A sponsor[8] submits an Investigational New Drug (IND) application to the US FDA prior to initiating drug testing in humans. Besides this type, there are other types of INDs, as discussed below [37]:

- An *investigator IND* is submitted by a physician who both initiates and conducts an investigation and, under whose immediate direction, the investigational drug is administered or dispensed. A physician might submit a research IND to propose studying an unapproved drug or an approved product for a new indication or in a new patient population.
- *Emergency use IND* allows the FDA to authorize use of an experimental drug in an emergency situation that does not allow time for submission of an IND in accordance with the Code of Federal Regulations 21CFR 312 (Sec. 312.23 or Sec. 312.20). It is also used for patients who do not meet the criteria of an existing study protocol or if an approved study protocol does not exist.
- *Treatment IND* is submitted for experimental drugs showing promise in clinical testing for serious or immediately life-threatening conditions, while the final clinical work is conducted and the FDA review takes place.

An IND application typically includes information on investigator, manufacturing, data from animal pharmacology and toxicology studies, and clinical study protocols [37, 38]. Once an IND is submitted, the sponsor has to wait for 30 calendar days before clinical trials can be initiated. Meanwhile, FDA reviews the IND for safety in order to assure that human subjects are not exposed to unreasonable risk. Once an IND is approved, Phase I clinical trials can be initiated on a small population of healthy subjects with the goal to determine dose tolerability and obvious side effects. For example, a silencing RNA (anti-EphA2)-based liposomal formulation (siRNA-EphA2-DOPC) has recently received FDA's approval for initiation of a Phase I clinical trial [39].

If the results of the Phase I trial demonstrate safety in healthy subjects, the drug can be tested in a larger population through Phase II and III clinical trials (see Fig. 12.1) with an objective to evaluate drug safety, efficacy, and toxicity in diseased patients [40].

If the results from clinical studies (end of Phase II or early Phase III) indicate that the benefits from drug efficacy outweigh the risk from drug toxicity(ies), a sponsor may submit a New Drug Application (NDA) to the FDA [40]. As per Section 505 of the *Food, Drugs and Cosmetic Act*, there are three types of new drug applications:

[8] "*Sponsor* is a person who takes responsibility for and initiates a clinical investigation. A sponsor could be an individual, government agency, pharmaceutical company, academic institute, private or other organization." Code of Federal Regulations 21CFR 312.3. For example, Janssen Products, LP is the sponsor for "Doxil."

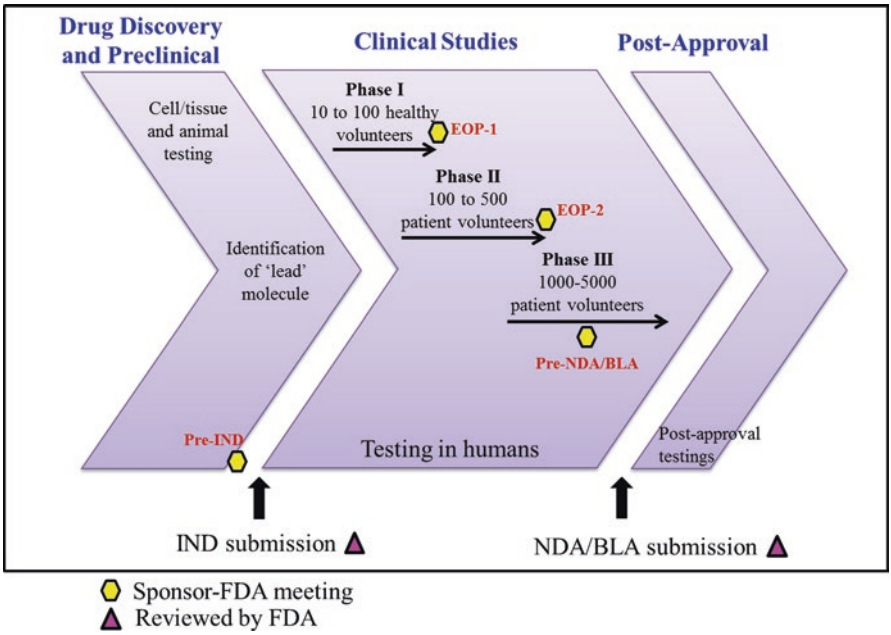

Fig. 12.1 Schematic of the regulation of drug products. IND Investigational New Drug, BLA Biologics License Application, NDA New Drug Application, EOP-1 end of Phase I, EOP-2 end of Phase II. (Modified from references [42, 64])

***505(b)(1)*:** This application is used for approval of a new drug (for clinical use) whose active ingredient has not been approved previously. The application contains full reports of investigations of safety and effectiveness [41].

505(b)(2): This application is used for approval of a new drug that relies, at least in part, on data not developed by the applicant. The application contains full reports of investigations of safety and effectiveness but where at least some of the information required for approval comes from studies not conducted by or for the applicant and for which the applicant has not obtained a right of reference [42]. Typically 505(b)(2) applications include change in dosage form, strength, or route of administration compared to an approved product or substitution of an active ingredient in an approved combination product [43]. 505(b)(2) applications are often used for products containing nanomaterials, especially products where the nanomaterial is used as a carrier for an already approved drug substance. For example, Taxol®, approved in 1998, uses paclitaxel as the active ingredient [44]. Another product, Abraxane®, contains paclitaxel bound to albumin (new formulation of paclitaxel). Abraxane® was approved by the US FDA in 2005 under section 505(b)(2) regulatory pathway [45].

505(b)(1) and 505(b)(2) applications require IND and NDA applications to be submitted to the FDA for review. Whereas regulatory requirements for IND applications have been discussed earlier in this chapter, the NDA is expected to include

chemistry, manufacturing, and control (CMC) information on drug substance and drug product, bioavailability data, analytical data, labeling, and packaging information, for each of the dosage forms, the sponsor intends to commercialize and any additional toxicological study reports that were not included in the IND application [46]. Detailed requirements for 505(b)(1) and 505(b)(2) applications are described at 21 code of Federal Regulations (CFR) 314.50. Additional requirements for certain 505(b)(2) applications are described at 21 CFR 314.54 as well as in the FDA draft guidance on applications covered under section 505(b)(2) [43].

Biologics License Application (BLA): This is a new drug application for biological products. Biological products are approved for marketing under the provisions of the Public Health Service (PHS) Act. This application is a submission, similar to an NDA, containing information on the manufacturing processes, chemistry, pharmacology, clinical pharmacology, and the medical effects of a biologic product [47]. For review, BLA applications are assigned to CDER or the Center of Biologics Evaluation and Research (CBER) depending on the nature of the biological product. Jurisdiction of CDER and CBER pertaining to BLAs is outlined in the cited reference [48].

505(j) (generics): A new drug, during its development and a few years post-approval, is often protected under a patent in order to give the sponsor the time to exclusively sell the drug to recover development costs. Once the patent expires, other companies can apply to the FDA to sell generic versions of the drug product by filing an Abbreviated New Drug Application (ANDA) with the FDA also known as 505(j) application [49, 50]. This application contains information to show that the proposed product is identical in active ingredient, dosage form, strength, route of administration, labeling, quality, performance characteristics, and intended use, among other things, to a previously approved product. This application is called abbreviated since an ANDA is generally not required to include nonclinical (animal) and clinical (human) data to establish safety and effectiveness. Instead, generic applicants scientifically demonstrate that their product is bioequivalent (i.e., performs in the same manner as the innovator drug) [50].

In all cases, FDA encourages timely, transparent, and effective communication with sponsors before and during the drug development process. This may result in more efficient and robust development programs considering that through FDA-sponsor communications, the key issues can be addressed in the early stages of drug development. This may also help FDA achieve its goal of early availability of safe, effective, and high-quality medicines to the American public. Sponsors can request meetings with FDA during drug development especially critical milestone meetings: pre-IND, end of Phase I, end of Phase II, and pre-NDA/BLA meetings (Fig. 12.1). More details on FDA-sponsor communication can be found in the FDA draft guidance on best practices for communication between IND sponsors and FDA during drug development [51].

Once a drug (new or generic) approaches the approval stage, the FDA requires the submission of additional information on the drug to ensure its continuous safety and efficacy for the period the drug is on the market. These are called Phase IV requirements (submitted pre-approval) and Phase IV commitments (usually submitted post-approval). As an example, post-marketing studies/clinical trials to demonstrate safety and efficacy of a drug approved under the accelerated approval requirement are a Phase IV requirement.

12.2.2 Review of Quality Attributes

> "A CQA is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality. CQAs are generally associated with the drug substance, excipients, intermediates (in-process materials), and drug product".*Guidance for Industry: Q8(R2) Pharmaceutical Development*, 2009. http://www.fda.gov/downloads/drugs/guidances/ucm073507.pdf

Clinical trials allow for an evaluation of the safety and efficacy of a new drug substance and product in humans. However, before a medication can be used in patients, quality of the product must also be ensured (e.g., the medicine can be manufactured reproducibly, and the doses produced are equivalent to each other irrespective of batch number or lot number). The evaluation of the process of developing a drug product, manufacturing process, stability protocols, etc. is also known as the quality review. In fact, the quality review is one of the major regulatory considerations for drug products containing nanomaterials, as per the risk assessment performed by the Agency (CDER) in the year 2013 [52].

Drug products containing nanomaterials can vary in their complexity. The more complex the product (containing nanomaterials or not), the more challenging it can be to demonstrate control of the manufacturing and the production of high-quality drugs with reliable batch-to-batch reproducibility. To facilitate the development of these complex products, the FDA often develops guidance for reviewers and for industry that specifically addresses manufacturing and characterization challenges. For example, from the experience reviewing applications for liposomal drug products, the Agency drafted a guidance on liposomal drug products that provides information to liposomal product manufacturers regarding development, manufacturing, pharmacokinetic aspects, and labeling of liposomal drug products [53]. From a quality perspective, the guidance points toward the importance of identification and characterization of critical physical, chemical, biological, and microbiological properties that may influence finished product quality or performance [54]. These properties are often called the critical quality attributes (CQAs) of the product. Some examples of CQAs for liposomal products are lamellarity, internal volume,

lipid-phase transition temperature, free and encapsulated drug proportions, lipid degradation products, zeta potential, particle size, and drug release kinetics [53, 55].

From the Agency's experience reviewing applications involving the use of nanotechnology across all platforms, certain quality issues were observed to be recurrent and are summarized as below.

Inadequate identification of CQAs: For product robustness and reproducibility, CQAs during formulation and manufacturing processes are identified and suitably controlled. For products using nanomaterials, particle size is often found to be a CQA as particle size distribution has been demonstrated to impact biodistribution, rate of drug release/dissolution, etc. Other examples of common CQAs for drug products containing nanomaterials include zeta potential and drug loading efficiency (for nanomaterials functioning as drug carriers). By definition, CQAs are product dependent. However, some CQAs can span across a product class. For example, lipid-phase transition temperature, which may influence drug loading, release, and overall stability, is a CQA specific to liposomes and not applicable to other (non-lipid) nanotechnology platforms such as dendrimers and iron colloids. By evaluating the applications submitted to FDA for drug products containing nanomaterials, CQAs for products often include (but are not limited to) the following:

- Size.
- Size distribution.
- Nanomaterial composition (e.g., lipids for liposomes).
- Crystal structure.
- Morphology/three-dimensional structure.
- API to nanomaterial ratio.
- State of API (e.g., encapsulated, bound, etc.)
- Surface functionalization and state of the surface.
- Ligands (if any).
- Zeta potential or surface charge.
- In vitro release rates (in vitro release studies under multiple conditions, including in biorelevant medium, which can be indicative of the physicochemical stability of the formulation [56]).

Inappropriate method and/or method validation: As with any drug product, the analytical methods used for characterization of CQAs is demonstrated to be fit for purpose (e.g., measures what it is supposed to in an accurate and reproducible fashion). Selection and validation of methods to characterize drug products containing nanomaterials may be challenging due to the complexity of the product and because the methods used for characterization of CQAs may not be as familiar for complex products as those used for small molecule drug products. Often these products require the use or development of novel techniques and methods to characterize these products. "Traditional" methods and novel methods should both be used within their capabilities. For example, both dynamic light scattering and static light

scattering can be used to determine particle size. However, the useful size range, the way the data are interpreted and analyzed, and other factors differ between the two techniques. In general, the analytical method is validated for sensitivity, accuracy, precision, robustness, and the ability to discriminate between acceptable and unacceptable batches. The use of an additional, orthogonal analytical technique to characterize the materials can often be beneficial to complete a data set prior to submission.

Lack of appropriate control strategies: Suitable controls are employed during product development to ensure that the CQAs are within an appropriate limit, range, or distribution in order to ensure the desired product quality. This may be achieved by including a CQA in either drug product release or in in-process specifications.

12.2.3 Special Regulatory Provision for Cancer Products

For therapies that address an unmet medical need in the treatment of a serious condition such as cancer, the FDA allows sponsors to request a faster review process through four FDA programs: *fast track designation, breakthrough therapy designation, accelerated approval, and priority review designation* [57]. Applications accepted into these *expedited programs* undergo an accelerated review (i.e., the review is completed faster, but with the same degree of scrutiny), thereby facilitating early availability of new therapies to the patients as soon as it can be determined that their benefits outweigh the risks. Expedited availability of new cancer therapies is crucial, especially in cases where there are no satisfactory alternative (existing) therapies. For a new cancer therapy, sponsors may apply for *fast track* and *breakthrough therapy designations* early in the development, for *priority review designation* during BLA or NDA submission, and for *accelerated approval designation* during BLA or NDA review. For example, *fast track designation* was granted to CRLX-101, a nanoparticle-drug conjugate currently under development (Phase I/II) by Cerulean Pharma for the treatment of platinum-resistant ovarian carcinoma and fallopian tube or primary peritoneal cancer [58]. *Breakthrough therapy designation* was granted by the FDA to CPX-351 or Vyxeos™ (by Celator Pharmaceuticals) for the treatment of acute myeloid leukemia based on the encouraging results from a Phase III clinical trial [59]. *Accelerated approval* that is granted based on a surrogate end point (since actual end point takes a long time to measure) was granted to Doxil® (liposomal doxorubicin) in 1995 for the treatment of Kaposi's sarcoma [60]. A *priority review designation* can abbreviate the review time from 10 months to 6 months and was assigned to Onivyde® (liposomal irinotecan) developed by Merrimack Pharmaceuticals for the treatment of advanced pancreatic cancer [61]. These modified time lines and approaches to review are designed to increase the number of novel therapeutics available to patients, which is in accord with FDA's mission to promote and protect the public health.

12.3 Future Perspective

At this time, the Agency anticipates continued interest in the development of products utilizing a variety of nanotechnology platforms, some familiar and some novel. In particular, it is likely that there will be an expansion of nanotechnology in cancer therapeutics designed to improve passive and active (triggered) targeting capabilities. Several products in clinical trials (Table 12.3) involve the use of multifunctional nanocarriers (PEGylated nano-sized particles for tumor targeting with or without an active targeting moiety) in the hope to achieve better efficacy and safety compared to the existing therapies. Industry is also investigating the use of nanotechnology-based delivery platforms for the delivery of anticancer drugs of biological origin (e.g., nucleic acids, peptides), potentially increasing the overall complexity of the products. These technologies could also be applied to therapeutics designed to target multiple tissues or active sites.

When faced with these new approaches, FDA has multiple options for response. For any new product drawing upon a novel platform or technology, the existing guidances apply as appropriate. In cases where new functionality or CQAs become relevant, these would be handled on a case-by-case basis, potentially resulting in the development of a product-specific guidance that could be extended to encompass a class of products at a later date. FDA can also draw upon related technologies. For example, the Agency has experience in reviewing monoclonal antibodies that can deliver a toxin or radioactive isotope in a targeted fashion. Targeted biologics have several parallels to drug products containing nanomaterials, and parallels may be drawn between the product classes. Such similarities have been reviewed previously [92].

In some cases, the new product or technology may require the sponsor to develop an innovative manufacturing process. To facilitate development and review of new manufacturing systems or processes, the FDA has created the Emerging Technology Program, which enables sponsors to discuss the process with Agency experts prior to a regulatory submission. These discussions are designed to identify potential concerns for the sponsor and to increase awareness of the new approach within FDA before the review process occurs to the benefit of both organizations.

As these technologies mature, FDA also anticipates that more generic versions of the products will appear as well. This is important to consider because complex formulations and manufacturing processes can impact the development of generic versions of drug products, and a lack of generic versions of medications can result in higher patient costs as well as a higher risk for drug shortages. The impact of nanotechnology on the generic drug process has been extensively reviewed [62].

12.4 Conclusion

It is anticipated that drug products will become more complicated in order to meet unmet medical needs. Such complexity spans all indications and routes of administration and includes both drug products containing nanomaterials and those taking

advantage of other technologies. With increased incorporation of nanomaterials in cancer therapeutics, both industry and regulatory authorities alike should strive for product understanding that involves adequate characterization of the nanomaterial, understanding of its intended use and application, and how it relates to the product quality, patient safety, and efficacy. By utilizing this framework, patients may gain access to new cancer medications.

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Chapter 13
Perspectives for Characterizing Drug Component of Theranostic Products Containing Nanomaterials

Christie M. Sayes and Anthony J. Hickey

13.1 Introduction

Nanomaterials used as (or in) drug products provide several unique therapeutic opportunities and regulatory challenges [1]. Some applications include the ability to direct therapeutic agents to particular cell types, perturb specific DNA or RNA pathways for targeted disruption, and increase inflammatory responses of tissues in an effort to elicit a metabolic response [2–4]. The regulatory challenges engage stakeholders from industry, academia, and government in conversations about efficacy, expense, and toxicology. The combinatorial approach of theranostics (also referred to as theragnostics) increases the complexity of novel nanomaterial drug products in terms of both therapeutic opportunities and regulatory challenges.

Theranostics can be defined as a form of diagnostic therapy that measures (qualitatively and quantitatively) the physiological and pharmacological reaction to that therapy of the patient. The term can also be defined as the use of molecular diagnostic techniques and treatment strategies for a particular patient in real time. This later definition can also describe the term "theragnostics." In fact, implementing treatment with a single drug designed to target a specific receptor is disadvantageous (REF?). Combination drug therapies designed for optimization of an individual patient's health condition are a major thrust in today's nanotechnology research agenda [5, 6].

Advances have been made in recent years to accelerate the safety and efficacy of nanomaterial drug products and their use as diagnostic agents and therapeutic

C. M. Sayes (✉)
Department of Environmental Science, Baylor University, Waco, TX, USA
e-mail: Christie_sayes@baylor.edu

A. J. Hickey
RTI International, Research Triangle Park, NC, USA
e-mail: ahickey@rti.org

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agents. The lofty goal of developing safe and effective theranostic agents can either be enabled by public-private partnerships (such as collaborations among industry, government, and academia) or inhibited by disparate philosophies among industry, government, and academia. The main technological thrust that drives the development of such complex drug products is the quest to produce sustainable and applicable "personalized medicine." Personalized medicine, in this context, is defined as the study of a particular patient and his/her unique biochemical and genetic profiles that define biomarkers of susceptibility to the onset of disease and/or his/her potential response to a treatment.

Ultimately, success of nanotheranostics will be defined by many crucial factors and will require a multidisciplinary strategy to reach drug safety and efficacy. Fig. 13.1 illustrates the six main critical attributes in the nanomaterial drug development pipeline for nanotheranostic agents:

1. Developing relevant treatment prototypes in nanotechnology-compatible therapeutic areas.
2. Understanding patient and healthcare provider compliance and willingness to utilize personalized medicine.
3. Analyzing economic drivers – Both financial and psychological.
4. Timeliness of R&D, preclinical, and clinical drug testing, formulation, and manufacturing.
5. Designing collaborative opportunities around intellectual property.
6. Defining regulatory filing strategies for an infinite number of drug treatments, patient conditions, and market prices.

The underlying effort associated with the six critical factors is the development of metrics designed to measure therapeutic quality, safety, and efficacy. Quality relates to the uniformity of the drug system (i.e., a homogenous particle system ensures reproducibility) and measurable control metrics in the formulation and manufacturing processes [1]. Safety refers to the assurance of contaminant-free drug products and side-effects testing at the clinical level [7]. Efficacy is related to the degree of effectiveness in terms of potency and dose [8–10].

Two entities that are most interested in nanotheranostic agent development are the US Food and Drug Administration (FDA) and the United States Pharmacopeia

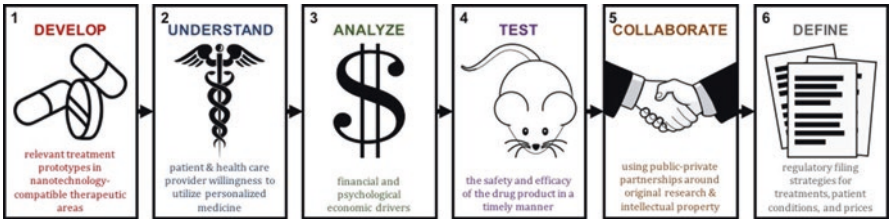

Fig. 13.1 The crucial factors that require a multidisciplinary strategy to reach drug safety and efficacy for nanotheranostics

(USP). Traditionally, the USP recommends standards for drug product quality for articles in commerce, whereas the FDA promulgates standards of manufacturing, characterization, and safety (REF). As of 2018, the USP and FDA are aligned for standards, testing requirements, and regulations related to nanomaterial drug products.

Traditionally, there are seven active agencies or organizations in the United States that are concerned with drug safety and efficacy, including drugs derived from nanomaterials (Table 13.2). Only one agency has regulatory authority (i.e., the US FDA). However, the USP produces monographs that are legally binding documents. When products use the term USP to describe the quality of their product, the substance has to meet USP standards. The mission of the USP is "to improve global health through public standards and related programs that help ensure the quality, safety, and benefit of medicines and foods" (www.usp.org), while the mission of the FDA is "to ensure the safety of human and veterinary drugs, biological products, and medical devices; ensure the safety of our food supply, cosmetics, and products that emit radiation; regulate the manufacturing, marketing, and distribution of tobacco products; and get the accurate, science-based information to maintain and improve health" (www.fda.gov).

The remaining agencies and organizations do not have regulatory authority, but do have mission statements that strongly support the development of safe and effective drugs designed for human health, therapy, and diagnostics (see websites; Table 13.1). There are many examples of coordination among and between these organizations [11–16]. This chapter will highlight some of the coordination efforts between the USP and the FDA. Perspectives of the USP joint subcommittee on nanotechnology are available online (http://www.usp.org/expert-committees/general-chapters-physical-analysis-expert-committee-work-plan).

Table 13.1 US agencies and organizations that are concerned with drug safety and efficacy

Agency or organization	Acronym	URL	Regulatory authority?
United states pharmacopeia	**USP**	www.usp.org	**No**[a]
Centers for Disease Control and Prevention	**CDC**	www.cdc.gov	**No**
Drug information association	**DIA**	www.diaglobal.org	**No**
National Institutes of Health	**NIH**	www.nih.gov	**No**
National Academy of medicine	**NAM**	www.nam.edu	**No**
US Food and Drug Administration	**USFDA**	www.fda.gov	**Yes**
Nanotechnology characterization laboratory[b]	**NCL**	https://ncl.cancer.gov	**No**

[a]USP monographs are legally binding documents enforced by the FDA. When products use the term USP to describe the quality of their product, by FDA-enforced law, the substance has to meet USP standards

[b]The European Union has established a counterpart to the US NCL, termed EU NCL (http://www.euncl.eu)

Table 13.2 Economic drivers that influence theranostic development

Economic driver	Paper
R&D costs	DiMasi, Joseph A., Henry G. Grabowski, and Ronald W. Hansen. "Innovation in the pharmaceutical industry: New estimates of R&D costs." *Journal of health economics* 47 (2016): 20–33
Clinical trial complexity	Hay, Michael, et al. "clinical development success rates for investigational drugs." *Nature biotechnology* 32.1 (2014): 40–51
Comparative effectiveness	Gargon, Elizabeth, et al. "choosing important health outcomes for comparative effectiveness research: a systematic review." *PloS one* 9.6 (2014): e99111
Expenditure for developed countries	Kantarjian, Hagop M., et al. "Cancer drugs in the United States: Justum Pretium—The just price." *Journal of clinical oncology* 31.28 (2013): 3600–3604
Expenditure for underdeveloped countries	Gelband, Hellen, et al. "costs, affordability, and feasibility of an essential package of cancer control interventions in low-income and middle-income countries: Key messages from disease control priorities." *The Lancet* 387.10033 (2016): 2133–2144
Patient safety	Mak, Isabella WY, Nathan Evaniew, and Michelle Ghert. "Lost in translation: Animal models and clinical trials in cancer treatment." *American journal of translational research* 6.2 (2014): 114
Patient privacy	Basch, Ethan. "Toward patient-centered drug development in oncology." *New England Journal of Medicine* 369.5 (2013): 397–400

13.2 Therapeutic Opportunities

When designing novel theranostic agents, two sets of properties should be considered. First, pharmaceutical properties (such as solubility, concentration, and formulation) are important characteristics to consider by the pharmaceutical engineers. Second, biopharmaceutical properties (such as dissolution rates, absorption, and toxicology) are important considerations for the pharmacists and physicians. Both categories of properties are critical during the development, manufacturing, and distributing phases of the drug product life cycle. Perspectives from analytical and manufacturing approaches create a collaborative advantage for any complex nanosystems, in particular nano-enabled theranostic agents [1].

The adoption of a uniform approach to the characterization of nano-enabled theranostic agents is partially addressed by curated databases that define, collate, and organize pharmaceutical and biopharmaceutical data [17–19]. The information organized in these databases promotes safety-by-design approaches that are technical to enable repeatability and transparent to enable comprehension. Analytical considerations of nano-drug products are connected to quality control, quality assurance, risk assessment, and risk management efforts. Manufacturing considerations are linked to the continuous network along the entire nano-drug product life cycle (from development to end of life). Scientific and regulatory scrutiny is also fundamental, as well as legal mechanisms, to ensure safe and effective nano-enabled theranostic agents as long as all stakeholders along the drug product life cycle are engaged.

Current opportunities for theranostic development include the incorporation of nanomaterial entities as nano-drug, nano-additive, or nano-carrier into a dosage form. These three important formulation options enabled by nanotechnology in further detail are [1]:

- Nano-drug (abbrev. ND), an example of a therapeutic agent that is the drug alone on the nanoscale; can be a hard solid (e.g., tablets, capsules), soft solid (ointments, suppositories), liquid (solutions, suspensions), or gas (aerosol); aka nano-pharmaceutical.
- Nano-additive (abbrev. NA), an inactive ingredient or excipient; excipients on the nanoscale that are added to dosage forms; used as a transport module for a drug.
- Nano-carrier (abbrev. NC), a non-drug component (additive) prepared as nanoparticles in which the drug is either dispersed (in a single particle) or to which drug is added (particles of drug and carrier); used as a transport module for a drug.

These formulation options generate new directions along many lines of research (stem cell transplant, hormone replacement, immunomodulation, precision and personalized medicine, and gene and radiation therapies) [20–27]. They can also be applied to cancer treatments in which drugs can either be the entire core of the nanoparticle (i.e., nano-drug), be embedded in a matrix (i.e., nano-carrier), or added to the surface (nano-additive) (Fig. 13.2). These simple structures are often functionalized or coated with a biocompatible shell that can be decorated with

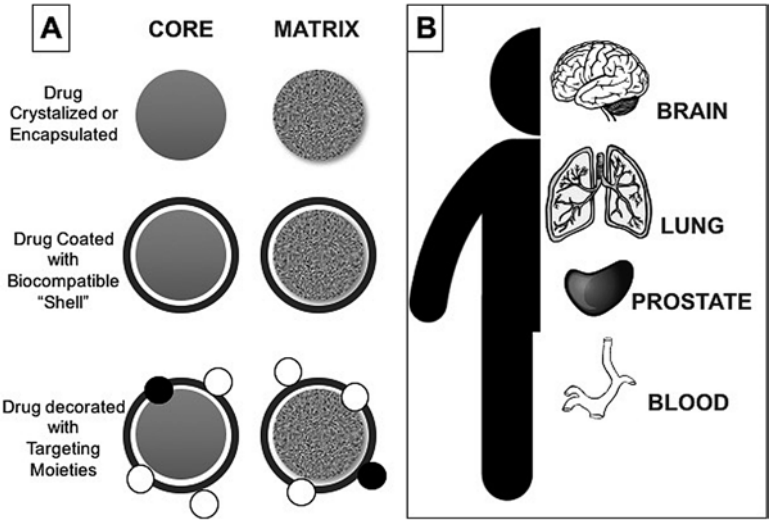

Fig. 13.2 Nanotechnology-enabled drug products used in cancer treatment. (**a**) Illustrations of the typical nanostructures used in nanomedicine today. (**b**) A diagram of a human and the major targets for cancer therapy

additional targeting groups or moieties. From cellular dysfunction to organism deterioration to susceptible populations, the concept of providing point-of-care testing that includes both diagnostic and therapeutic treatment options, simultaneously, is highly desired, needed, and possible [28–32].

13.3 Convergence of USP and FDA Considerations

The increasing interest in nanomaterials as components of drug formulations has resulted in greater regulatory and pharmacopeial scrutiny. Nanomaterials have been components of drug products for a century or more. The use of colloidal materials in drug products had various purposes such as a means to prepare thermodynamically stable drug suspensions; to increase the surface area and thereby improve dissolution and, consequently, drug bioavailability; and to improve flow properties of solid powders, using fumed silica, for example, as an aid to filling in solid dosage form manufacture. The appearance of nanotechnology as a separate area of scientific and engineering endeavor was a corollary to the biotechnology revolution of the last decade of the previous century and the first decade of the new millennium. Additional functionality has been ascribed to nanomaterial-based formulations, which requires consideration to assure quality, efficacy, and safety.

The FDA has a broad brief to assess and regulate the quality, safety, and to some extent efficacy of drug products. Primarily the focus is on quality and safety to ensure the health of US citizens. In this regard, the guidance promulgated by the FDA is intended to assure the product quality and performance on release from commercial manufacturing sites. In contrast, the USP contains a set of standards and protocols, first developed in the nineteenth century to prevent adulteration of drugs sold or dispensed to the public, ordinarily considered items in commerce. As a consequence, there is a subtle but meaningful difference between the objectives of each organization. As a general guide, the USP standards are considered minimally acceptable to the FDA, and frequently the latter has more extensive requirements of quality and performance related to drug product manufacturing.

The FDA has taken steps to define their position on the use of nanomaterials in drug products. The USP has aligned closely with the FDA to establish national consistency.

A wide range of nanomaterial formulations exist that are being considered for use in drug delivery. The most common examples of nanomaterials are drug alone, natural and synthetic polymeric particles, liposomes, micelles, and emulsions [33–41]. Inorganic nanoparticles have been considered most frequently as imaging agents or for vaccine delivery [42–51].

Nanomaterials have unique physicochemical properties that from a regulatory standpoint may need to be specified to ensure the quality and safety of the product and, of greatest interest to the manufacturer, efficacy. Besides composition and structure, particle size and distribution are of primary importance as several key properties are related to these parameters including specific surface area, intrinsic

solubility, dissolution, and drug release. Greater attention may be required for other key features such as surface functionality, morphology, encapsulation efficiency, and stability. It is notable that the key measure of nanoparticle size measurement, which is usually performed by dynamic light scattering with complementary transmission or scanning electron microscopy, is acknowledged by both the FDA and USP but has yet to appear as a general chapter in the latter.

Among nanomaterial products approved by the FDA, liposomes and polymeric nanoparticles dominate for drug delivery, and inorganic particles have been approved for imaging contrast agents [52–54]. The impact of these systems in recent years has been predominantly in cancer therapy where the physicochemical properties of the drug substance, in particular poor solubility, require a drug delivery technology for delivery and in some cases specific targeting.

13.4 Challenges Presented by Nanomaterial Drug Products

The challenges presented by nanomaterial drug coproducts are not new or unique. The same six previously mentioned factors that require a multidisciplinary strategy to reach drug safety and efficacy for other novel drugs are still applicable for nanotheranostic agents. The following paragraphs review examples of each crucial factor reported upon in the current literature.

Developing treatment prototypes is a necessary step in the development of nanotechnology-compatible therapeutic agents. Tools, techniques, and assays have been established over the last decade that are specifically aimed at measuring physicochemical characteristics, biological effects, therapeutic efficacy, and toxicological potency. In 2008, Sumer and Gao described features of highly desirable nanomedicine platforms. “Ideal nanomedicine platforms should be small in size, provide high drug-loading densities, be efficient in targeting to the tumor tissues with minimal nonspecific uptake, provide responsive release mechanisms to improve drug bioavailability and also imaging ultrasensitivity to pre-validate and monitor therapy.” The authors go on to say that advances in both material science and composite chemistry are needed to make multifunctional platforms on the nanometer-size scale a realty. Arguably, the best mechanism to develop and evaluate treatment prototypes is to produce and test variations and publish the findings in the peer-reviewed literature [55–57].

The industry’s willingness to use personalized medicine is another factor needed to be overcome when developing nanomaterial-based theranostics [58]. In this regard, two criteria must be met. First, compliance by the healthcare provider is imperative to the successful implementation of personalized therapies and diagnostics. Second, patient’s readiness to utilize personalized medicine must be assessed and evaluated throughout duration of treatment. Hamburg and Collins state that the challenges associated with “developing and using diagnostic tests based on genetics or other molecular mechanisms to better predict patients’ responses to targeted therapy” are vast; but, one factor rises above the others, i.e., timeliness. Research argues

that focusing on prescribing the right drug to the right patient at the right dose at the right time is not only beneficial but should also be regulated [59–61].

Technical feasibility of novel therapeutic and diagnostic agents is an overarching challenge. Economic feasibility is another [57, 62–64]. This challenge revolves around comprehensive analyses of economic, financial, and psychological drivers. Omics technologies, the major technical driver for personalized medicine and theranostic development, are restricted to affluent regions [65]. In 2015, Alyass et al. concluded that "personalized medicine is likely to widen the growing gap in health systems between high and low-income countries." While the economic value of omics technologies is immeasurable in terms of hastening the implementation of theranostic agents, the access equality is hindered due to the lack of health insurance, personal funds, education, and understanding [66]. There have been many papers published in this specific area of evolving healthcare. Table 13.2 summarizes some of the recent research.

Characterizing Nanomaterial Drug Products It is important in considering nanomaterials that they are biocompatible and safe. The standard approach to the safety of molecules, of which the nanomaterials are composed, is well understood in classical toxicology. However, the use of nanomaterials, specifically nanoparticles, raises the issue of disposition dictated by particle size that might introduce the component molecules to different trafficking pathways, cellular and subcellular compartments, and potential sites of concentration than the molecules alone would experience. It is essential that the potential for adverse effects resulting from these unique toxicokinetic and dynamic phenomena is addressed in evaluating the performance of nanomaterials.

The characterization of nanomaterial drug products requires nontrivial sample preparation, use of control and standard reference materials, and tedious result interpretation. Various physicochemical properties have been used to facilitate interpretation of data collected in the research lab, preclinical and clinical trials, and environmental health endpoints. These properties include descriptions of the product's size (primary particle, aggregate, agglomerate, and size distribution) as well as description of the product's surface (area, charge, intended functionalization, and unintended modification) [54, 67, 68]. The most commonly employed analytical techniques used to characterize properties of the drug product are dynamic light scattering (DLS, a measure of particle size in an aqueous suspension), transmission electron microscopy (TEM, a measure of particle size in its dry state), bright field (BF) and dark field microscopy (DF, a measure of aggregation or agglomeration), zeta potential (ZP, a measure of particle surface charge in an aqueous suspension), and inductively coupled plasma-mass spectrometry (ICP-MS, a measure of inorganic chemical composition).

Each of these analytic methods has advantages and limitations. For instance, some methods are quantitative and based on large populations, such as DLS and zeta potential. Other methods are qualitative because of the limited sample size, such as microscopy. However, even though microscopic methods tend to deliver qualitative data sets, the results are valuable since micrographs visually show the particle's morphology and heterogeneity/homogeneity across the sample population.

DLS is also known as photon correlation spectroscopy or quasi-elastic light scattering. The techniques measure the fluctuations of scattered light passed through a particle suspension. The software can extrapolate the average particle size from the detected scattered light. While this indirect method of measuring particle size has drawbacks, its versatility is unparalleled. DLS is cheap, can measure a wide range of sizes, and is used on multiple particle types throughout the entire drug product development pipeline.

DLS is widely accepted in the nanomedicine field if it is accompanied by TEM images. TEM images provide a two-dimensional representation of a three-dimensional object. A beam of electrons is passed through the prepared sample, and scattered electrons are detected and converted to a micrograph at high magnification and resolution. Particles as small as individual atoms have been visualized using TEM techniques. Its limitations are based on the ability to develop contrasting shades of gray, i.e., capability to distinguish among the background, the sample of interest, and artifacts. However, if proper sample preparation techniques are utilized (such as staining, mounting, preserving, and slicing), then nano-scopic features of a drug product can be visualized and assessed for quality.

Other microscopy techniques can be used to characterize the drug product. Bright field and dark field microscopy use visible light and magnifying lenses to examine objects not visible to the naked eye. These techniques illustrate shape and aggregation characteristics of samples at low magnifications. Bright field images provide convenient preliminary data to indicate which microscopy technique with higher-resolution capabilities should be followed up, such as electron microscopy. Imaging in dark field mode enables the inherent emission of light of the specimen to be easily viewed, imaged, and recorded.

Zeta potential, also referred to as electrokinetic potential, is a popular way to measure the stability of a dispersed drug system. The measurement is actually the electrical potential between the dynamic dispersion matrix (i.e., water) and the fixed dispersion molecules absorbed onto the surface of the drug. The magnitude (either positive or negative) of the ZP indicates the relative stability of the drug suspension. The lower the value (i.e., between 0 and ± 10), the more unstable the particle suspension (i.e., high likelihood to aggregate or agglomerate). The higher the value (i.e., between ±10 and ± 50), the more stable the particle suspension (i.e., not likely to coagulate or flocculate).

ICP-MS is a routinely used quality control technique that ensures the absence of trace metals. In some cases, when the nano-enabled drug product is metal-based, ICP-MS provides verification that the metal is present in its correct oxidation state. The technique uses plasma to ionize components within the sample and mass spectrometry to detect the chemical identity of the ionized portions. Over the past 10 years, ICP-MS has evolved to become one of the most versatile, element-specific detection techniques. Although it was initially used for the total quantification of trace metals in samples, ICP-MS is now used to detect organic compounds, organometallic compounds, and even more complex biomolecules, such as nucleic acids, phospholipids, and proteins.

Considerations in Manufacturing Processes As the interest in nanomaterial formulations in drug therapy increases, engineering strategies are being developed that might improve the potential of these to aid dosage form design. Particle replication in non-wetting templates (PRINT) and 3D-printing technologies are among the methods that may expand the capability to rapidly and efficiently manufacture nanoparticles at a scale that will support product development activities [69, 70].

Nanoparticles are produced by a range of processes, each of which involves input parameters that impact the physicochemical properties of the final product. Manufacturing processes must be assessed through quality-by-design (QbD) strategies and application of multivariate statistical methods to identify critical process parameters. For example, crystallization processes require steady control over input variables such as analyte concentration, stir speed or agitation rate, and temperature. For a milling method, particle suspension concentration (aka particle load), fluid velocity, and dimensions of apparatus components (i.e., nozzles) might be considered [1, 71, 72].

The key to addressing the regulatory considerations associated with nanomaterials is to quickly establish if the nanoscale component of the formulation is critical to its performance and if so, which of the properties requires measurement to establish manufacturing specifications sufficient to control its quality. It is unlikely in the short term that new analytical methods will be developed to characterize the product's quality and performance. However, it is possible that measuring as many physicochemical properties as possible may result in the identification of either primary critical quality attributes (CQAs) or variable that might be confounded in second- or third-order interactions that are critical to the product quality and performance. In this regard, it has recently been suggested that the accumulation of data in repositories or registries from which metadata can be derived may be the most rapid path to predicting which of the properties in any circumstance will be important [1, 17–19].

13.5 Broad Regulatory Perspective

Regulatory interest in nanomaterials originates from its importance to the safety and efficacy of the product. If the role of the nanomaterial in product performance cannot be defined, then it is unlikely to be of routine interest from a regulatory standpoint although the manufacturer may still have an interest from a development standpoint.

All drug products are subject to formal safety assessment, and in this respect, nanomaterial-based products will be treated in the same manner. The intricacies of classical safety testing will account for the disposition and effect of nanotechnology products. However, it is incumbent on the manufacturer to incorporate into the assessment anything that may be known about unusual disposition of a nanomaterial that might require specific modifications to the toxicology protocols to fully

address the potential for toxicity, for example, cell-specific uptake by the mononuclear phagocytic system and related concentrations in anatomical compartments.

Organizations such as caNanoLab, the NIH Nanomaterial Registry, and nanoHUB are attempting to curate sufficient data with quality metrics to allow for generalization of nanomaterial disposition and effect [19, 73, 74]. This objective has yet to be fully met, but there has been enormous progress over the last decade. As the opportunities for data entry into nanotechnology-specific databases on drug products increase and are supplemented by high-quality research data, it can be anticipated that new quality and safety protocols will be developed that more thoroughly manage the risk of new nanomaterials.

13.6 Future Considerations

In the field of theranostics, much consideration has been placed on conceptual model development and preliminary efficacy data collection. Efforts in nano-enabled theranostic agent development as personalized medicine for oncology patients are attractive due to decreased costs, dosing concentrations, and time while undergoing treatment. While recent advances in drug delivery systems have the potential to make these attractions a reality, more considerations are needed to ensure safe and effective theranostic drugs.

(a) First, availability of full study information and sharing participant-level data for drug development research should be endorsed by all stakeholders involved in nanotheranostic agent research and development efforts.
(b) Second, development of standards for safety and efficacy must be established and followed by drug developers throughout the development pipeline.
(c) Third, robust methods and techniques should be used and validated against orthogonal approaches to verify results.

These three considerations can be applied to many aspects of nanomaterial-enabled drug product development. Robust methods, implemented standards, and disclosed study information are valuable at all stages of the process (Table 13.3). Researchers

Table 13.3 Lists of primary characteristics of major nanotechnology-enabled approaches to cancer treatment

Nanomaterial	Modification	Target	Agent	Example
•Liposome •Amorphous •Polymer •Crystalline •Organic, inorganic	•Targeting moieties •Solubility •Time-release	•Brain •Lung •Prostate •Blood	•Therapeutic •Diagnostic •Imaging agent •Immuno-modulator	•Taxol, Doxil •Cisplatin •Magnevist, Feridex I.V. •Rapamune

should measure specific physical, chemical, and biological properties of their specific nanomaterial. Formulators can optimize specific modifications to the surface of nano-drugs, nano-carriers, and nano-additives. Physicians can record the effectiveness of the drug in various target tissues and organs, while pharmacologists can monitor the use of the nanotechnology-enabled approach based on use-case scenarios [53, 75–78].

In addition to particle size, an assessment of the particle's morphology (i.e., shape and surface roughness), charge, and functionality are also key critical quality attributes that have been identified for nanomedicines during their manufacturing and quality control processes. The USP has no general chapter on nanotechnology; the organization has simply published a stimuli article indicating that the recommended guidelines follow the FDA's lead on the subject. There are no specifications on manufacturing nanomedicines since there are so many different types of nanoparticles used in this field. The literature has demonstrated that each nanomaterial used in a drug product has unique properties that require variable critical process parameters (CPP). The most prominent examples of a nanoparticle used in nanomedicine applications are liposomes, followed by polymeric nanoparticles and colloidal iron (used primarily as an imaging agent). The USP approach to specifications can be generally summarized as is that each drug product containing nanomaterials would appear in specific monographs for any given product and would be designated for that particular product [79].

13.7 Conclusions

Some of the important perspectives involved in characterizing drug products containing nanomaterials, including nanotheranostic agents, have been discussed. The success of nanotheranostics will be defined by many crucial factors and will require a multidisciplinary strategy to establish drug safety and efficacy. In addition, the underlying effort associated with this strategy is the development of metrics designed to measure therapeutic quality, safety, and efficacy.

The primary characteristics of major nanotechnology-enabled approaches to cancer treatment, beyond the drug itself, include selection of the nanomaterial type, type of surface modification, intended target tissue or organ, and desired pharmacological effect. It is the control of all of these properties that ultimately dictate the theranostic effect. Control is continually improved with increasingly robust methods, implemented standards, and disclosed study information. An authoritative nanomaterial-oriented data registry can enable an iterative process necessary for effective implementation of nanotheranostic agents [80].

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Part IV
Cancer Nanotheranostics

Chapter 14
Engineering Multifunctional Nanomedicine Platforms for Drug Delivery and Imaging

James Grant, Mana Naeim, Youngshin Lee, Darron Miya, Theodore Kee, and Dean Ho

14.1 Introduction

The field of nanotechnology involves the synthesis, manipulation, and utilization of materials within the dimensions of 0.1–100 nm [1]. In recent years, this field has made significant advances in medicine related to uses in enhancing targeted drug delivery, increasing efficacy in immunotherapy, imaging techniques, and comprehensive disease management. More specifically, the field of nanomedicine has realized substantial advances to understand pathology, diagnose disease, and target and treat abnormalities and may ultimately improve the quality of life for patients [2]. Nanomedicine platforms have included metallic nanoparticles such as gold and

James Grant, Mana Naeim, and Youngshin Lee are contributed equally to this work.

J. Grant · M. Naeim · Y. Lee · D. Miya
School of Dentistry, University of California, Los Angeles, Los Angeles, CA, USA
e-mail: jamesgrant@ucla.edu; mnaeim@ucla.edu; youngshin@ucla.edu; dmiya90@ucla.edu

T. Kee
SINAPSE, Departments of Biomedical Engineering and Pharmacology, and Biomedical Institute for Global Health Research and Technology (BIGHEART), National University of Singapore, Singapore, Singapore
e-mail: bietwk@nus.edu.sg

D. Ho (✉)
SINAPSE, Departments of Biomedical Engineering and Pharmacology, and Biomedical Institute for Global Health Research and Technology (BIGHEART), National University of Singapore, Singapore, Singapore

Department of Bioengineering, University of California, Los Angeles, Henry Samueli School of Engineering and Applied Science, Los Angeles, CA, USA

Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA, USA

California NanoSystems Institute, Los Angeles, CA, USA
e-mail: dean.ho@ucla.edu; biedh@nus.edu.sg

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silver [3, 4], liposomes (small lipid vesicles) [5–7], dendrimers (novel polymers) [8], and carbon structures such as nanodiamonds (NDs) [9]. Furthermore, nanomedicine agents are purposefully designed in a broad range of dimensions that enable controlled localization for enhanced treatment specificity [10]. These particles have generated substantial interest due to their ability to integrate therapeutic and diagnostic capabilities, treatment modalities, and imaging techniques when varying conjugations are made using diverse classes of drug/imaging compounds, antibodies, peptides, hormones, or ligands. This chapter will focus on multifunctional nano-drug delivery and nanodiagnostic imaging systems of four nanomedicine platforms, nanodiamonds, liposomes, dendrimers, and gold nanoparticles, and illustrate their variability in potential therapeutics and clinical utilization. The safety and biocompatibility of each platform will be overviewed, and the challenges and future directions of developing multifunctional nanomedicines as a whole will be discussed.

Emerging nanomedicine platforms possess unique intrinsic properties and chemical structures. Their abilities to co-load pharmacologic drugs, imaging agents, and targeting moieties in parallel combinations confer novel functionality and significant advantages to traditional cancer imaging and therapy. The poor blood solubility and bioavailability of many anticancer therapeutics [11] may be overcome by conjugating the anticancer drugs to surface functional groups or by enveloping them within the cores of soluble nanocarriers. The simultaneous functionalization of combinations of actively targeting compounds [6, 7], anticancer drugs, and imaging/contrast agents has revealed the multifunctional versatility of nanomedicine platforms and their possible theranostic applications toward a broad spectrum of disease indications. These multifunctional nanomedicines may result in substantial advantages in cancer theranostic applications compared to standard of care approaches. Preclinical studies have even shown that drug-loaded nanoparticles are capable of overcoming drug resistance in tumors. As such, combinations of anticancer drugs with targeting compounds carried on the same nanoparticle platform may improve the selectivity and specificity of the nanoparticle and anticancer drug activity [12, 13]. Other nanomedicine functionalization approaches with multimodal imaging probes may allow for complementary diagnostic imaging with systems such as optical/CT, optical/PET, PET/CT, and PET/MRI [14]. As a result, combinations of anticancer drugs and imaging agents may then mediate image-guided therapy. Combinations of multiple imaging agents and pharmacologic drugs on a single nanocarrier have been used to monitor drug release, delivery, and therapeutic efficacy [15]. Additionally, nanomedicines loaded with multiple anticancer drugs and pharmacologic agents show synergistic effects that may improve therapeutic efficacy and safety compared to monotherapy [16, 17].

As multifunctional theranostics continue to be developed for the clinic, important issues such as production, scalability, and the characterization/control of properties including polydispersity, biocompatibility, and other physicochemical properties are being addressed [18]. In this chapter we review the properties of multifunctional nanomedicines and highlight applications of these systems in four nanoplatforms, liposomes, dendrimers, gold nanoparticles, and nanodiamonds.

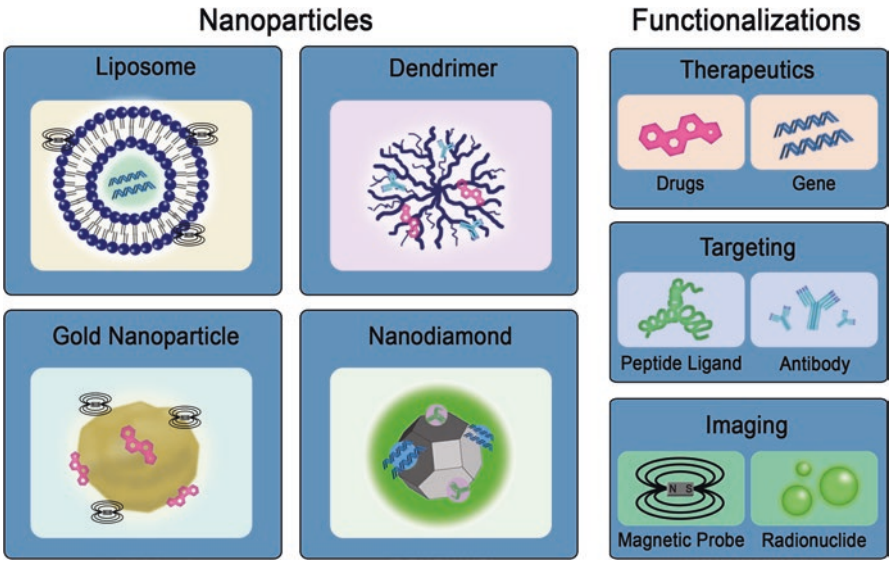

Fig. 14.1 Four multifunctional nanomedical platforms (liposomes, dendrimers, gold nanoparticles, nanodiamonds) illustrating example combinatorial therapeutic payloads and surface conjugations including targeting and imaging moieties [10]

These four platforms have been widely explored for theranostic applications due to their ability to simultaneously deliver therapy, targeting, and imaging agents (Fig. 14.1). Finally, we will discuss the challenges facing the development and application of multifunctional platforms as well as their future potential in personalized medicine [9].

14.2 Multifunctional Nanoparticles

14.2.1 Function

Multifunctional nanoparticles are distinguished from their monofunctional counterparts by the potential to deliver two or more therapeutic benefits utilizing a single nanoparticle modality [19]. Relevant medical applications include their combined use as (1) therapeutics, either as carriers for drugs or via their inherent therapeutic potential, (2) targeting devices to deliver therapy in a disease-specific manner, and finally (3) imaging agents to aid in evaluating and diagnosing pathologies. The advantages of combinatorial approaches are particularly relevant in the context of cancer, where treatments are frequently toxic by design and challenging to sequester. Advances in multifunctional nanomedicines promise to improve therapeutic indices and reduce off-target toxicity while allowing clinicians to visualize treatment delivery in real-time, diagnose malignancies at earlier stages, and track

treatment progression [20]. Reducing the number of treatment modalities can mitigate procedural invasiveness, decrease the number of office visits, and ultimately improve patient and clinician satisfaction. For example, a current monofunctional non-pharmaceutical therapeutic application of nanoparticles in oncology is the use of solid gold nanospheres in hyperthermia-induced ablation. Once injected interstitially into solid tumors, gold nanospheres can be stimulated externally to their tuned resonance frequencies and via photothermal conversion, which induce apoptosis [21]. Alternatively, the same particles can be functionalized with antibodies targeting tumor-specific cell surface markers that when administered systemically, self-target malignancies that have distantly metastasized or are undetectable due to their small size. In this instance, the nanoparticles and functionalized antibodies are an example of combined therapeutic and targeting mechanisms [22].

Nanoparticle capacities are not solely limited to bifunctional approaches and may yet assume more complex roles. Nanodiamonds in particular are an appealing medium for multimodal approaches due to their biocompatibility and surface chemistry that allows for versatile functionalization and adsorbance of a wide range of biomolecules including drugs, genes, and imaging markers [9]. For instance, nanodiamonds that have been simultaneously conjugated with chemotherapeutic agent paclitaxel, an anti-EGFR antibody, and a fluorescent oligonucleotide have demonstrated increased uptake by EGFR-upregulated breast cancer models via fluorescence microscopy [20]. This highlights their potential in drug delivery, payload targeting, and confirmation by direct imaging. Finally, nanoparticle versatility can be harnessed to provide synergistic effects even in the context of one application. For example, within the domain of biomedical imaging, a single nano-core can be functionalized with probes serving multiple imaging modalities, overcoming the deficiencies while retaining the advantages of any one platform. A magnetic core chemically conjugated to optical and radionuclide moieties can be imaged using MRI, optical fluorescence, and positron emission tomography (PET) simultaneously, overcoming the low target sensitivity, low tissue penetration, and poor spatial resolution, respectively, that limits each modality on its own [23]. Similarly, hyperbranched polymers (HBP) of the dendritic family have been used for tumor-specific dual-modality imaging. Combining the high spatial resolution and anatomical accuracy of 19F overlaid 1H MRI, the sensitivity of fluorescence imaging and targeting properties of conjugated folate ligands allow for the in vivo detection and measurement of B16 melanoma cells, which commonly overexpress the folate receptor (Fig. 14.2) [24]. Winning combinations of therapeutic functionality seem inexhaustible with tactical design and engineering, and once realized, multifunctional nanomedicines can impart a substantial advantage over singular consecutive approaches.

14.2.2 Architecture

While reviewing the state of multifunctional nanoparticles, it is essential to consider their intended applications as well as the physical characteristics required for their rational design. Nanoparticles are well suited to multifunctionality due to

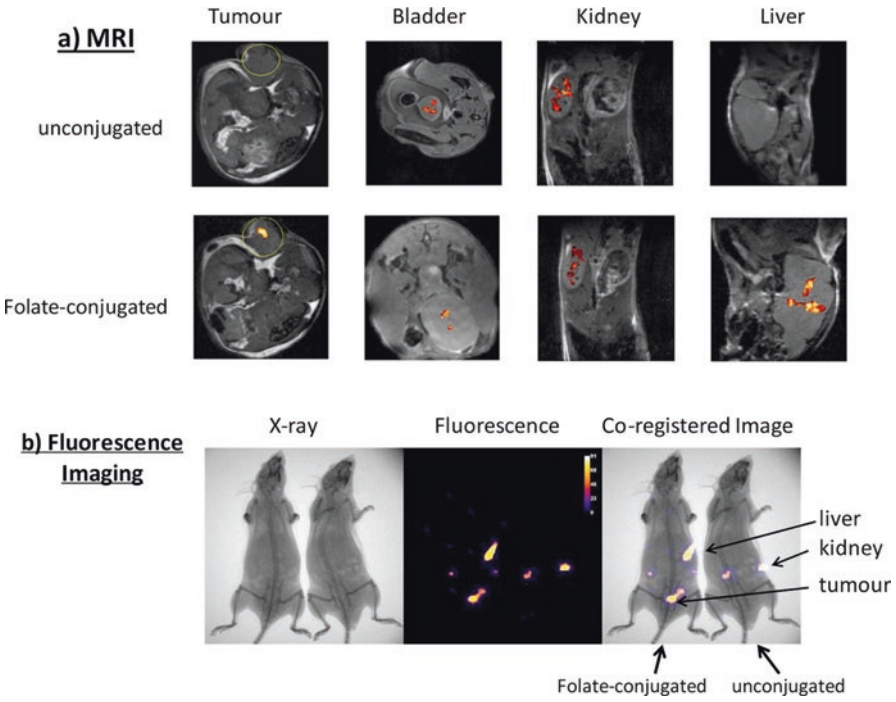

Fig. 14.2 (**a**) 19F MRI overlaid on 1H MRI in mouse melanoma models 1 h post-IV injection of control HBP and folate-conjugated HBP, depicting folate receptor targeting in tumors (yellow circle). Folate receptors are also normally overexpressed in liver cells, while bladder and kidney accumulations depict renal excretion. (**b**) Fluorescence overlaid on X-ray imaging on the mice 1 h post injection. (Reprinted with permission from [24]. Copyright (2014) American Chemical Society)

characteristics such as small size, large surface area to volume ratio, and programmable surface or carrier load modifications [22]. While these shared qualities collectively confer nanomedicines their unique functionality, it is important to note that they comprise a staggeringly diverse range of materials, beyond the four platforms we will subsequently discuss here. Newer classes of nanomedicines are under constant development and include nanoporphyrins, the first biophotonic multifunctional nanomedicine; ultrasmall quantum dots, which can interact with their targets at the atomic level; and flavonoids, touted for their "green" plant-derived synthesis [25–27]. This list is by no means exhaustive considering that, in the strictest sense, the hallmark of nanoparticles is particle size. However, certain aspects of their form and architecture determine nanoparticles' suitability for use as nanomedicines and more specifically as multifunctional nanomedicines.

Broadly speaking, nanoparticles are generally classified either as organic polymers, a class that includes liposomes and dendrimers, or as inorganic with an elemental core, including nanodiamonds and metal derivatives such as gold nanospheres [28]. Polymeric nanoparticles are noted for their ability to deliver drugs systemically by self-assembly into an amphipathic bilayer around an encapsulated drug

payload with surface chemistry amenable to functionalization. They are distinguished by their biodegradability, higher encapsulation yields, structural flexibility, and relatively larger size [29–31]. Inorganic nanoparticles are noted for their biocompatibility, inherent magnetic or electronic properties useful for imaging modalities, programmable surface modifications, superior stability and drug solubilization, and comparatively small size. However, their rigid crystalline structures require their payloads to be transported as surface conjugations rather than as encapsulations [32, 33]. Within these classes, nanomedicines can be further classified depending on key physical characteristics that govern not only their function as therapeutics and imaging agents but also their interaction within the biological system. Size, shape, and surface properties including valence and the nature of moiety conjugation guide nanoparticles' biodistribution, clearance, and toxicity in vivo. These factors are of elevated significance in light of multifunctional design.

The principal limitation of the therapeutic efficacy of most pharmaceutical platforms is the ability to overcome physiological barriers upon administration. Particularly in anticancer applications, the physical parameters of size and shape of the nanoparticle are intimately linked to tumor penetration and thus efficacy. For example, the targeting functions of nanomedicines are mediated via either active mechanisms, which rely on ligand-receptor interactions, or passive mechanisms, attributed to the enhanced permeability and retention (EPR) effect intrinsic to the tumor microenvironment. The range in size of certain nanomedicines, which lies intermediate to that of healthy vasculature pores (~5 nm) and abnormal endothelial fenestrations (up to 780 nm), comprises the basis of EPR [34, 35]. Furthermore, particle size has a primary role in regulating particle metabolism and distribution. Rapid renal clearance is observed with nanoparticles smaller than 5 nm, while those between 150 and 300 nm are processed by slower hepatic processes [36]. Those smaller than 12 nm in size are capable of crossing the blood-brain barrier to the central nervous system, allowing them to access malignancies typically difficult to reach by traditional means [34]. Nanoparticle shape has also demonstrated an effect on the adhesive strength between particles with targeting ligands and the target cell surface. Studies have shown that given the same volume, oblique, rather spherical nanoparticles bind with higher affinity and exhibit greater carrying capacity for drugs or imaging agents [37]. Once bound, however, spherical particles have demonstrated greater and faster cell internalization, owing to lower free energy requirements for endocytic membrane wrapping [38]. The addition of multifunctionality can also complicate dimensional determinations, particularly if optimization involves conflicting trends. In magnetic imaging applications, larger nanoparticle size has been shown to correlate with both higher saturation magnetization values, resulting in greater sensitivity and higher r_2 relaxivity values for greater negative contrast ability [39, 40]. Additional studies have demonstrated shape-dependent differential theranostic ability in metallic nanoparticles, with high aspect ratio gold nanorods exhibiting the greater photoabsorption than their spherical counterparts [41]. While size and shape are by no means the only architectural considerations, both have wide-ranging implications in modulating pharmacokinetic behavior and efficacy as therapeutic platforms. Designing multifunctionality

into these platforms further entails thoughtful analysis on potential trade-offs and synergies that can occur between modalities based on structural composition.

14.2.3 Liposomes

Liposomes have been widely studied for decades owing to their clinically proven ability to enhance treatment efficacy while reducing complications associated with cardiotoxicity. Importantly, liposomal drug formulations have been approved for clinical administration [42]. Liposomes are composed of a single lipid bilayer (micelle) or double lipid bilayer composed of amphipathic phospholipids that enclose an aqueous core. The aqueous core is held together via hydrogen bonding, while the hydrophobic tails are held together via van der Waals forces. This unique chemistry allows for liposomes to be loaded with both hydrophilic and hydrophobic drugs. When drugs are effectively encapsulated within the lipid bilayer, the hydrophobic tails offer protection from the external environment. This preserves the drug from native enzymatic and host immune degradation while also reducing negative cytotoxic effects of the unmodified drug. This unique physical architecture makes liposomes potential drug-delivery vehicles for areas in the body that are notoriously difficult to target, such as the human brain [5]. Targeted drug therapies have historically proved to be challenging due to the inherent impermeability of the blood-brain barrier (BBB). A variety of cells, astrocytes, pericytes, neurons, and endothelial cells create tight junctions that make transport across the BBB extremely limited [43]. Prior techniques consisted of systemically administering a drug with the knowledge that only a minor fraction would be able to penetrate the blood-brain barrier while the majority would transfer to other nontargeted sites [5]. This is not only inefficient, but it also increases the likelihood of negative side effects. Notable formulations include Doxil® (doxorubicin) and liposomal irinotecan [44]. Polyethylene glycosylated liposomes loaded with doxorubicin were found to significantly reduce tumor volume in Lewis lung carcinoma tumors, while micelle nanoparticles used to deliver paclitaxel showed increased encapsulation efficiencies and a longer drug release profile [45]. Patients experienced fewer negative side effects such as vomiting, cardiotoxicity, and nausea when given drugs encapsulated within liposomes compared to the free drug alone [46, 47]. Another recent clinical translation and application of a liposomal injectable formulation is CPX-351 (Vyxeos™), a fixed 5:1 molar ratio combination of two chemotherapy drugs cytarabine and daunorubicin. Early animal studies of human leukemia CRM-CCF xenografts demonstrated that the CPX-351 liposomes carrying the synergistic dose were internalized in a tumor-specific manner and resulted in myelosuppression that was longer lived than the response seen with a free-drug cocktail (Fig. 14.3) [48]. Clinical trials with FDA-approved Vyxeos™, indicated for adults newly diagnosed with high-risk acute myeloid leukemias, demonstrated that the drug increased estimated survival rates by an average of 4 months in comparison to those of control groups receiving monotherapy. Another study observed that of 13 patients enrolled,

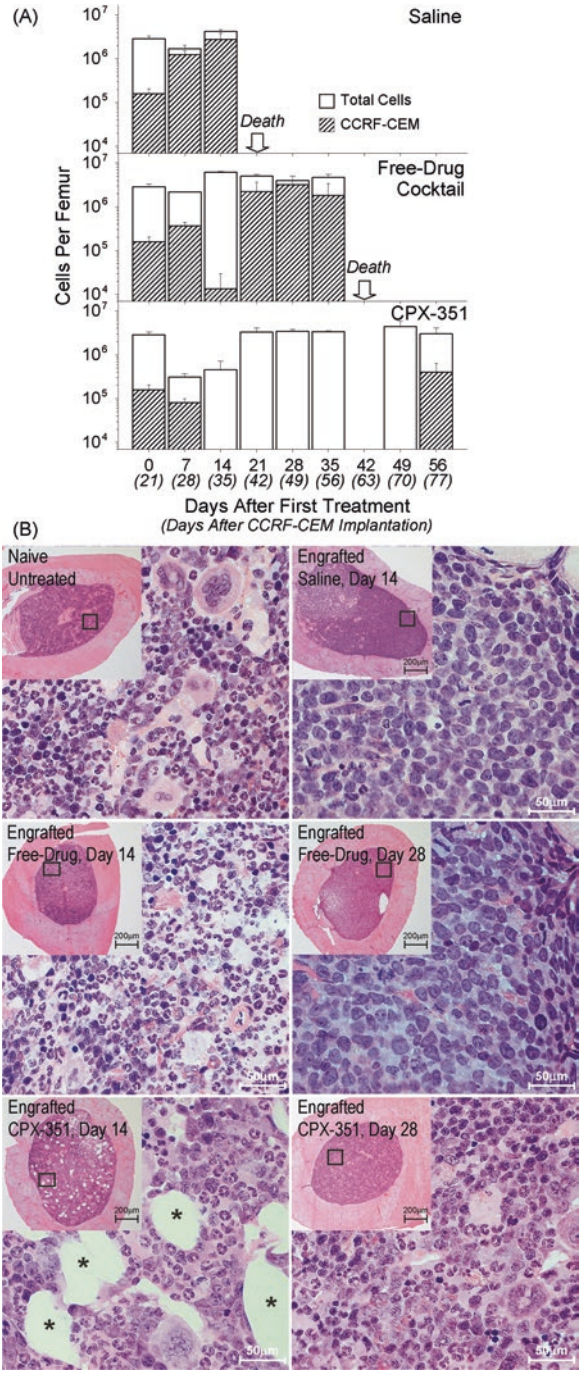

Fig. 14.3 (**a**) Rag2-M mice grafted with CCRF-CEM human leukemia cells and subsequently administered control (0.9% NaCL), free-drug (300:4.5 mg/kg), or CPX-351 (10:4.4 mg/kg). Femoral marrow samples were analyzed to determine ratios of normal (white) to CCRF-CEM (shaded) cells at 7-day intervals. (**b**) H&E staining of ungrafted and saline control, free-drug, and CPX-351 samples at 14 and 28 days under 10× and 60× magnification. At +14 days, CPX-351-treated sections display greater hypocellularity of leukemic cells than do the free-drug sections, while the saline control samples instead show increased leukemic transformation. At +28 days posttreatment, CPX-351 sections show repopulation by non-leukemic cells, while free-drug sections display a rebound of CCRF-CEM cells. (Reprinted with permission from [48])

10 patients achieved near or complete remission, facilitating eligibility for transplantation. It was also found that treatment with Vyxeos™ was independent of renal function, indicating that patients with impaired renal function did not require dose adjustments, all promising features of the combinatorial drug strategy [49, 50].

Liposome nanoparticles loaded with various drugs have also shown to be very effective in targeted therapy in difficult to access areas such as the brain. For example, a heat shock protein, [HSP]72, delivered via liposomes to patients suffering from ischemic stroke showed superior aggregation of the drug in the ischemic brain area compared to delivery as a free agent [51]. In addition, MRI assessment demonstrated a greater reduction in ischemic severity of subjects that received the targeted liposome-drug complex when compared to the control group. By harnessing the effectiveness of liposome-mediated doxorubicin delivery, prospective therapies in neuro-oncology and treatment management of tumors residing in the brain are promising. In addition, liposome-encapsulated doxorubicin introduced in vivo had longer circulating cytotoxic capabilities within brain tumors of Fischer rats compared to doxorubicin alone, which resulted in improved therapeutic efficacy [52]. These studies emphasize the true multifunctionality of nano-drug delivery systems in targeting a diverse range of pathologies, such as cancers and neurodegenerative diseases, and the variability of the nanoparticle drug delivery complexes.

An overarching concern across oncological disease treatment has consistently been the development of MDR in patients. Once a patient is resistant to a number of drugs, the next course of action is usually to increase the dosage in an effort to overwhelm the disease, which leads to a variety of systematic side effects in many cases [41]. A major biological contributor to MDR is the plasma membrane glycoprotein, P-gp, which is responsible for effluxing or removing therapeutic drugs from tumor cells. As such, the drugs cannot be adequately retained within the cancer cells, resulting in substantially reduced efficacy [53]. Thus, a P-gp inhibitor is often combined with traditional cytotoxic drugs in order to effectively address the patient's cancer and simultaneously prevent MDR. Recently, liposomes have been used as the platform to deliver both a P-gp inhibitor and therapeutic drug via EPR. When tariquidar (a P-gp inhibitor) and paclitaxel were both incorporated into liposomes, the formulation increased paclitaxel concentration within cancer cells compared to an equivalent dose of paclitaxel given without a P-gp inhibitor. Therefore, the use of a liposome platform co-loaded with multiple drugs has shown improved drug retention and efficacy.

Liposomes offer a possible means to address tumor formation due to their inherent nanoparticle size. The vasculature of tumors often includes endothelium pores that are larger than the particle size of liposomes, allowing for the accumulation of the liposomal drug agents within the interstitial space of the tumor [35]. For example, improved safety and drug localization resulted from the liposomal combination of the anticancer drug vincristine with quinacrine, an agent with intrinsic MDR-reversing properties [12, 17, 54]. Liposomal delivery of these two drugs to murine xenografts harboring drug-resistant K562 cells responded more favorably than unmodified quinacrine and vincristine delivery. In a similar study, topotecan, an ovarian cancer medication [55], and amlodipine, a calcium channel blocker

with MDR-inhibiting properties [56], were integrated within liposomes. Enhanced synergistic effects of both drugs were seen, and in vivo results showed lessened cardiotoxicity due to the liposomal encapsulation of both drugs, which reduced their blood exposure [16]. Liposomes represent a potential combinatorial nanoplatform allowing for the integration of multiple therapies to effectively target and treat human cancers while mitigating MDR.

14.2.4 Dendrimers

Dendritic macromolecules or "dendrimers" are serially branched synthetic nanoparticle polymers that are symmetric around a multivalent core [57]. Dendrimers are characterized depending on their three distinct zones: the multivalent core, the interior, and the periphery [58]. Their versatile properties allow for the simultaneous delivery of multiple classes of targeting and therapeutic/imaging agents [59]. Drug molecules and imaging agents may be covalently attached to the dendrimer's numerous surface sites and functional groups or encapsulated within the core by non-covalent forces [60]. The latter can help solubilize hydrophobic anticancer drugs [61]. Among the most studied structures for delivering anticancer drugs are poly(propylene imine) (PPI), polyamidoamine (PAMAM), poly-L-lysine (PLL), polypeptide, polyesters, polyether dendrimers, and polyethylene glycol (PEG)-based dendrimers [62, 63]. Their well-defined particle sizes/architectures, highly branched construction with many surface site functional groups for drug conjugation, and interior voids for drug encapsulation make them possible candidates for anticancer applications [64–66]. One study found dendrimers functionalized with magnetic resonance and fluorescence imaging probes were successfully able to cross the blood-brain barrier and accumulate in RG-2 malignant glioma cells. These particles were imaged in vivo via dynamic contrast-enhanced MRI (DCE-MRI), and ex vivo confirmation of delivery was achieved via fluorescence imaging [67].

Folic acid (FA) is a ligand that is commonly conjugated to dendrimers to target the overexpression of the high-affinity FA receptor observed in many cancer lines. When activated, the FA receptor stimulates the internalization of folic acid and its conjugated molecules. Multiple studies successfully utilized FA-conjugated dendrimers to target tumor cells and to internalize the dendrimer complexes both in vitro and in vivo. For example, one study conjugated the anticancer drug methotrexate to FA-conjugated dendrimers to target KB cells (epidermal carcinoma cells) in vitro [68–70]. Another study conjugated methotrexate to FA-conjugated dendrimers along with radiolabeled/fluorescent indicators to target and image tumors in mice in vivo [69]. PAMAN and PPI dendrimers with Gd(III)-loaded surfaces and PEG chains were successfully used to interrogate newly formed angiogenesis vasculature via EPR targeting [71, 72]. Furthermore, tumor-associated angiogenesis has been successfully monitored by utilizing radiolabeled bromine dendrimers functionalized with αvβ3 integrin, a molecular marker associated with angiogenesis [73].

Dendrimer-based carriers have overcome many of the challenges associated with traditional disease treatment and monitoring. Both conventional and newly developed anticancer formulations are plagued by poor bioavailability due to low water solubility, cell membrane permeability, and systemic toxicity [74, 75]. Dendrimers may overcome these obstacles as they have been shown to achieve targeted anticancer drug delivery, higher solubility, lower toxicity, and multifunctional capabilities [76–78]. Another major obstacle in combating cancer is overcoming drug-resistant tumor cells due to the presence of drug efflux transporters such as the adenosine triphosphate-binding cassette (ABC) transporter and the P-gp transporter [79–81]. Some evidence indicates that dendrimers may be conjugated to efflux pump substrates and function as pump inhibitors, subsequently enabling them to avoid efflux-mediated reduction in treatment efficacy [82]. Additionally, propanol-conjugated dendrimers have been reported to potentially bypass the P-gp efflux transporter instead of inhibiting it [83].

14.2.5 Gold Nanoparticles

Gold nanoparticles can possess many architectures, ranging from nanospheres and nanorods to nanoshells and nanocages. Gold nanoparticles possess high surface area to volume ratios, and their surfaces can be readily modified with functional groups such as thiols, phosphines, and amines. Moreover, these functional groups allow the conjugation of the gold nanoparticle to many biologically relevant ligands including citrates, amines, oligonucleotides, peptides, antibodies, and lipids [84]. This wide array of surface ligands can mediate attachment of targeting moieties, anticancer drugs and gene therapies, and imaging and diagnostic agents.

The surface plasmon resonance (SPR) optical property is a unique feature of gold nanoparticles that enhances their use in disease imaging and photothermal ablation therapy [85]. Nanoshells possess a thin gold shell and a dielectric core. This structure allows for the application of scattering properties, which are useful for imaging, as well as absorption properties, which enable photothermal ablation [86]. By manipulating the nanoshell structure, the resonant wavelength, and relative scattering, the absorption efficiency can effectively be optimized [87]. Photothermal ablation is conducted by harnessing the strong absorbance in the visible and near infrared light regions (NIR) of the gold nanoparticles due to their SPR oscillations. These properties can then be used to heat the particles and ultimately lead to death of malignant cells through hyperthermia damage. Nanospheres, nanorods, nanoshells, and nanocages of gold have been explored for photothermal ablation [88]. Ablation treatment in a murine colon carcinoma model using PEG-coated gold nanoshells resulted in healthy and tumor-free subjects 90 days after treatment [89]. In addition to nanoshell-based ablation, optical coherence tomography (OCT)-based imaging was also achieved in later studies [90]. Gold nanoparticles have also been functionalized with surface targeting moieties to enhance photothermal ablation specificity. Gold nanoshells conjugated to VEGF resulted in a twofold increase

in accumulation in intracerebral glioma tumors in mice [91]. Similarly, anti-EGFR antibody conjugated gold nanoparticles selectively ablated HaCaT epithelial carcinoma cell lines [3].

Nanoshells offer great potential for early cancer detection due to their optical properties, which may improve commonly used imaging techniques such as OCT, used for prostate, gastrointestinal, oral mucosal cancers, etc., as well as reflectance confocal microscopy (RCM) [86, 90]. Similar to other nanomedicine platforms, nanoshells can utilize the EPR effect to enhance tumor accumulation [92]. For example, nanoshells conjugated with anti-HER2 antibodies were administered to HER2+ breast adenocarcinoma cells. Imaging and in vitro photothermal therapy studies were then conducted, and a greater enhancement in imaging efficacy and photoablation therapy-mediated treatment outcomes were observed in the nanoshell group versus the control [86]. In a separate study, gold nanoshells conjugated to trastuzumab, an FDA-approved monoclonal anti-HER2 antibody, demonstrated successful targeting and photothermal ablation of trastuzumab-resistant breast cancer cells [93]. Moreover, metal nanoshells tuned to absorb NIR in solid tumors achieved higher maximum temperatures causing irreversible tissue damage more efficiently than controls without nanoshells under magnetic resonance guidance [94].

Gold nanoparticles have been utilized to deliver drug and gene cancer therapies as well. For example, methotrexate conjugated to gold nanoparticles suppressed tumor growth in a mouse model of Lewis lung carcinoma [95]. One study showed that intravenously injected TNF-targeting cAu-PEG-TNF had a greater degree of accumulation in colon carcinoma tumors compared to other healthy organs. A later experiment demonstrated that paclitaxel conjugated cAu-PEG-TNF reduced tumor size in the same model [96, 97]. Additionally, gold nanoparticles used to cross the blood-brain barrier to mediate RNA interference against the Bcl2L12 oncogene in a mouse glioblastoma model demonstrated markedly enhanced efficacy compared to the control cohort [98]. Finally, a synthetic oligo(ethylenediamino)-beta-cyclodextrin-modified gold nanoparticle (OEA-CD-NP) successfully delivered DNA plasmids to breast cancer cells (MCF-7) [99].

As imaging agents, gold nanoparticles functionalized with targeting moieties may enhance translationally relevant applications [100]. Gold nanoparticles possess many advantages compared to standard modalities, including longer imaging times, improved blood vessel delineation, and less toxicity, among others [13]. As many epithelial cancers overexpress the epidermal growth factor receptor (EGFR), one study demonstrated the feasibility of using anti-EGFR antibodies conjugated to gold nanoparticles to optically image precancers in real time [101]. Additionally, dendrimer-entrapped gold nanoparticles (Au-DENPs) simultaneously targeted and imaged a human epithelial carcinoma cell line (KB cells). Folic acid was conjugated to a PAMAN dendrimer surface to target overexpressed folate receptors on KB cells, while the entrapped gold nanoparticles served as the imaging agent [102].

Recently, gold nanoparticles have been explored for potential immunotherapy applications [103]. Gold nanoparticle cancer vaccines have been shown to increase antibody titer levels against cancer markers, stimulate T-cell proliferation, and

substantially reduce tumor sizes in mice in vivo [104]. A previous study synthesized a Tn-antigen glycan-conjugated gold nanoparticle cancer vaccine, which may be applicable toward a broad range of cancers [105].

14.2.6 Nanodiamonds

Carbon-based nanostructures represent another class of inorganic nanostructures being explored for their multitherapeutic potential and can be engineered in a variety of structural formations, including carbon nanotubules, carbon dots, graphene, and fullerenes [106]. Colloidal suspensions of nanodiamond (ND) particles, with single-digit nanoscale diameters, have been actively investigated as platforms for localized drug delivery, scalable gene therapy, targeted chemotherapeutics, and biocompatible imaging (Fig. 14.4) [107–111]. NDs are an effective means of delivering drugs, ranging from cancer therapeutics to growth factors, for a multitude of reasons. They possess faceted surfaces that can carry a broad spectrum of compounds, and their surfaces also possess diverse chemical groups which allow for multifunctional conjugation with targeting moieties, drug compounds, and imaging agents [112]. Also, NDs can also carry both water soluble and insoluble compounds

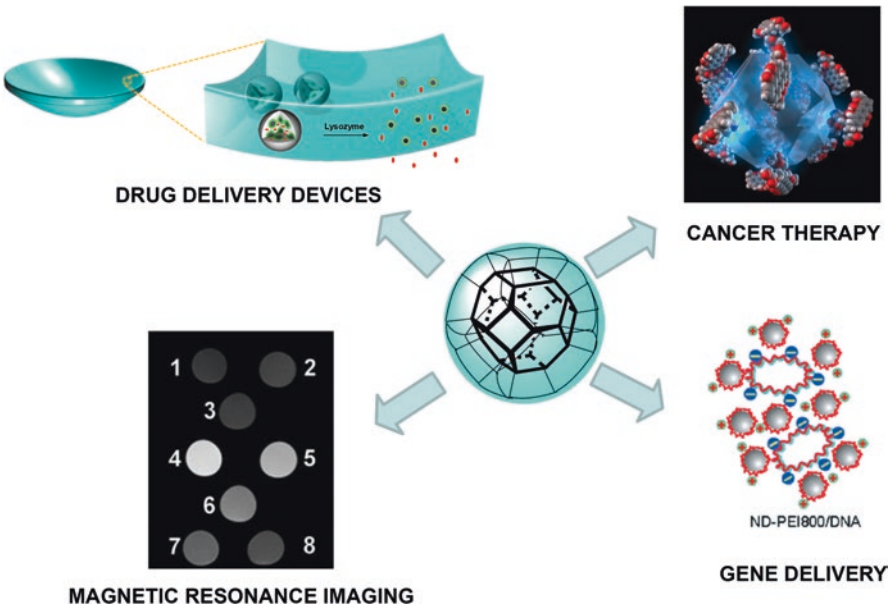

Fig. 14.4 Nanodiamonds have been harnessed for a broad array of applications, ranging from chemotherapeutic delivery to gene therapy and contact lens-based release of glaucoma treatment compounds. (Adapted with permission from [107–110]. Copyright (2009, 2010) American Chemical Society)

while preserving efficacy [113]. With regard to biocompatibility, NDs are well tolerated once introduced in vivo and are considered among the least toxic of the nanoparticles in the carbon family demonstrated in both in vitro and in vivo studies [114]. For instance, NDs implanted within the trachea had low pulmonary toxicity in transplanted hosts, intravenously introduced NDs resulted in no significant deviation in liver serum markers, and real-time polymerase chain reaction (RT-PCR) assays revealed no indication of inflammatory cytokines [109, 115, 116]. Furthermore, while ND-injected *Caenorhabditis elegans* worms transferred the NDs onto their progeny, the presence of NDs alone did not hinder reproduction or cause mortality for the offspring [117]. Another appealing aspect of NDs is their cost-effectiveness. NDs are created by the controlled detonation of carbon-based explosives in closed metallic chambers and thus are inexpensive to mass-produce. Following the detonation, the soot is collected, purified of graphite carbon and incombustible impurities such as metals and oxides, and subsequently processed for surface modification [111, 112]. To mediate unimpaired activity, chemotherapeutic drugs may require a vehicle that is capable of carrying compounds that exhibit low solubility. Chemotherapeutic drugs of interest, such as purvalanol A, dexamethasone, and 4-hydroxytamoxifen (4-OHT) used to treat a number of different cancers, are combined with the ND particles by forming electrostatic and physisorption interactions, allowing for the timely release of the drugs. Purvalanol A, a drug that promotes apoptosis in cells that overexpress the *myc* oncogene; 4-hydroxytamoxifen (4-OHT), used for treating breast cancer; and dexamethasone (Dex), used as an anti-inflammatory, have all been successfully complexed to ND particles. These drugs in their uncomplexed form are not dispersible in water; however, after bridging with ND particles, all three improved water solubility and dispersion and decreased particle size [113]. Furthermore, transmission electron microscope analysis of 4-OHT revealed qualitative images of the 4-OHT residue occupying the surface area of ND particles, visually confirming their physisorption [118]. Following the synthesis of these and other ND-based drug complexes, the EPR effect may serve as a mechanism for localizing ND-based therapy following administration [119–121], which thereby improves the safety of the chemotherapeutic drugs by sparing healthy neighboring cells. These three drugs are examples of similar therapeutics, which have been interfaced with NDs, such as doxorubicin and epirubicin [109, 122]. Therefore, the potential of NDs as a translationally impactful drug delivery agent is clear.

In addition to improving upon the effectiveness of various therapeutics, NDs are also the topic of research involving their potential applications in biomedical imaging, specifically magnetic resonance imaging (MRI) [123]. Contrast media or dyes are intravenously injected to improve the visibility of pathology (such as inflammation and tumors) and the overall diagnostic quality of the scan. The effectiveness of any contrast agent is measured in terms of its relaxivity, the time it takes for water protons to relax after stimulation. Most contrast media include paramagnetic gadolinium(III) (GD(III)), a soft earth metal whose seven unpaired electrons allow it to bond to water [124]. When ND surfaces were covalently modified and attached

to Gd(III) complexes, it was found that the per-Gd(III) relaxivity of the complex was increased by tenfold [110]. This finding strongly suggests that biocompatible NDs may improve the diagnostic quality of clinical MRI scans. Fluorescent NDs (FNDs) have also been investigated in terms of improving lymph node mapping in vivo. Due to a point defect within their lattice structure, known as the negatively charged nitrogen-vacancy center (N-V center), FNDs exhibit photoluminescence in the infrared range when excited, making them appropriate for various bioimaging applications. FNDs also improve the contrast of various preclinical imaging modalities due to longer fluorescent lifetime compared to that of normal cells [114]. In addition, for mice injected with FNDs intradermally in the right paw, it was found via fluorescence imaging that FNDs accumulated primarily in the lymph node associated with the injection region, in this study the right axillary lymph node. This finding demonstrates that, due to their molecular architecture and biocompatible properties, NDs may be used for fluorescent imaging and sentinel lymph node mapping, a routine technique performed in cancer treatment to identify the lymph node most intimately associated with a patient's tumor. Thus, the application of NDs in predicting prognosis and aiding in treatment planning of patients diagnosed with various malignancies is promising [114].

NDs play key roles in multifunctional applications. For example, NDs have been used in conjunction with poly-L-lactic acid (PLLA), a biopolymeric compound used for bone scaffolding, due to its biocompatibility [125]. One drawback of PLLA is that when used alone, it can demonstrate low resistance to mechanical deformation. However, when PLLA was combined with NDs containing octadeclamine-functionalized surface modifications, this improved the dispersion of PLLA and increased bone mineralization. The multifunctional ND platform of ND-ODA/PLLA has potential for use as a bone scaffolding material, for musculoskeletal tissue engineering, and for monitoring the appearance of newly formed bone following implantation [126]. This exemplifies NDs' ability to operate both as a multifunctional carrier and as a modality for tracking treatment progress due to their bright fluorescence and clinically relevant imaging capabilities.

14.3 Challenges Facing Multifunctional Nanomedicines

The promise of multifunctionality in nanomedical platforms is well established, with implications that reach beyond simply additive capabilities in delivery, targeting, and theranostics. Equally exciting is the potential for incorporating strategies addressing multidrug resistance (MDR), stealth mechanisms to evade immunogenicity, controlled "triggered" release in response to temporal or environmental stimuli, and nonvector-mediated gene therapy [127–129]. A review of the significance of multifunctional nanomedicines would be incomplete, however, without a discussion of some of the challenges associated with increasing sophistication. For example, the success of the targeting mechanism is dependent on

various factors such as the qualitative and quantitative nature of the targeting moiety, the method of moiety conjugation, and surface engineering strategies that can protect or stabilize the particles in vivo. The process of advancing functionality by adding targeting moieties and imaging probes can introduce changes in confirmation, orientation, and biorecognition that compromise the intended therapeutic activity. For instance, while monoclonal antibodies can offer increased specificity in recognition compared to peptide ligands, their larger size may obstruct the activity of adjacent functionalizations [130]. Similarly, while there exists an initial correlation between ligand density and target cell internalization, it has been shown that nanoparticles with a ligand density above a certain threshold level exhibit lower binding levels, likely due to steric strain. Other physical considerations include a concomitant increase in particle size with increasing surface conjugation, which impedes distribution through tissues in which diffusion is the principal route of entry (i.e., solid tumors) [131]. Increased specificity in targeting ligands can also display a paradoxical "anchoring" effect in which the strength of carrier-target interactions at the cell surface obstructs internalization of the payload. This leads to either an accumulation of the drug in the extracellular environment without effective delivery inside the cell of interest or an arrest of the delivery of the drug within the surface layer of solid tumors without effective penetration depth [120]. Finally, the incorporation of targeting mechanisms escalates the immunogenic potential of the system [132]. The consequent increased opsonization and phagocytosis can alter biodistribution, rendering drug delivery and imaging functions unreliable.

This trade-off is next illustrated in the context of theranostics, which allows clinicians to image, diagnose, track, and treat disease in real time. Targeting of imaging materials decreases the requisite dosage of functionalized contrast agents, many of which are associated with their own adverse reactions and toxicity concerns [133]. Given this appeal, however, integrating modalities can compromise or even negate their intended actions. The capacity of multimodal nanoparticles to transport contrast agents or imaging probes is often orders of magnitude lower than that of dedicated delivery vehicles, resulting in image quality or resolution below the threshold required for accurate diagnosis [134]. Differences in optimal circulation times may also pose a temporal challenge for drug delivery-probe conjugations, whereas maximizing circulation time of nanoparticle-mediated chemotherapeutics results in enhanced distribution, less off-site toxicity, and overall higher therapeutic indices, and the delayed clearance of imaging probes interferes with follow-up imaging by increasing background noise [135, 136]. Another concern of linking therapy to imaging is the prospect of acquiring MDR essentially eliminating any utility of the associated imaging agent [137].

Because the transition from single to multimodal applications can involve a compromise in efficacy, engineering of novel nanotherapeutics must proceed with the knowledge that added functionality does not necessarily translate to optimal functionality. There are practical constraints that can hinder translation of novel therapeutics into the clinic as well. Highly complex nanoparticles incur additional expense in development and synthesis, with multiple conjugations

requiring repeated purification, producing lower yield, and introducing greater product heterogeneity at the junction of successive steps [137]. Multicomponent nanoparticles also entail higher intellectual property costs and greater regulatory challenges, which collectively present challenges to commercialization [138]. In light of these considerations, an examination of the costs and benefits of added functionality of nanomedicines is not only prudent but necessary before the immense anticancer potential of these platforms can be fully realized.

14.4 Future Directions: Optimizing Nanomedicine Through Combination Nanotherapy

A broad spectrum of nanomaterial platforms has been explored for therapeutic delivery, targeting, and imaging applications. The next phase of nanomedicine will harness emerging technologies such as artificial intelligence (AI) to systematically optimize nanoparticle-based therapeutics based on differential drug and dose combinations (Fig. 14.5) [138–140]. This is critical since multidrug nanoparticles are intended to enhance drug synergy while minimizing antagonism, and these effects are variable depending on dosage, over time, and for each individual. One such informatics-based approach (Latin hypercube sampling) has already been utilized to determine the therapeutic window of chemotherapeutics functionalized to nanodiamond carriers, determining the global optimum for both drug-drug pairs and drug-dose ratios on breast cancer cell lines featuring variable drug resistance (MDA-MB-231, MCF-7, and BT-20) and controls (IMR-90, MCF-10A, and H9C2) (Fig. 14.6) [139]. Importantly, optimized nanodiamond-modified drug combinations (ND functionalized doxorubicin, mitoxantrone, and bleomycin) as well as stand-alone drugs (paclitaxel) outperformed optimized and unmodified drug combinations, as well as nano-modified and unmodified monotherapy and arbitrarily designed monotherapy. Many of the arbitrarily designed

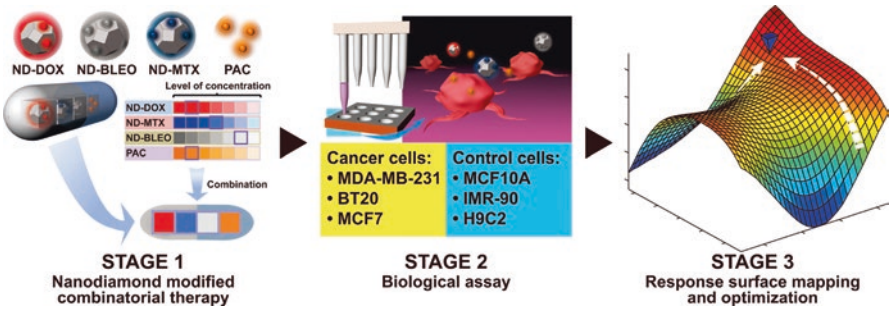

Fig. 14.5 Powerful technology platforms can be used to systematically design combination nanotherapy that can globally optimize treatment outcomes. (Reprinted with permission from reference [139]. Copyright (2015) American Chemical Society)

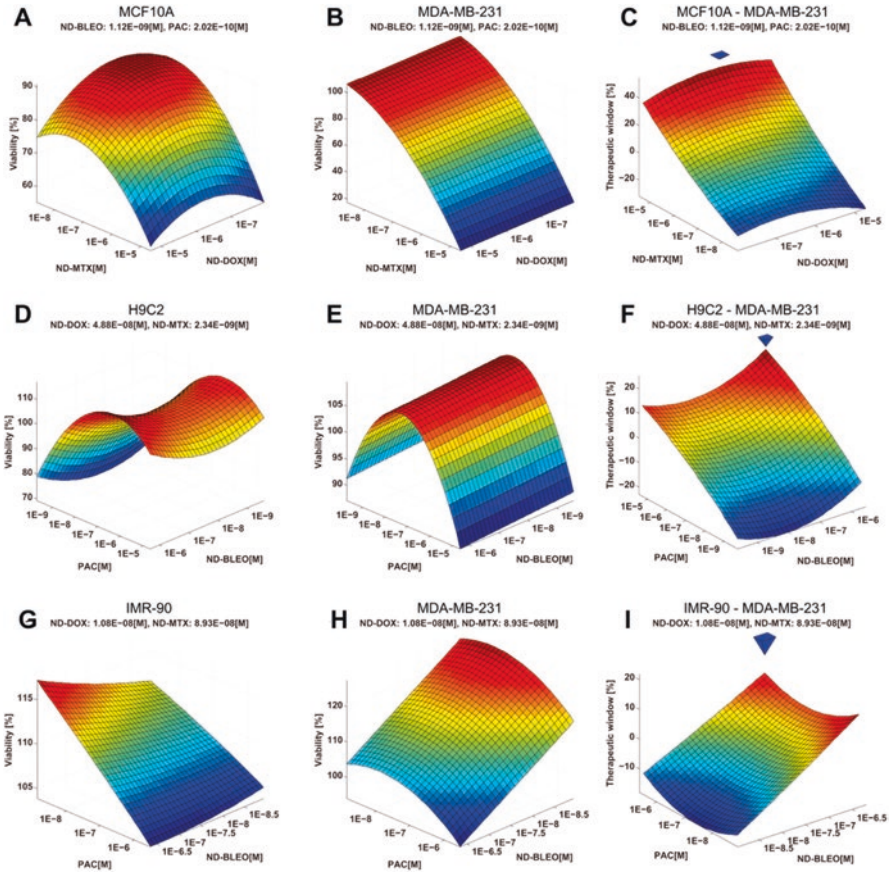

Fig. 14.6 Determining the optimum therapeutic window between pairs of four drug combinations, ND-DOX, ND-BLEO, ND-MTX, and PAC, from cellular response surfaces of a drug-resistant breast cancer cell line, MDA-MB-231, and three normal control cell lines, MCF10A (mammary gland epithelial), H9C2 (heart myoblast), and IMR-90 (lung fibroblast), using Latin hypercube sampling. ND-BLEO and PAC dosages are held constant with phenotypic response surfaces of ND-MTX and ND-DOX shown for cell lines (**a**) MCF-10A (control), (**b**) MDA-MB-231, with the therapeutic window calculated from the responses of (**c**) MCF-10A and MDA-MB-231. ND-DOX and ND-MTX dosages are held constant with phenotypic response surfaces of PAC and ND-BLEO shown for cell lines (**d**) H9C2 (control), (**e**) MDA-MB-231, with the therapeutic window calculated from the responses of (**f**) H9C2-MDA-MB-231. ND-DOX and ND-MTX dosages are held constant with phenotypic response surfaces of PAC and ND-BLEO shown for cell lines (**g**) IMR-90 (control), (**h**) MDA-MB-231, with the therapeutic window calculated from the responses of (**i**) IMR-90-MDA-MB-231. (Reprinted with permission from reference [139]. Copyright (2015) American Chemical Society)

nano-modified and unmodified combinations were also more toxic to the healthy cells than they were efficacious toward the cancer cells. This approach demonstrates the importance of identifying globally optimized combinations, where synergy is implicit.

The nanomedicine field has already made substantial improvements over conventional standard of care therapies across the preclinical to clinical domains. Improving drug retention, targeting, imaging efficiency, and other treatment outcomes using nanomedicine has served as a promising gateway toward the clinical translation of AI-optimized multi-nanotherapeutic regimens that will ultimately transform the way that medicine, particularly in anticancer applications, is practiced.

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Chapter 15
Image-Guided Drug Delivery

Camila Gadens Zamboni, Keyvan Farahani, and Jordan J. Green

15.1 The Rationale for Image-Guided Drug Delivery

Image-guided drug delivery (IGDD) utilizes advances in imaging techniques to facilitate drug therapy through image guidance of delivery, active monitoring of drug carriers upon administration, and quantification and validation of biological responses to therapy. Increasingly, the IGDD field has developed in the context of theranostic platforms, systems that employ biocompatible carriers to combine diagnostic and therapeutic elements. Theranostic platforms have grown to play an important role in preclinical validation of drug delivery systems (DDSs), enabling the observation of biological response to therapeutics, the assessment of biodistribution and pharmacokinetics, and even the visualization or facilitation of drug release. To a much lesser extent, but also of importance, theranostic systems have also been tested in clinical trials. However, the perhaps most promising application of IGDD in the clinical setting is yet to be investigated: the utilization of nanotheranostics to predict drug-related responses on a case-by-case basis, potentially guiding proper patient selection for individualized therapy and precision medicine. Improving efficacy and mitigating off-target toxicity of therapeutic agents are the primary motivations that drive the IGDD field [1–4].

Classical chemotherapeutic agents, including many anticancer drugs, are low molecular weight compounds (<1500 Da) that, upon administration, may be prematurely cleared out from circulation due to rapid renal excretion and substantial

C. G. Zamboni · J. J. Green (✉)
Department of Biomedical Engineering, Translational Tissue Engineering Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA
e-mail: green@jhu.edu

K. Farahani
National Cancer Institute, National Institutes of Health, Rockville, MD, USA
e-mail: farahank@mail.nih.gov

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extravasation to perivascular tissues. As a result, these drugs do not effectively accumulate at the cancer site(s) of interest and cause unwanted toxicity for healthy cells. In this context, DDSs can be enabling to increase circulation time and improve the pharmacokinetics and biodistribution of pharmaceuticals. DDSs are complexes of macromolecular structures (e.g., polymers and lipids) with small-molecule drugs that work as vehicles/carriers of the pharmaceutical to the site of interest. In cases in which an imaging agent is also present, the DDS or carrier is also denominated a theranostic system (or platform). While an extended circulation half-life is often beneficial, the effectiveness of DDSs at extending circulation time is often highly dependent on their structure and physical parameters. Small carriers (<10 nm) bring about the same shortcomings of free small molecules, including clearance through the kidneys. Large (>100 nm) and/or charged structures are typically quickly recognized and eliminated from the circulation by the reticuloendothelial system (RES). On the other hand, carriers with hydrodynamic sizes between 10 and 100 nm and neutral surface charge are able to avoid sequestration into the RES, enable extended blood circulation time, and are well suited to facilitate tissue targeting [3, 5–10]. DDSs, including theranostic platforms, can benefit from surface shielding with biologically inert materials, such as polyethylene glycol (PEG), which offers additional protection against the RES and other degrading mechanisms, prolonging circulation half-lives of carriers [11]. Long-circulating DDSs can take advantage of the leaky vasculature and insufficient drainage system found in the tumor environment to better accumulate into the tumor parenchyma. This phenomenon, termed enhanced permeability and retention (EPR) effect [12, 13], represents an important strategy available to nanocarriers for passive targeting and plays a key role in IGDD for cancer therapy. When the EPR effect is insufficient to ensure appropriate concentration of theranostic agents into the tumor site, ligand-receptor systems can be applied to promote active tissue targeting [10, 14]. Antibodies [15–17], peptides [18], small molecules [19], and aptamers [20] are commonly used moieties that have been conjugated to DDSs to enable active cancer targeting [14]. These ligands are directed to biochemical markers of the carcinogenic process and, when incorporated into a nanotheranostic platform, enable tumor treatment and molecular imaging of cancer-related alterations [21].

Molecular imaging is a noninvasive approach that allows real-time visualization, characterization, and measurement of tissue, cellular and subcellular (molecular) events that take place under physiological and pathological condition [21, 22]. Techniques that have been applied in molecular imaging include ultrasonography, radionuclide-based imaging (positron emission tomography and single-photon emission computed tomography), optical imaging, and magnetic resonance imaging. These modalities differ in characteristics such as penetration depth, sensitivity of detection, temporal resolution, and spatial resolution. In some cases, the need for external stimuli to enable targeting or therapeutic effect also drives the selection of the imaging method [10]. Ultrasound (US), for example, is widely known for its diagnostic capabilities as an imaging tool, but, at suitable intensities it can be used to remotely trigger drug delivery using US probes (e.g., microbubbles). The choice of the signaling agent to be incorporated into an IGDD nanotheranostic platform is dictated by its characteristics and by constraints of the intended IGDD application (Table 15.1). In the past few years, several multimodal IGDD systems [23–26] have

Table 15.1 Strengths and limitations of different molecular imaging modalities

	Strengths	Limitations
MRI	High spatial resolution Unlimited penetration depth Whole-body imaging High soft tissue contrast Triggered drug release	Poor sensitivity Limited temporal resolution High cost
Radionuclide-based imaging	High sensitivity Unlimited penetration depth Whole-body imaging	Limited spatial resolution Limited temporal resolution High cost Ionizing radiation
Optical imaging	High sensitivity High temporal resolution Low cost Triggered drug release	Limited spatial resolution Low penetration depth No whole-body imaging Limited clinical translation
US	High spatial resolution[a] High temporal resolution High sensitivity Low cost Triggered drug release	Low penetration depth[a] No whole-body imaging Limited applications Operator dependence

Adapted from [21, 27–31]
[a]Trade-off between spatial resolution and penetration depth

been developed to overcome the limitations of single imaging techniques and to accommodate the inclusion of multiple therapeutic options within a DDS as well. Along with the development of theranostic nanoplatforms, molecular imaging and probes are also playing a key role in the improvement and advancement of surgical and radiation therapy techniques, mapping body structures and helping to plan and perform the therapeutic procedures.

This chapter presents an overview of molecular imaging modalities used for IGDD, highlighting preclinical and clinical applications of theranostic nanoplatforms (Fig. 15.1) and cancer targeting strategies involved in each system (Table 15.2). In this chapter, we also describe broader IGDD applications beyond the use of theranostic systems, also providing a brief discussion in image-guided surgery and radiation therapy.

15.2 Molecular Imaging Modalities for Image-Guided Drug Delivery

15.2.1 Magnetic Resonance Imaging (MRI)

15.2.1.1 Basic Principles of MRI

MRI is a molecular imaging modality that combines a strong magnetic field and radiofrequency pulses to, respectively, align and excite spins (rotating nuclei) present in atoms with unpaired proton number (e.g., hydrogen [^{1}H] from water molecules). In the presence of a strong magnet, the magnetic dipole moment produced

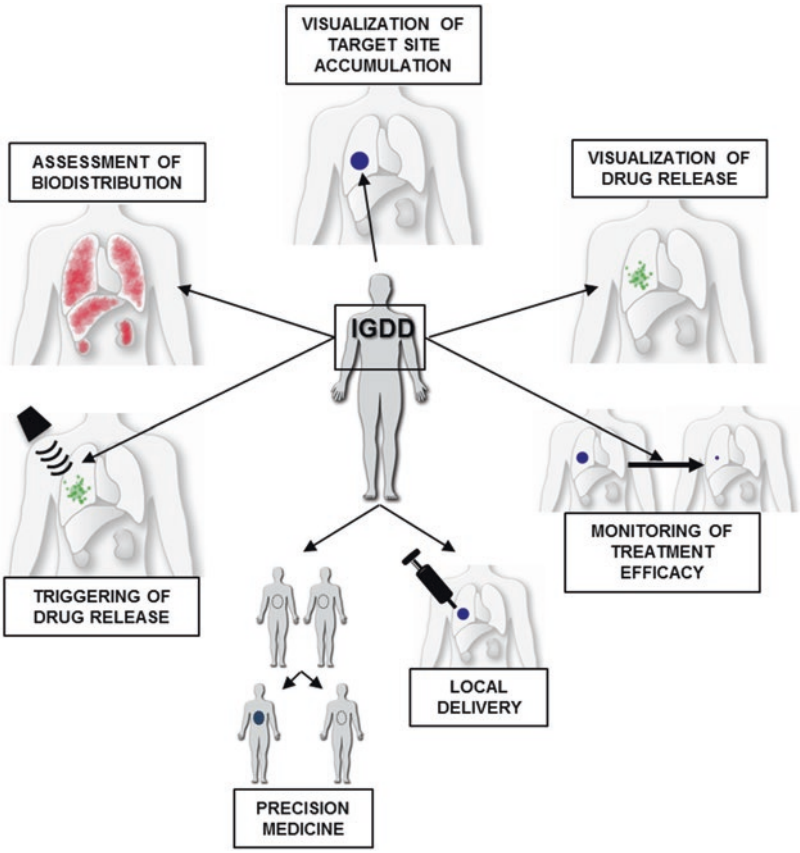

Fig. 15.1 Preclinical and clinical applications of theranostic nanoplatforms used for IGDD

by rotating nuclei causes these spins to change their orientation (process) and to align in parallel to the field [63, 64]. Radiofrequency waves disturb this resting state, causing the orientation of the alignment to change. When returning to the original equilibrium level, the signal emitted through two different relaxation processes can be captured by receiver coils and reconstructed into images. The rates in which these relaxations happen can influence the image contrast and are termed T1, longitudinal or spin-lattice relaxation time, and T2, transversal or spin-spin relaxation time [65, 66]. Since different tissues have different proton densities, T1, and T2 relaxation times, these three parameters determine the strength of the MRI signal and can create endogenous contrast to distinguish neighboring tissues [65]. In order to further increase the contrast between adjacent structures, magnetically active substances can be delivered to a target site. MRI contrast agents can be divided into paramagnetic or superparamagnetic [67].

Table 15.2 Representative studies of preclinical IGDD applications in animal models

Imaging type	Nanocarrier	Therapeutic agent	Imaging agent	Application	Type of targeting	Cancer type	Description	Reference
MRI	Polymeric-based	siRNA (siCD44v6)	IONPs	Biodistribution	Passive	Gastric cancer	SQ mice xenografts IV treated with LNCs	[32]
MRI	Polymeric IONPs	Gold NPs[a]	IONPs	Tumor targeting	Passive	Fibrosarcoma	SQ mice xenografts IV treated with LNCs and/or RT	[33]
MRI	IONPs	Paclitaxel	IONPs	Tumor targeting	Passive	Liver cancer	SQ mice xenografts IV treated with LNCs	[34]
MRI	IONPs	Anti-EGFRvIII antibodies	IONPs	Tumor targeting (particle dispersion and tumor penetration)	Active (BM: EGFRvIII receptor)	GBM	Orthotopic mice xenografts treated via CED with LNCs	[16]
MRI	Polymer-coated IONPs	Gemcitabine	IONPs	Tumor targeting Monitor treatment efficacy	Active (BM: uPAR receptor)	Pancreatic cancer	Orthotopic mice xenografts IV treated with LNCs	[35]
MRI	Polymer-coated IONPs	Doxorubicin	IONPs	Tumor targeting Monitor treatment efficacy	Active (BM: PSMA)	Prostate cancer	SQ mice xenografts IV treated with LNCs	[20]
MRI NIRF	Polymer-coated IONPs	Doxorubicin	IONPs DiR	Visualize drug release Tumor targeting	Passive	Prostate cancer	SQ mice xenografts IV treated with LNCs	[36]
MRI	Liposomes	Doxorubicin	Mn	Visualize drug release	Passive	Fibrosarcoma	SQ rat xenografts IV treated with LNCs	[37]

(continued)

Table 15.2 (continued)

Imaging type	Nanocarrier	Therapeutic agent	Imaging agent	Application	Type of targeting	Cancer type	Description	Reference
MRI	IONPs	Doxorubicin	IONPs	Tumor targeting	Passive	Liver cancer	VX2 rabbit models IA treated with LNCs and embolization	[38]
SPECT/CT	Polymeric-based micelles	Paclitaxel	^{125}I	Tumor targeting Biodistribution	Passive	Ovarian carcinoma	Orthotopic mice xenografts IV treated with LNCs	[39]
SPECT	CNTs-based	HCPT	^{99m}Tc	Tumor targeting Biodistribution	Passive	Liver cancer	SQ mice xenografts IV treated with LNCs	[40]
SPECT/CT	Polymeric-based NP	Doxorubicin	^{123}I	Tumor targeting Biodistribution	Active (BM: folate receptor)	Cervical cancer	SQ mice xenografts IV treated with LNCs	[41]
Micro-SPECT/CT	Liposomes	Doxorubicin ^{186}Re	^{186}Re	Tumor targeting Biodistribution	Passive	Head and neck cancer	SQ rat xenografts IV treated with LNCs	[42]
Micro-SPECT/CT	Gold NPs	Doxorubicin Gold NPs	^{111}In	Tumor targeting Biodistribution	Active (BM: EphB4 receptor)	Ovarian carcinoma	SQ mice xenografts IV treated with LNCs and/or PTA	[43]
PET/CT	Polymeric-based micelles	Doxorubicin	^{64}Cu	Tumor targeting Biodistribution	Active (BM: $\alpha_v\beta_3$ integrin receptor)	GBM	SQ mice xenografts IV treated with LNCs	[44]
Micro-PET/CT	Gold NRs	Doxorubicin Gold NRs	^{64}Cu	Tumor targeting Biodistribution	Active (BM: $\alpha_v\beta_3$ integrin receptor)	GBM	SQ mice xenografts IV treated with LNCs	[45]
PET	$mSiO_2$ NPs	Doxorubicin	^{64}Cu	Tumor targeting Biodistribution	Active (BM: CD105 endoglin)	Breast cancer	SQ mice xenografts IV treated with LNCs	[17]

PET	Polymeric-based NPs	Paclitaxel	[^{18}F]NPB4	Tumor penetration	Passive	GBM	Orthotopic rat xenografts treated via CED with LNCs	[46]
NIRF BLI	Lipid NCs	pHSV-tk/GCV	DiD	Biodistribution Monitor treatment efficacy Tumor targeting	Passive	Melanoma	Orthotopic mice xenografts IV treated with LNCs	[47]
NIRF	Polymeric-based micelle	siVEGF	Cy5.5	Tumor targeting	Passive	Prostate cancer	SQ mice xenografts IV treated with LNCs	[48]
NIRF	Polymer-lipid NPs	Doxorubicin Sorafenib	Cy7	Tumor targeting	Passive	Colon cancer	SQ mice xenografts IV treated with LNCs	[49]
NIRF	Liposomes	Doxorubicin	Alexa Fluor 750	Biodistribution Tumor targeting	Active (irradiated tumors)	Lung cancer	SQ mice xenografts IV treated with LNCs	[18]
NIRF	Liposomes	Doxorubicin	ICG Doxorubicin	Tumor retention Visualize/trigger drug release	Passive	Breast cancer	SQ mice xenografts IT treated with LNCs and/or PTT	[50]
NIRF	Dendrimers	Pc	Pc	Tumor targeting	Passive	Ovarian carcinoma	SQ mice xenografts IV treated with LNCs and PDT	[24]
NIRF	Polymeric-based NPs	CPT	DCM	Visualize drug release	Passive	Breast cancer	SQ mice xenografts IV treated with LNCs	[51]
NIRF Vis	Polymeric-based NPs	pHSV-tk/GCV	Cy5 GFP	Tumor targeting (particle dispersion and tumor penetration)	Passive	GBM	Orthotopic mice xenografts treated via CED with LNCs	[52]

(continued)

Table 15.2 (continued)

Imaging type	Nanocarrier	Therapeutic agent	Imaging agent	Application	Type of targeting	Cancer type	Description	Reference
BLI	Liposomes	pHSV-tk/GCV Doxorubicin	pFLuc pRLuc	Tumor targeting Monitor treatment efficacy	Active (BM: CD44 receptor)	Liver cancer	Orthotopic mice xenografts IV treated with LNCs	[15]
BLI	Polymeric gold NPs	Cisplatin Gold NPs	pLuc	Monitor treatment efficacy	Active (BM: folate receptor)	Breast cancer	Orthotopic mice xenografts IV treated with LNCs and/or PTT	[53]
US NIRF intravital	Polymeric-based NPs	Doxorubicin	PFP FPR-675 Nile red	Tumor targeting Visualize/trigger drug release	Passive	Squamous cell carcinoma	SQ mice xenografts IV treated with LNCs	[54]
US	Polymeric-$mSiO_2$ NPs	CPT11 Microbubbles	PFH	Tumor targeting Visualize/trigger drug release	Active (BM: CD44 receptor)	Liver cancer	SQ mice xenografts IV treated with LNCs	[55]
Multimodality NIRF ^{18}F MRI	Polymeric NPs	Doxorubicin	ICG PFOB	Tumor targeting Biodistribution	Active (BM: folate receptor)	Nasopharyngeal carcinoma	SQ mice xenografts IV treated with LNCs	[19]
Multimodality NIRF MRI	IONPs	Doxorubicin	NIR830 IONPs	Tumor targeting Monitor treatment efficacy	Active (BM: IGF1 receptor)	Pancreatic cancer	Orthotopic mice xenografts IV treated with LNCs	[56]
Multimodality NIRF MRI	Polymeric IONPs	Doxorubicin Paclitaxel	QDs IONPs	Biodistribution Tumor targeting Visualize/trigger drug release	Active (BM: tumor vessels)	Breast cancer	SQ mice xenografts IV treated with LNCs and/or hypertermia	[57]
Multimodality NIRF MRI	IONPs	Ce6	IONPs Ce6	Tumor targeting	Active (MF)	Breast cancer	SQ mice xenografts IV treated with LNCs and/or PDT	[58]

Multimodality MR-HIFU SPECT	Liposomes	Doxorubicin	Gd ^{111}In	Tumor targeting Trigger/visualize drug release Biodistribution	Passive	Gliosarcoma	SQ rat xenografts IV treated with LNCs and hypertermia	[59]
Multimodality FUS MRI	IONPs	Doxorubicin	MBs IONPs	Tumor targeting Trigger/visualize drug release	Active (MF)	GBM	Orthotopic rat xenografts IV treated with LNCs	[60]
Multimodality US MRI	Polymeric IONPs	ROS	O_2 IONPs	Trigger/visualize drug release	Active (MF)	Cervical cancer	SQ mice xenografts IV or IT treated with LNCs	[61]
Multimodality US ^{19}F MRS intravital	Polymeric NPs	Paclitaxel	PFCE RFP	Trigger drug release Tumor targeting Biodistribution Monitor treatment efficacy	Passive	Pancreatic cancer	SQ or orthotopic mice xenografts IV treated with LNCs	[62]

siRNA small interfering RNA, *siCD44v6* siRNA for cluster of differentiation 44 (CD44) variant 6, *IONPs* iron oxide nanoparticles, *SQ* subcutaneous, *IV* intravenous, *LNCs* loaded nanocarriers, *NPs* nanoparticles, *RT* radiotherapy, *EGFRvIII* epidermal growth factor receptor variant III, *BM* biomarker, *GBM* glioblastoma multiforme, *CED* convection-enhanced delivery, *uPAR* urokinase plasminogen activator receptor, *PSMA* prostate-specific membrane antigen, *DiR* 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide, *Mn* manganese, *IA* intra-arterial, *^{125}I* iodine-125, *CNTs* carbon nanotubes, *HCPT* 10-hydroxycamptothecin, *^{99m}Tc* technetium-99m, *^{123}I* iodide-123, *^{186}Re* rhenium-186, *^{111}In* indium-111, *EphB4* ephrin type-B receptor 4, *PTA photothermal ablation*, *^{64}Cu* copper-64, *$mSiO_2$ NPs* mesoporous silica nanoparticles, *CD105* cluster of differentiation 105, *[^{18}F]NPB4* *N*-(4-[^{18}F]fluorobenzyl)propanamido-PEG_4-Biotin, *pHSV-tk* herpes simplex virus thymidine kinase, *GCV* ganciclovir, *DiD* 1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine, *siVEGF* siRNA for vascular endothelial growth factor, *ICG* indocyanine green, *IT* intratumoral, *PTT* photothermal therapy, *Pc* phthalocyanines, *PDT* photodynamic therapy, *CPT* camptothecin, *DCM* dicyanomethylene-4*H*-pyran, *Vis* visual, *GFP* green fluorescent protein, *pFLuc* firefly luciferase plasmid DNA, *pRLuc* Renilla luciferase plasmid DNA, *pLuc* luciferase plasmid DNA, *PFP* perfluoropentane, *CPT11* irinotecan hydrochloride trihydrate, *PFH* perfluorohexane, *PFOB* perfluorooctyl bromide, *IGF1* insulin-like growth factor 1, *QDs* quantum dots, *Ce6* chlorin e6, *MF* magnetic field, *HIFU* high-intensity focused ultrasound, *Gd* gadolinium, *MBs* microbubbles, *ROS* reactive oxygen species, *MRS* magnetic resonance spectroscopy, *FUS* focused ultrasound, *^{19}F MRS* fluorine magnetic resonance spectroscopy, *PFCE* perfluoro-15-crown-5-ether, *RFP* red fluorescent protein

[a]Gold NPs applied as a radiosensitizer

Paramagnetic Agents

Paramagnetic contrast agents are formed by metal ions containing unpaired electrons (e.g., gadolinium, Gd^{3+}, and manganese, Mn^{2+}) complexed with chelating materials [67–69]. Electron spins and their large dipole magnetic moments interact with water protons, enhancing the T1 relaxation rates without substantially interfering with T2 relaxation time [70]. With seven unpaired electrons, gadolinium ions generate one of the largest dipole magnetic moments among paramagnetic elements, effectively promoting T1 relaxation of water protons [68, 71]. Due to the tissue brightening resultant from the reduction on T1, metal chelates are called positive or T1 contrast probes [67]. Low molecular weight metal chelates can diffuse from the circulation to the extracellular space and have been extensively applied as extracellular contrast agents in clinical practice [68].

Superparamagnetic Agents

Superparamagnetic probes (T2 agents), when under the influence of a magnetic field, generate induced dipole magnetic moments that are much larger than the ones created by paramagnetic elements. These large induced dipoles are capable of increasing the existing inhomogeneities (lack of uniformity) in the field, which results in the dephasing of surrounding water protons and the consequent shortening of the T2 relaxation time [70, 72, 73]. As T2-enhanced tissues classically appear dark in MR images, superparamagnetic materials are also called negative agents [68]. T2 probes are synthesized as nanoparticles (NPs) containing a superparamagnetic core and an external coating to promote stability in aqueous environment. While iron oxide NPs (IONPs) (e.g., magnetite, Fe_3O_4) are by far the most commonly studied T2 agents [16, 32, 74], metal alloy and metal-doped IONPs have also been described as alternatives [67, 75, 76]. The versatile nature of NPs enables superparamagnetic agents to be optimized for active targeting and adapted for different applications. For instance, by manipulating the hydrodynamic size, charge, and type of coating of IONPs, one can modify its susceptibility for RES sequestration and therefore create macrophage-vulnerable formulations for liver imaging or long-circulating blood pool agents for visualization of tissues with abnormal endothelium (e.g., tumors) [68]. Taking advantage of the leaky vasculature of tumor sites, blood pool IONPs have shown to offer a great option for the development of cancer imaging agents. To date, however, no such particles were approved for use in humans [77]. FDA approved liver imaging agents Feridex™ and Resovist™, on the other hand, which were shown to be ineffective in differentiating human hepatocellular carcinoma lesions from the normal liver parenchyma [77] and had their production discontinued in the United States [78, 79].

15.2.1.2 Applications of MRI-Based IGDD Nanoplatforms for Cancer Therapy

MRI-Based Assessment of Biodistribution and Visualization of Target Site Accumulation

MRI has unlimited penetration depth and is capable of generating images with high spatial resolution (from 10 to 100 μm) [29]. These characteristics enable MRI probes to be applied for varied purposes when incorporated into IGDD nanosystems, including biodistribution and drug release visualization, tumor targeting, and therapeutic efficacy monitoring. Chen and collaborators utilized a superparamagnetic iron oxide (SPIO) core to incorporate tracing capacity into PEGylated polymeric NPs intended for siRNA delivery. The authors observed a remarkable, iron concentration-dependent, T2-shortening of the liver signal following intravenous administration of the carrier, validating the vector as an effective MRI tracer. The density at the tumor site, however, did not clearly change post NP administration, suggesting poor vector-mediated targeting, as confirmed by the histological findings [32]. Successful accumulation of passively targeted NPs carrying MRI contrast agents could be observed in other studies [33, 34, 80]. For instance, radiosensitizers such as gold (Au) NPs have been successfully delivered by PEGylated carriers containing MRI contrast agents. McQuade and collaborators described PEG-PCL micelles loaded with SPIO and gold (Au) NPs as possible nanotheranostic carriers for IGDD. T2-weighted MRI demonstrated a significant signal intensity drop 24 h after treatment, suggesting intratumoral localization of the carrier. Additionally, the tumor contrast enhancement had a strong correlation with the complete response to radiotherapy in animals radiosensitized with AuNPs [33]. A more detailed discussion on radiosensitizers and radiotherapy is provided in Sect. 15.6 of this chapter.

MRI-Based Monitoring of Therapeutic Efficacy of Interventions

MRI allows reliable assessment of tumor sizes and, thus, is highly suitable for monitoring the therapeutic efficacy of DDSs' interventions. This imaging modality was applied by Lee and collaborators to monitor tumor regression in orthotopic human pancreatic xenografts in mice (Fig. 15.2). SPIO NPs carrying a gemcitabine (Gem) payload and urokinase plasminogen activator receptor (uPAR) ligand (ATF) as a targeting agent were systemically delivered every 3–4 days for 5 weeks. Animals were followed with T2-weighted MRI for signs of tumor progression 1 and 2 weeks after the first treatment cycle. The authors observed a significant T2 signal reduction from the tumor site in mice treated with ATF-SPIO-Gem NPs and signal enhancement in animals receiving no treatment or injected with free Gem. No change was detected in animals treated with NPs

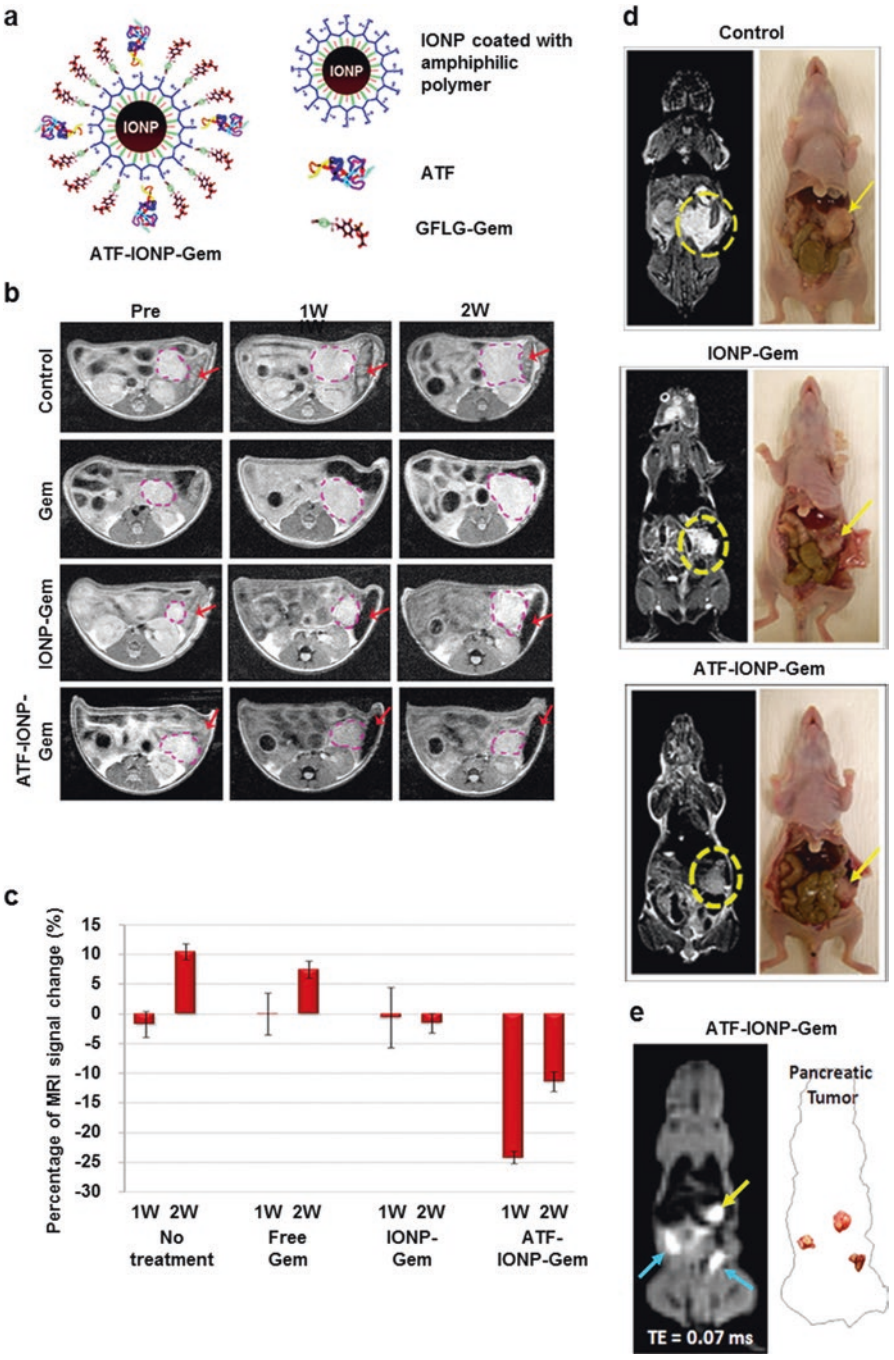

Fig. 15.2 MRI-based IGGD nanoplatform for evaluation of tumor targeting and therapeutic efficacy. uPAR-directed IONPs for concurrent MR imaging and chemotherapy of human

lacking the targeting agent [35]. Yu et al. developed another successful example of a SPIO-based nanosystem for active tumor targeting and treatment efficacy assessment. Here, prostate-specific membrane antigen (PSMA) was the biomarker of choice to enable selective doxorubicin delivery to the prostate cancer tissue. MRI demonstrated that particles carrying PSMA-specific aptamers accumulated better into the tumor site than their nontargeting counterparts and lead to a significant drop in T2 after the conclusion of the treatment. In addition, MRI demonstrated smaller tumor sizes in the PSMA-targeted group when compared to Dox-free, nontargeted NPs and control animals, which was confirmed by measurement of the tumor volume [20].

MRI-Based Visualization of Drug Release

In addition to tracking nanotherapeutics and monitoring their efficacy, MRI can also be utilized for monitoring drug delivery dynamics in real time. Kaittanis et al. demonstrated that loaded IONPs (ferumoxytol) have higher relaxation times than unloaded ones, which enabled the authors to successfully visualize doxorubicin release from SPIO carriers by observing T2 shortening. Animals were treated with intravenous injection of doxorubicin-loaded ferumoxytol or empty NPs. Two hours following administration, tumors appeared with a higher T2 in animals that received

Fig. 15.2 (continued) pancreatic cancer in orthotopic mice xenografts [35]. (**a**) Schematic representation of ATF-IONP-Gem NPs. (**b**) Axial T2-weighted MR images obtained before and 48 h following the second (1 week from the beginning of treatment) and fourth injections (2 weeks from the beginning of treatment) of ATF-IONP-Gem, IONP-Gem, and Free Gem. Animals injected with saline buffer were used as controls. *Dotted circles* and *arrows* show the location/size of the cancer lesions and MRI contrast change in the spleen, respectively. (**c**) Percentage MRI signal change (from the pretreatment baseline) in the tumor site during treatment. Quantitative data was based in region of interest (ROI) measurements. MR images and ROI analysis demonstrate significant (Student's t test, $p < 0.01$) T2 signal reduction in the tumor of animals treated with uPAR-targeted IONPs (ATF-IONP-Gem) 48 h from the second injection, indicating NP accumulation in the target site. Nontargeted particles did not lead to significant signal reduction. (**d**) Coronal T2-weighted MR images and corresponding dissection pictures of tumor-bearing mice 2 weeks after the introduction of the treatment, i.e., 48 h after the fourth therapeutic dose. These images reveal improved tumor accumulation of ATF-IONP-Gem when compared to nontargeted NPs (4.8-fold using muscle as baseline) in areas that match the anatomical location of the lesions, as per dissection images. MR images were also able to demonstrate the tumor growth inhibition by ATF-IONP-Gem, as well as the evolution of the lesions in control animals and mice treated with free Gem or IONP-Gem. Findings matched the postmortem anatomical observations. *Dotted circles* and *arrows* show the location of primary tumor lesions in the MRI and dissection images, respectively. (**e**) Ultrashort echo time (UTE) MRI were used for capturing T1 signal from residual tumors after treatment. This sequence is able to mitigate some of the limitations of T2 agents, such as poor contrast in areas with low background (e.g., peritoneal cavity), increasing the sensitivity of detection [81, 82]. Histological analysis with Prussian blue staining confirmed increased IONP accumulation in tumor of animals treated with targeted platforms (not shown). *ATF* amino-terminal fragment, *uPAR* urokinase plasminogen activator receptor, *Gem* gemcitabine, *W* week, *GFLG-Gem* Gly-Phe-Leu-Gly (GFLG) tetrapeptide linker. (Adapted from Lee et al. [35]. Copyright © 2013, American Chemical Society)

particles carrying doxorubicin. The signal dropped over the course of 24 h and reached that of the groups treated with empty NPs, showing that in vivo real-time drug release visualization is possible with MRI technology [36]. Ponce and collaborators applied temperature-sensitive water-impermeable liposomes to controllably deliver doxorubicin to fibrosarcoma rat models under hyperthermia conditions (Fig. 15.3). Manganese was incorporated into the nanotheranostic platform as the MRI contrast agent. The authors were able not only to monitor drug release but also detail the temporal and spatial distribution of doxorubicin throughout the tumor via T1-weighted MRI. T1 maps were used to quantify the drug distribution pattern. Tumors that achieved the highest doxorubicin concentration according to MRI analysis were the same ones that had the greatest treatment response [37]. The application of manganese as the MRI probe was particularly useful in this case since its signaling capacity depends on a closer interaction with surrounding water protons than occurs for superparamagnetic materials [73]. Therefore, manganese enabled a differential positive contrast enhancement when released from the water-impermeable vesicles [37].

15.2.2 Radionuclide-Mediated Imaging

15.2.2.1 Basic Principles of Radionuclide-Mediated Imaging

Radioactive nuclides are naturally occurring or artificially produced unstable isotopes that emit ionizing radiation as their energy levels decay to restore nuclear stability [83]. Such atoms can be used to label nanocarriers [17, 25, 26, 59, 84] and biological molecules [85] involved in physiological and pathological mechanisms. The radioactive emission can then be traced for imaging or applied for therapeutic purposes [86]. Therefore, nuclear imaging is based on radiation produced from inside the body. Two main types of radioactive emission form the basis of nuclear medicine: gamma and beta decays. While the former involves the

Fig. 15.3 (continued) of each tumor. (**c**) T1-based assessment of mean doxorubicin concentration in the tumor following LTSL injection before HT, during HT or split dose (half before and half during HT). Mean doxorubicin concentration is shown as a function of the normalized tumor radius for each rat. (**d**) Total doxorubicin delivered at different time points for the three different groups of LTSL plus HT. *Vertical lines* in the graphs (**c**) and (**d**) represent the 95% confidence intervals. T1-weighted MRI demonstrated that a higher amount of doxorubicin could be delivered when Dox-/Mn-loaded LTSLs were administered during hyperthermia, or as a split dose, in agreement with the measurements from high-performance liquid chromatography (data not shown). LTSL injection led to a central, peripheral, or homogeneous doxorubicin accumulation pattern following injection before HT, during HT, or at a split dose schedule, respectively. These findings reflect the tumor perfusion pattern and enabled the assessment of drug delivery in a spatiotemporal manner. *LTSL* lysolipid-based temperature-sensitive liposomes, *Dox* doxorubicin, *Mn* manganese, $MnSO_4$ manganese sulfate, *PEG* polyethylene glycol, *DPPC* 1,2 dipalmitoyl-*sn*-glycerol-3-phosphocholine, *MSPC* 1-stearoyl-2-hydroxy-*sn*-glycerol-3-phosphocholine. (Adapted from Ponce et al. [37]. Copyright © 2007, Oxford University Press)

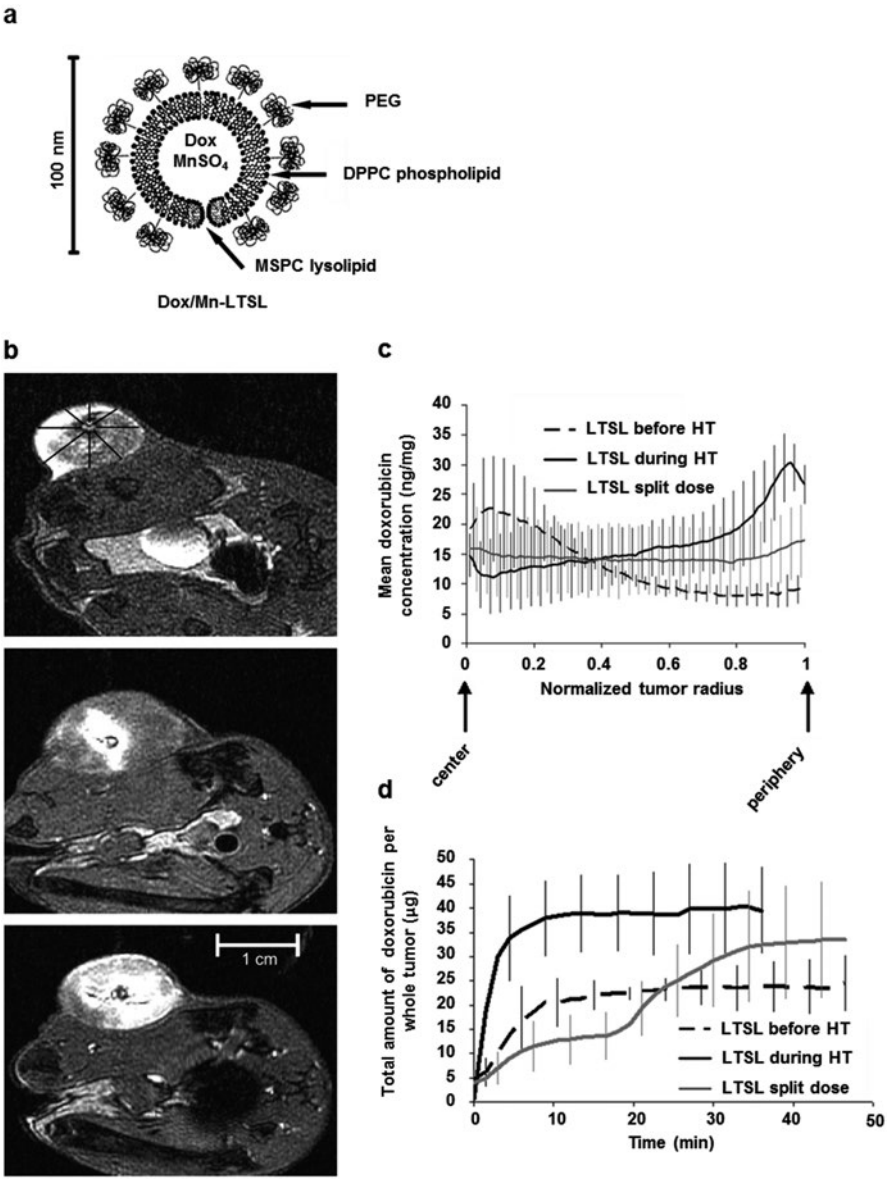

Fig. 15.3 MRI-based IGGD nanoplatform for visualization of drug release. LTSLs for concurrent MR imaging and on-demand chemotherapy of human fibrosarcoma in SQ rat xenografts [37]. (**a**) Schematic representation of Dox/Mn-LTSLs. (**b**) Axial pelvic MR images of fibrosarcoma-bearing rats showing release of the liposomal content (*light color contrast*) following intravenous administration of LTSLs before hyperthermia (*top picture*), during steady-state hyperthermia (*middle picture*), or in two equal doses, half before and half during steady-state hyperthermia (*bottom picture*). *Radial lines* in (**b**) (*top picture*) show the orientation of doxorubicin concentration profiles in (**c**) and (**d**). Hyperthermia (HT) was generated by a heating system placed near the center

production of high-frequency electromagnetic waves (gamma rays), beta decay occurs through the ejection of beta particles (electrons or positrons) from the parent nucleus [86, 87]. Isotopes that undergo gamma decay are applied as radiotracers in planar scintigraphy and single-photon emission computed tomography (SPECT) [83, 88]. Beta decay is useful for treatment of some cancer types due to the cytotoxic effects of beta particles and also represents the basis of positron emission tomography (PET) imaging [86]. In the past years, X-ray computed tomography (CT) has been increasingly applied to incorporate structural anatomical information from CT to SPECT and PET exams, creating combined SPECT/CT and PET/CT images [59, 89, 90].

SPECT

Radiotracers administered into the organism originate gamma photons that travel through the tissues in all directions, exit the body, and can be detected by a gamma camera. During SPECT imaging, the energy of the photons deposited into a scintillation crystal is converted into electrical pulses by a photomultiplier. Images are produced by 3D reconstruction of multiple planar data (projections) collected from different angles and represent the activity distribution of the radiotracer in the body [91, 92]. Since the SPECT signal is produced by single photons reaching the camera as independent events, the correlation between the photon detected and its origin within the body relies on the ability to map the direction of the photons that interact with the crystal. This is achieved by the incorporation of a collimator into the system, which works by absorbing photons that reach the camera at random angles, allowing only photons traveling in a preselected direction to be detected by the crystal [93].

PET

Unstable isotopes carrying a proton excess can restore stability by ejecting positrons from the parent nucleus, a process called beta plus decay. Following emission, a positron travels for short distances until it encounters a free electron; the resultant interaction leads to the destruction (annihilation) of both particles and is accompanied by energy release in the format of two gamma rays of opposing directions [86, 94]. These two photons travel at a 180° angle from each other and reach the gamma camera simultaneously. The detection of coincident events provides positional information regarding the origin of the photons within the body, eliminating the necessity of the physical collimators required in single-photon imaging. Positron emitters have extremely short half-lives; therefore cyclotrons need to be present at the PET facility to produce the isotopes soon before its administration into the body [94].

15.2.2.2 Applications of Radionuclide-Based IGDD Nanoplatforms for Cancer Therapy

SPECT-Based Assessment of Biodistribution and Visualization of Target Site Accumulation

In general, radionuclide-based imaging has high sensitivity of detection and unlimited penetration depth. These characteristics benefit biodistribution and drug targeting studies and have been exploited in the development of nanoplatforms for IGDD. PET offers a better spatial resolution and higher quantitative capacity than SPECT, but its high costs, limited accessibility, and narrow availability of approved radiotracers have allowed SPECT to be maintained as a valuable tool in the molecular imaging field [89, 95].

In a SPECT-based DDS study, Zhang *et al*. engineered a long-circulating and biodegradable multiblock HPMA (*N*-[2-hydroxypropyl] methacrylamide) copolymer carrier with potential to promote systemic paclitaxel delivery. Iodide 125 (^{125}I) labeling allowed the copolymer-drug conjugates to be imaged with SPECT/CT over the course of 21 days. Mice bearing orthotopic human ovarian carcinoma xenografts were treated with either high or low molecular weight (M_w) copolymer-paclitaxel conjugates. SPECT/CT images demonstrated that the nanocarriers with higher M_w had a prolonged circulation time, potentially increasing tumor exposure to the chemotherapeutic drug, as suggested by *ex vivo* analysis. Since the signal intensity of the conjugates decreased significantly between days 1 and 21, the biodegradability of the carrier could also be verified by SPECT/CT [39]. Similarly, Wu *et al*. applied SPECT imaging to monitor the circulation time and tumor accumulation of a carbon nanotube-based DDS labeled with ^{99m}Tc [40]. Lu and collaborators applied SPECT/CT to investigate the role of folic acid (FA) ligand in tumor accumulation of iodide 123 (^{123}I)-labeled polymeric micelles (Fig. 15.4). SPECT results showed that the accumulation of FA-conjugated particles into the tumor (subcutaneous xenograft) was time-dependent and occurred in parallel to the signal decay in the liver. These findings suggest that successful tumor accumulation was mediated by the active targeting of the folate-binding protein (FBP) [41].

SPECT radiotracers have also been incorporated into nanoplatforms designed for multiple therapeutic modalities, such as chemoradiotherapy and photothermal chemotherapy. Soundararajan and collaborators described a liposomal doxorubicin formulation (Doxil) loaded with a beta and gamma emitter radionuclide (^{186}Re) as a potential nanosystem for imaging and chemoradiotherapy [42]. You et al. exploited the near-infrared (NIR)-driven heat generation capacity of gold NPs to synthesize doxorubicin-loaded hollow gold nanospheres to combine chemo- and photothermal therapies. The nanospheres were labeled with ^{111}In and directed to EphB4, a receptor from the tyrosine kinase family overexpressed in several cancer types and angiogenic vessels. Animals that received targeted nanospheres had a higher tumor signal as measured by SPECT/CT, which led to a better tumor response after photoablation therapy (PTA) than groups injected with nontargeted particles [43].

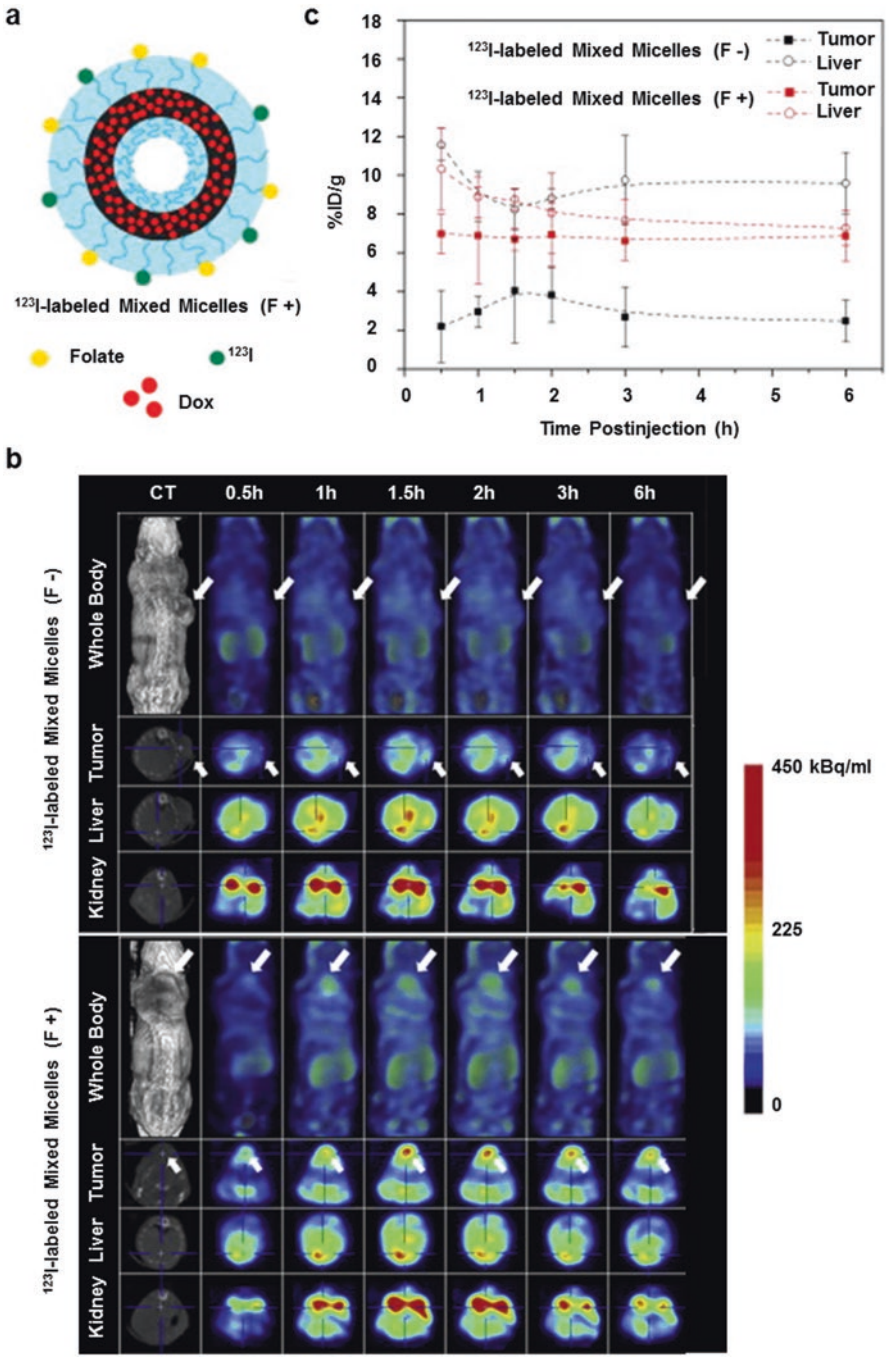

Fig. 15.4 SPECT/CT-based IGDD nanoplatform for evaluation of biodistribution and tumor targeting. Folate-targeted hollow NPs for concurrent SPECT/CT imaging and chemotherapy of human cervical cancer in SQ mice xenografts [41]. (**a**) Schematic representation of folate-targeted

PET-Based Assessment of Biodistribution and Visualization of Target Site Accumulation

PET-based nanotheranostic systems are also effective for visualizing the distribution and accumulation of DDSs. Xiao et al. designed a ^{64}Cu-labeled polymeric micelle for combined doxorubicin delivery and PET imaging. Nanocarriers were actively targeted to $\alpha_v\beta_3$ integrin-expressing tumor neovasculature by conjugation to cRGD peptides. PET and PET/CT studies were performed from 30 min to 24 h after systemic injection of either cRGD-conjugated or non-conjugated micelles in mice bearing heterotopic human glioblastomas. Images and quantitative region of interest (ROI) analysis demonstrated significant higher tumor uptake of actively targeted particles than those lacking cRGD peptides. Administration of a blocking dose of free cRGD also resulted in low tumor accumulation of $\alpha_v\beta_3$ integrin-targeted micelles. Additionally, carriers had low off-target uptake (e.g., muscles) according to ROI measurements [44]. The same authors demonstrated that ^{64}Cu-based PET/CT scans can be used to monitor and quantify the biodistribution of $\alpha_v\beta_3$ integrin-targeted gold nanorods as carriers for doxorubicin delivery. Even though targeted particles did not result in enhanced tumor uptake compared to nontargeted controls, when optimized, this system has the potential to combine the benefits of both chemo- and photothermal therapies [45]. In another study, Chen et al. employed ^{64}Cu-labeled silica mesoporous NPs to deliver doxorubicin to human breast cancer in subcutaneous mouse xenografts (Fig. 15.5). The platform included antibodies to target endoglin (type I integral membrane homodimer protein) and could be successfully used to monitor tumor accumulation by PET/CT [17].

15.2.3 Optical Imaging

15.2.3.1 Basic Principles of Optical Imaging

Optical imaging studies are based on photoactivation events (photophysical and photochemical) that follow the interaction between light (ultraviolet, visible, and infrared wavelengths from the electromagnetic spectrum) and biological matter [96]. Extra- and intracellular structures interfere with light propagation through living tissues, causing wavelength-dependent deflection (scattering) or absorption

Fig. 15.4 (continued) ^{123}I-labeled mixed micelles. (**b**) SPECT images showing the biodistribution and tumor accumulation of folate-targeted (F+) and folate-nontargeted (F−) ^{123}I-labeled NPs. (**c**) SPECT-based quantification of radioactivity intensities (volumes of intensity) in the liver and tumor site of mice treated with (F+) and (F−) NPs. Over the course of 6 h post intravenous treatment, SPECT-based results demonstrated that (F+) micelles led to a higher and more stable tumor radioactivity than (F−) NPs. In addition, high liver signal was related with particle sequestration into the RES. *Dox* doxorubicin, *^{123}I* iodine-123, (*F+*) folate-targeted, (*F−*) folate-nontargeted, *CT* computed tomography. (Adapted from Lu et al. [41]. Copyright © 2010, Elsevier Ltd. All rights reserved)

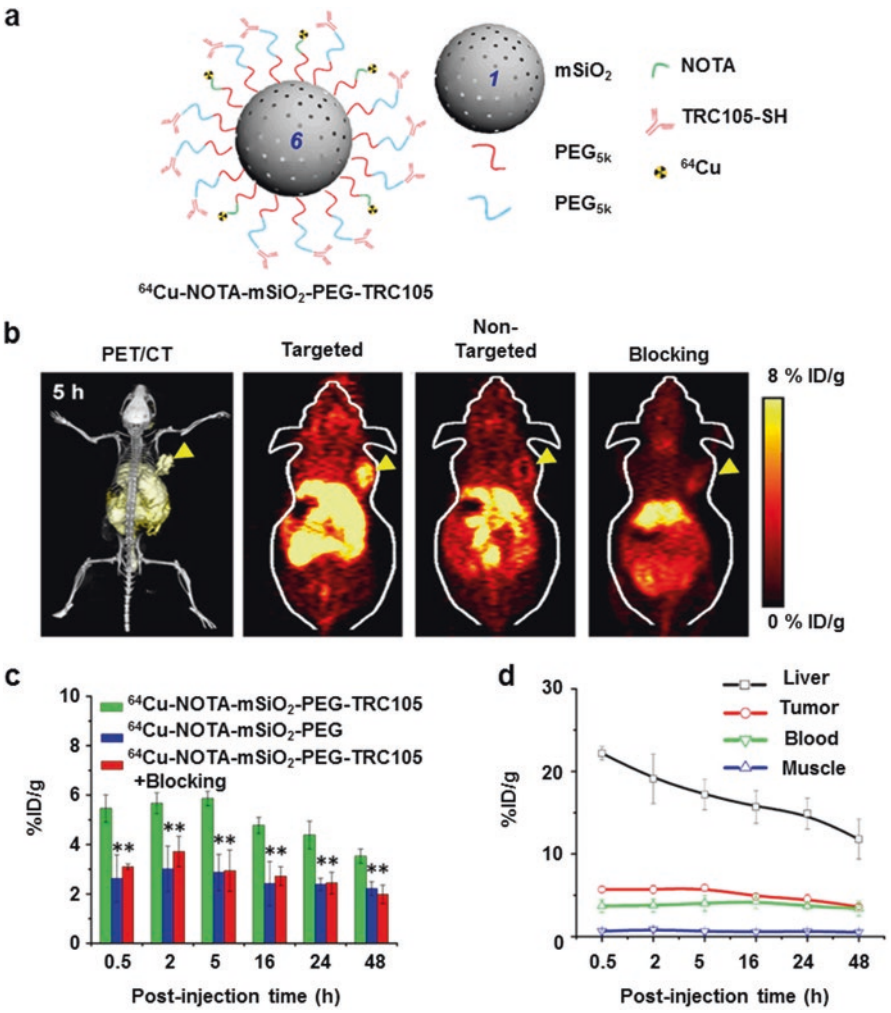

Fig. 15.5 PET/CT-based IGDD nanoplatform for evaluation of biodistribution and tumor targeting. CD105-targeted $mSIO_2$ NPs for concurrent PET/CT imaging and chemotherapy of human breast cancer in SQ mice xenografts [17]. (**a**) Schematic representation of ^{64}Cu-NOTA-$mSiO_2$-PEG-TRC105 NPs. (**b**) Coronal PET/CT images of tumor-bearing mice acquired 5 h after intravenous administration of CD105-targeted ^{64}Cu-labeled $mSiO_2$ NPs, CD105-nontargeted ^{64}Cu-labeled $mSiO_2$ NPs, or CD105-targeted ^{64}Cu-labeled $mSiO_2$ NPs with a blocking dose of TRC105. (**c**) Quantitative data from the tumor site obtained from ROI analysis. PET images and ROI data could evidence the significantly ($p < 0.05$) higher tumor-targeting capacity of TCR105-conjugated NPs when compared to their PEGylated only counterparts. (**d**) PET-based time-activity curves of ^{64}Cu-NOTA-$mSiO_2$-PEG-TRC105 biodistribution demonstrating low off-site concentration (e.g., muscles). *$mSIO_2$* mesoporous silica, *CD105* endoglin (type I integral membrane homodimer protein), *TCR105* human/murine chimeric IgG1 monoclonal antibody against both human and murine CD105, *PEG* polyethylene glycol, *NOTA* chelator, *^{64}Cu* Copper-64. (Adapted from Chen et al. [17]. Copyright © 2013, American Chemical Society)

of incident photons. While cellular membranes, intracellular organelles, and connective tissues are strong scattering centers [97–99], light absorption is carried out by endogenous and exogenous chromophores. Following absorption of light photons, chromophores decay from the acquired excited state by dissipating energy as heat and/or by re-emitting radiation. Lower-energy longer-wavelength photons originating from the radioactive decay of chromophores are responsible for the autofluorescence (endogenous chromophores) and fluorescence (exogenous chromophores) phenomena [96, 100]. Autofluorescence and scattering are inherited tissue properties that enable acquisition of valuable information [100, 101] but are also involved in decreasing optical imaging resolution [102, 103] and limiting penetration depth of the modality [98, 103]. The utilization of near-infrared fluorescence and bioluminescence imaging techniques can overcome such drawbacks and demonstrate the utility of optical imaging for nanotheranostics.

Near-Infrared Fluorescence (NIF) and Bioluminescence Imaging (BLI)

Near-infrared wavelengths (650–900 nm) are poorly absorbed by endogenous chromophores, which creates a favorable window in the optical spectra for imaging deeper structures [96]. NIF has also contributed to the development of tumor-specific photothermal and photodynamic therapies. While the former induces tumor damage by heat-mediated cell killing, the second relies on photosensitizers to produce reactive oxygen species (ROS) [24, 58, 104, 105].

The bioluminescence signal is the result of an enzyme-substrate reaction accompanied by photon emission. Unlike fluorescence, no external excitation source is required to trigger light production, and, thus, no background noise occurs [96]. Additionally, photon emission through bioluminescence does not involve energy dissipation in the format of heat [106].

Emerging Modalities of Optical-Based Imaging Techniques

Alternative imaging approaches offer additional options to assess the events that follow drug administration in living subjects. A more detailed description of emerging optical imaging techniques falls beyond the scope of this chapter but can be found in James and Gambhir (2012) [21]. Here we briefly describe two techniques that broaden the applicability of fluorescence-based imaging by adding functionalities and overcoming some of the limitations of the method.

Intravital microscopy (IVM) incorporates fluorescence light microscopy, fluorescent probes, and unique animal models to create highly spatially resolved images of tissues, cellular events, and biological processes directly from live subjects [107]. In the past two decades, the advent of multiphoton microscopy has also made possible in vivo visualization of subcellular events [108]. Multiphoton microscopy is guided by the nonlinear optics principle that a fluorophore can be

excited by simultaneous absorption of two low-energy photons at a coincident focal plane, which reduces autofluorescence artifacts and increases the penetration depth of the modality [107]. Collectively, nonlinear microscopy techniques now represent a growing area of interest within IVM and, unlike other molecular modalities (e.g., radionuclide-based imaging and conventional NIRF), provide high spatial resolution when interrogating cellular and subcellular events [108]. The microscopic resolution also enables IVM to quantify the biological processes under investigation, which is not possible with other conventional optical-based techniques. In the oncology field, IVM has been successfully employed to study biological barriers for drug delivery, evaluate the interaction of nanomedicines with cancer cells or their microenvironments, and assess how the therapy affects processes such as cellular division and death [107, 109]. In order to gain access to the site of interest, IVM requires the animals to be surgically prepared to create proper optical windows, which are key determinants of the final imaging quality. Since long-term windows can be implanted, longitudinal study designs that enable the researcher to obtain a series of IVM images over time are also possible [110]. Examples of commonly used optical windows are dorsal skinfold chambers for subcutaneous xenograft models [111] and cranial window for brain cancer [112].

Fluorescence molecular tomography (FMT) is another method that adds quantitative capabilities to an otherwise non-quantitative technique. Earlier in this section, we describe the utilization of fluorescence, especially NIRF, in the creation of planar images (conventional NIRF). In planar fluorescence imaging, quantification of the chromophore's distribution is not possible due the inability of the method to differentiate the origin of photons detected at the surface but emitted from chromophores localized at different depths within the tissue. This happens because the nonlinear propagation of light in biological tissues prevents an accurate discrimination between independent events when fluorescent molecules are simultaneously illuminated by a single field light source, as occurs in the planar technique [113]. The incorporation of tomographic approaches is capable of solving this issue. With the utilization of multiple light sources-detector pairs, mathematical models of light propagation in tissues, and three-dimensional reconstruction algorithms, fluorescent emission events can be independently recorded, providing localization and sizing information that can be used to quantify the pattern (concentration, depth) of the chromophore's distribution [114]. Similarly to other imaging modalities, FMT is also being incorporated into hybrid imaging systems to compensate some of the limitations of the stand-alone technique and provide researchers with images that tell a more complete story about structures and events under investigation. FMT-CT co-registration can incorporate valuable anatomical information to the FMT technique [115]. Using FMT, Nahrendorf et al. demonstrated that fluorescent-labeled protease sensors can detect the presence of protease in atherosclerotic lesions and that co-registration with CT angiography was able to reliably map the vascular territories with higher protease activity [116]. FMT and CT images were reconstructed using fiducial markers (objects placed on the imaging field) that could be visualized by both

methods as points of reference [116]. The anatomical content acquired with CT imaging can also be applied to feed FMT reconstruction algorithms and improve even further the sensitivity and accuracy of the method [117]. In subcutaneous xenograft models of breast cancer, Ale et al. observed that the utilization of anatomical information to parameterize FMT reconstruction algorithms in FMT-CT images results in better correspondence with the ex vivo method used as gold standard when compared to FMT alone [117].

15.2.3.2 Applications of Optical-Based IGDD Nanoplatforms for Cancer Therapy

NIR-Based Assessment of Biodistribution and Visualization of Target Site Accumulation

While the capacity of optical imaging to interrogate deeper body structures is limited, the high sensitivity and high temporal resolution of the method enables quality assessment of superficial tumors, frequently used in preclinical research (e.g., subcutaneous models). Mieszawska and collaborators utilized Cy7 labeling to track poly(lactic-co-glycolic acid) (PLGA)/lipid NPs for tumor accumulation in subcutaneous xenografts of colon cancer [49]. In this case, ex vivo NIR was employed to observe the biodistribution of the particles [49]. Similarly, NIR emission from Alexa Fluor® 750 enabled comparison of tumor accumulation and biodistribution between targeted (TL) and nontargeted liposomes (nTL) by Lowery et al. The authors used HVGGSSV peptides as targeting moiety against irradiated tumors [18].

Assessment of gene therapy has also been accomplished by incorporation of NIR probes into IGDD nanosystems. Kim et al. applied NIR fluorescence to evaluate the biodistribution and tumor targeting of a siRNA-containing DDS vehicle. Polyelectrolyte complex (PEC) micelles resultant from polyethylenimine (PEI) and PEGylated siVEGF (siRNA for interference of vascular endothelial growth factor expression) self-assembling were labeled with Cy5.5 dye and delivered intravenously to a human prostate carcinoma model. In vivo fluorescence demonstrated that the tumor-targeting capacity of the micelles was superior to naked siRNA and PEI-siRNA particles. This finding supported the results on treatment efficacy, which showed a significant difference in the tumor volumes of micelle-treated animals and the other groups [48].

In addition to enabling fluorescence imaging, some NIR fluorophores can be incorporated into nanotheranostic systems as absorbents for photothermal therapy and/or photosensitizers for photodynamic therapy. Taratula and collaborators, for instance, developed PEGylated polypropylenimine (PPI) dendrimers for photodynamic therapy using phthalocyanines (Pc) as both photosensitizers and imaging agents. In a proof-of-concept study, tumor accumulation of the carriers could be observed in mice bearing ovarian carcinoma via NIR fluorescence imaging of subcutaneous xenografts [24].

Visualize and/or Trigger Drug Release

Wu et al. developed a nanotheranostic platform that relies on the reducing cytosolic environment to concomitantly release an anticancer prodrug and an imaging agent inside the cell. Dicyanomethylene-4*H*-pyran (DCM), a NIR fluorophore, was linked to the drug camptothecin (CPT) by a disulfide bond (S–S), and the conjugate was then encapsulated into PEGylated polymeric-based NPs. In vitro, the DCM fluorescence and CPT toxicity from DCM-S-S-CPT conjugates increased significantly in the presence of the reducing agent glutathione. Therefore, DCM fluorescence could be used in vivo to noninvasively monitor the cytoplasmic drug release from the DCM-S-S-CPT conjugate via NIR fluorescence imaging. The authors demonstrated that tumor-bearing mice treated with DCM-S-S-CPT showed greater and longer tumor fluorescence retention than control animals that received DCM-CPT conjugates not containing disulfide bonds [51]. Zhao et al. applied thermo-sensitive liposomes to control the release of a doxorubicin payload through NIR-driven hyperthermia (Fig. 15.6). Indocyanine green (ICG) loaded into the vector acted as the phototherapeutic agent, converting NIR irradiation into heat, and also allowed for fluorescence-based visualization of the vector's retention into the tumor. Doxorubicin release upon liposome disruption could be traced through the fluorescence signals of both ICG and doxorubicin itself [50].

BLI-Based Monitoring of Tumor Targeting and Treatment Efficacy of Therapeutic Interventions

Bioluminescence imaging has the advantage of not requiring a light source for excitation and the ability of detecting deeper tumors than fluorescence imaging. Feng et al. developed a folic acid (FA)-conjugated theranostic platform for active targeting and combined chemo-/photothermal therapy of breast cancer. Gold nanorods (GNRs) and cisplatin were employed as the photoabsorbent and anticancer drug elements, respectively. The utilization of luciferase-positive cells to establish the orthotopic mice xenograft enabled treatment efficacy to be monitored using BLI. The authors observed complete eradication of the tumor bioluminescence signal after NIR irradiation in animals treated with either FA-targeted or nontargeted GNRs-cisplatin particles [53]. Wang et al. described liposome-mediated delivery of a triple fusion (TF) gene encoding for herpes simplex virus truncated thymidine kinase (HSVttk) and two optical probes (Renilla luciferase and red fluorescent protein [RFP]) (Fig. 15.7). In a gene therapy approach, exogenously expressed herpes simplex virus thymidine kinase (HSVtk) can phosphorylate a systemically administered prodrug, ganciclovir (GCV), ultimately forming GCV triphosphate, a cytotoxic agent for cancer cells. Since in vivo studies were performed in orthotopic hepatocellular carcinoma models established with firefly luciferase-positive (fLuc+) cells, both tumor targeting and treatment efficacy could be followed independently using Renilla and firefly bioluminescence, respectively. The authors found that, even though CD44-targeted TF/GCV treatment is able to delay tumor growth in comparison to nontargeted TF/GCV controls, the treatment did not lead to complete

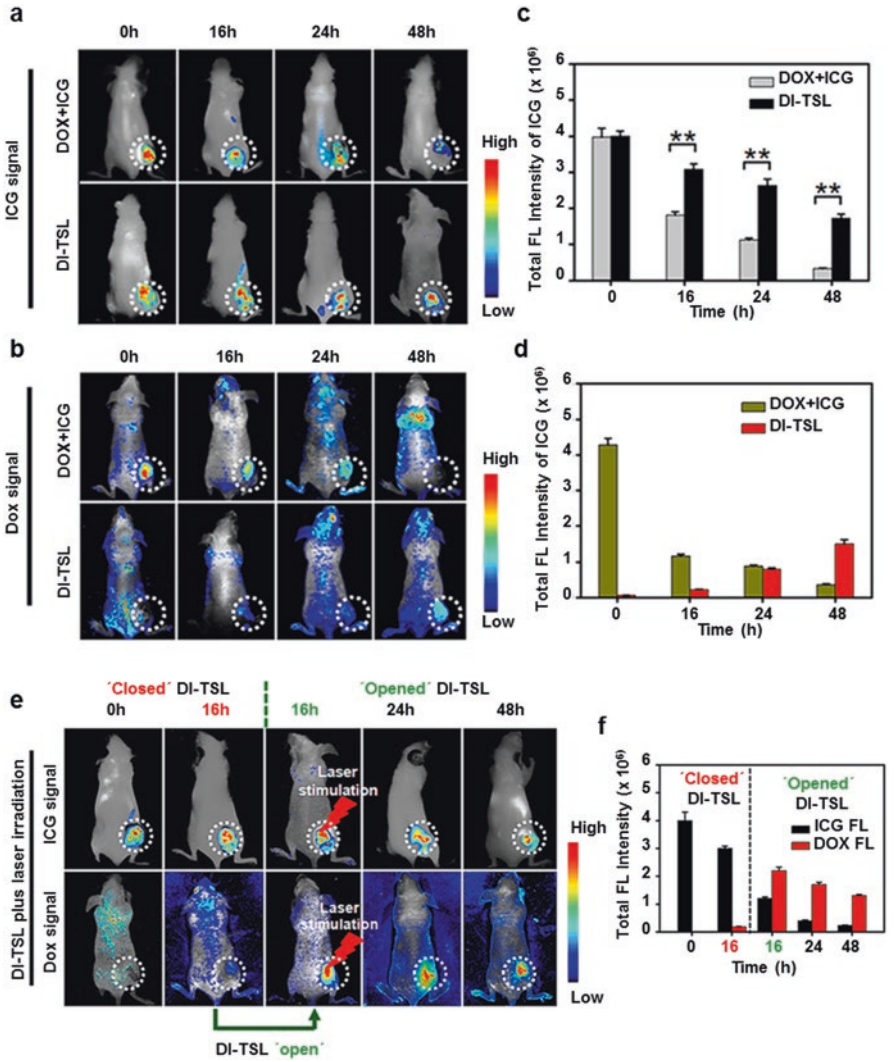

Fig. 15.6 NIRF-based IGDD nanoplatform for triggering/visualizing drug release and evaluating tumor retention. TSLs for concurrent fluorescence imaging, chemo- and photothermal therapy of human breast cancer in SQ mice xenografts [50]. (**a**) ICG and (**b**) Dox fluorescence images and respective signal intensities (**c**, **d**) of tumor-bearing mice following intratumoral injection of Dox-/ICG-loaded TSLs (DI-TSLs) or free Dox + ICG. Images and quantitative data evidenced a significant difference ($p < 0.01$) in the ICG signal between DI-TSLs and free Dox + ICG groups. The ICG signal from animals treated with DI-TSLs was better maintained over the course of 48 h, suggesting pronounced tumor retention of the liposomal formulation. Similarly, the Dox signal was quickly washed away in animals treated with free Dox + ICG, but not in the ones injected with DI-TSLs. (**e**) and (**f**) ICG and Dox signals showing ICG fluorescence decay and Dox signal enhancement following NIR irradiation. These results indicate increased ICG metabolism and Dox de-quenching upon release from disrupted liposomes. *TSLs* temperature-sensitive liposomes, *ICG* indocyanine green, *Dox* doxorubicin, *DI* DOX/ICG. (Adapted from Zhao et al. [50]. Copyright © 2015, Macmillan Publishers Limited)

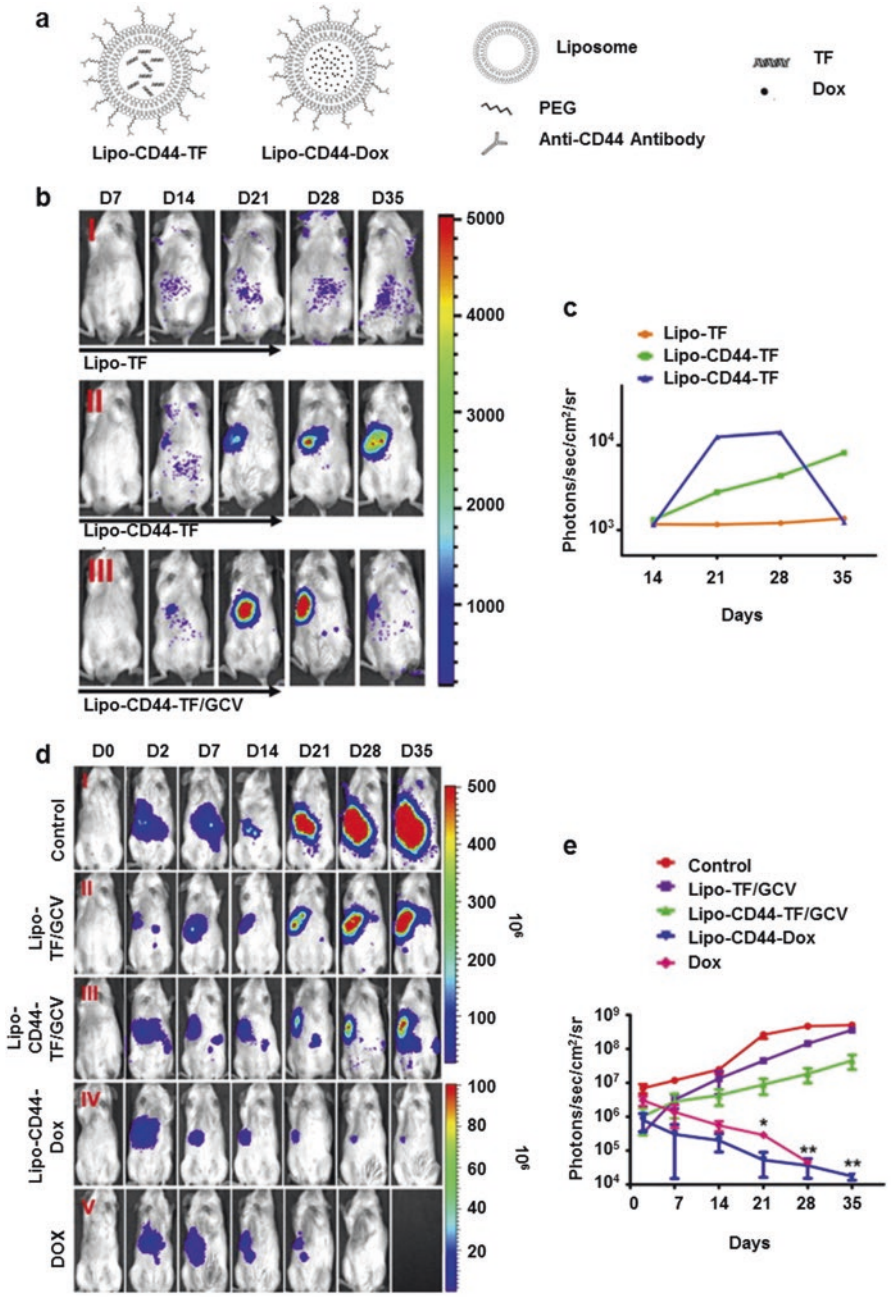

Fig. 15.7 BLI-based IGDD nanoplatform for evaluation of tumor targeting and therapeutic efficacy. CD44 antibody-targeted liposomal NPs for concurrent BLI imaging and chemo- or gene therapy of human hepatocellular carcinoma (HCC) orthotopic mice xenografts [15]. (**a**) Schematic representation of Lipo-CD44-TF and Lipo-CD44-Dox. (**b**) BL images and (**c**) Quantitative analysis of RLuc signal showing tumor targeting of HCC cells by CD44-targeted or nontargeted lipo

tumor remission. In order to test the efficacy of the CD44-targeted liposomal system itself, doxorubicin-loaded liposomes were used, in which case the nanoplatform led to significant treatment improvement when compared to free-dox [15].

Multimodal NIR and BLI Imaging to Monitor Tumor Targeting and Treatment Efficacy of Therapeutic Interventions

Both NIR and BLI can be combined to enable multimodal imaging. In a study by David and collaborators, an HSVtk-encoding plasmid and a NIR fluorophore (DiD) were encapsulated into lipid nanocapsules (LNCs), PEG-coated or not, and intravenously delivered to orthotopic melanoma xenografts. In vivo fluorescence imaging evidenced increased circulation time of PEGylated LNCs when compared to their non-PEGylated counterparts. Tumor regression upon GCV administration was successful and evaluated by quantitative bioluminescence analysis, which was made possible by applying luciferase-positive melanoma cells to establish the xenograft model [47].

15.2.4 Ultrasound (US)

15.2.4.1 Basic Principles of US

Medical US imaging exploits the transmission of high-frequency sounds (1–30 MHz) through soft tissues in order to generate echoes (reflected sound) that can be detected, processed, and displayed as images [119]. Sound waves are generated in pulses by a transducer equipped with piezoelectric crystals that vibrate in response to electrical signals. Resting periods between pulses allow the probe to also detect returning echoes. When ultrasound pressure waves reach the interface between tissues of different acoustic impedances, some waves are propagated forward, some lose energy, and some are reflected or scattered, producing echoes [120]. Acoustic impedance is a tissue property determined by the velocity of the wave propagation

Fig. 15.7 (continued) somes loaded with TF plasmids. Animals injected with CD44-Lipo-TF followed by GCV treatment are also shown. (**d**) Bioluminescence images and (**e**) Quantitative analysis of FLuc signal showing tumor development and treatment response. RLuc expression in TF transfected cells enabled BL imaging of tumor targeting and indirect assessment of treatment response (decreased TF transfection following TF/GCV treatment). The utilization of firefly luciferase (FLuc) expressing HCC cells to establish orthotopic xenografts allowed direct evaluation of tumor growth inhibition or lesion regression through FLuc-based BLI. *CD44* CD44 protein, class I transmembrane glycoproteins [118], *Lipo* liposome, *PEG* polyethylene glycol, *TF* triple fusion gene (HSVttk, RLuc, and RFP), *HSVttk* herpes simplex virus truncated thymidine kinase, *RLuc* Renilla luciferase, *RFP* red fluorescent protein, *Dox* doxorubicin, *GCV* ganciclovir. (Adapted from Wang et al. [15]. Copyright © 2012, Elsevier Ltd. All rights reserved)

and tissue density. Since the velocity of wave propagation throughout most tissues can be considered similar, variations in acoustic impedance are mainly a result of differences in tissue densities [121]. The amount of waves reflected, and therefore the brightness of signal created, is proportional to the acoustic impedance gradient in the interface of two different tissues: the higher the gradient, the more waves are reflected, the brighter the signal [120]. Extremely high gradients (e.g., bone or air/soft tissue interfaces), however, can impair ultrasound imaging acquisition. In these cases, the waves are mostly reflected, decreasing the sound transmission to deeper structures and resulting in important artifacts [121].

In ultrasonography, penetration depth and spatial resolution are highly dependent on the frequency of the wave propagation. Low-frequency waves offer poorer resolutions but can penetrate deeper into the tissues, being better suited for imaging internal structures. On the other hand, waves of high frequency have a superficial penetration depth but can generate images with relatively higher resolutions [122].

Microbubbles and NP-Based US Contrast Agents

Microbubbles are coated gas-filled structures [123] that serve as blood pool contrast agents for US imaging [124]. While coating substances (e.g., polymers, lipids, or proteins) are necessary for structural stability, the acoustic impedance mismatch at gas/blood interfaces creates tecidual contrast [124]. Since these gaseous structures expand and contract in response to low-intensity acoustic pulses, the use of special techniques enables nonlinear echoes emitted by oscillating microbubbles to also be exploited as contrast signal [125]. In addition to their application in US imaging, microbubbles can behave as cavitation nuclei under high ultrasound intensities, a phenomenon that has been investigated for US-triggered drug release [126].

Liquid substances such as fluorocarbon can function as microbubbles' precursors and have been employed in the synthesis of NP-based US contrast agents [62, 84, 127]. Under thermal activation, these NPs are capable of undergoing a liquid-to-gas phase change into their cores, and the result is a temperature-dependent microbubble formation, which not only enables stable NPs structures to be injected and circulate previous to activation but also creates the possibility of on-demand drug release [128]. The incorporation of stable microbubbles' precursors into the US technology has been particularly enlightening for researchers pursuing effective drug delivery to the brain. NPs containing a fluorocarbon core can be designed to readily cross the blood-brain barrier (BBB) after systemic administration and to release their therapeutic cargo into a small area (limited by the US focus) upon sonication with transcranial focused ultrasound (FUS) [127]. While FUS has been widely exploited to deliver chemotherapy to brain cancer [62, 129], very recently Airan et al. demonstrated how this same technology can be used in neuromodulation [127]. In this study, FUS triggered the release of propofol (small-molecule anesthetic) from perfluoropentane nanoemulsions to the brain, stopping drug-induced seizures in rat models. Perfluoropentane was chosen as the perfluorocarbon core due to its relatively high boiling temperature and consequent lower

susceptibility for spontaneous gas formation [127]. The ability of microbubbles to disrupt the BBB when exposed to high-intensity focused ultrasound (HIFU) is also inspiring the development of new DDSs for brain diseases [130, 131]. Mesiwala et al. demonstrated that HIFU can cause reversible, nondestructive BBB disruption by opening tight junctions of capillary endothelial cells [132]. The temporary permeability created with HIFU enables systemically injected nanomedicines to cross the BBB and deliver their therapeutic payloads to the tumor site [130, 133]. Besides microbubbles' precursors, alternative NP-based US contrast agents, such as bubble liposomes [134–137] and solid NPs [138–140], have also been reported.

15.2.4.2 Applications of US-Based IGDD Nanoplatforms for Cancer Therapy

Visualization and Triggered Drug Release

Wang et al. developed an ultrasound-sensitive and stimuli-responsive drug delivery system that enabled US imaging intensification and controlled drug release. The nanoplatform was based on mesoporous silica nanocapsules ($mSiO_2$ NCs) containing ultrasound-sensitive perfluorohexane (PFH) encapsulated in a redox-responsive copolymer. Hyaluronic acid (HA) ligands were incorporated as the tumor-targeting elements to direct the carrier to CD44-overexpressing tissues. Tumor accumulation of the carriers in animal models could be evaluated in vivo by US imaging. The echogenicity in the tumor site was enhanced following exposure to high-intensity focused ultrasound (HIFU), validating the PFH responsiveness to US stimuli. In addition, US images demonstrated that HIFU had a synergistic effect with the reducing tumor environment to trigger the release of the drug payloads [55].

US-Based Multimodal Nanoplatforms for IGDD

US has generally been applied in multimodal nanotheranostic approaches to concurrently trigger and visualize drug release. In this section we describe IGDD nanoplatforms that incorporate US technology and other molecular imaging modalities.

US and MRI

IONPs and microbubbles can be incorporated in multifunctional nanoplatforms not only as MRI and US contrast agents but also as mediators for magnetic-targeting and on-demand drug release. Fan and collaborators studied the response of brain tumor-bearing orthotopic rat models to systemic treatment with doxorubicin-loaded SPIO-conjugated microbubbles. US and MR images were used to

monitor in vivo lifetime and contrast-enhancement capability of DOX-SPIO-MBs. A craniotomy performed beforehand allowed US transmission into the brain. Under external focused ultrasound (FUS) sonication, SPIO-conjugated microbubbles were able to locally disrupt the BBB and trigger doxorubicin delivery. Additionally, an external magnetic field improved the deposition of IONPs and doxorubicin into the tumor parenchyma. MRI signal enhancement after FUS and magnetic exposure demonstrated the synergic effect of these two elements on IONP deposition [60]. Alternatively, Li et al. showed that diagnostic US can also benefit magnetic targeting, as revealed by MRI and US imaging. In this study, IO-coated H_2O_2-loaded polymersomes provided not only magnetic contrast and targeting capacity but also US signaling and therapeutic abilities through the generation of echogenic (O_2) and therapeutic (ROS) elements initiated by exposure to US (Fenton reaction) [61]. Rapoport et al. utilized PFCE (perfluoro-15-crown-5-ether) as a contrast precursor in the development of paclitaxel nanoemulsions (NE) for pancreatic cancer theranostics. NE biodistribution in orthotopic models was assessed by ^{19}F MRI and US, using fluorine signal and FUS-triggered bubble formation from PFCE as respective contrast agents. Treatment efficacy was evaluated in subcutaneous models. In this case, tumors were established using red fluorescent protein (RFP) cells, which enabled IVM-mediated imaging of dead tissues (non-fluorescent) [62].

US and Optical Imaging

Min and collaborators engineered echogenic glycol chitosan nanoparticles (CNPs) loaded with doxorubicin for imaging and US-triggered drug release (Fig. 15.8). Perfluoropentane (PFP) was incorporated into the CPNs as a microbubble precursor to enable ultrasound (US) contrast and to mediate the drug release process. Following intravenous CNP administration, external acoustic irradiation was applied over the tumor. The authors hypothesized that the US energy causes the nanocarriers to burst along with their cargo of oscillating microbubbles, which can release the drug in a spatiotemporal-specific manner. Initially, effective tumor accumulation of Echo-CPNs was assessed by US imaging. Following US irradiation, NIRF of Flamma™-labeled Echo-CPNs were used to demonstrate the incremental effect of acoustic cavitation in tumor targeting by Echo-CNPs. The drug release

Fig. 15.8 (continued) NIRF imaging of Flamma™ emission enabled assessment of biodistribution and tumor accumulation of Echo-CNPs. The US-treated group showed a higher NIRF signal in the tumor than US non-treated animals, indicating that acoustic cavitation can improve the penetration of Echo-CNPs into the cancerous tissue. (**e**) IVM of subcutaneous tumors after intravenous injection of Nile red-loaded Flamma™-labeled Echo-CNPs accompanied or not by US irradiation. Effective US-mediated NP penetration and drug release could be observed through visualization of NIRF and RFP signals, respectively. *Echo-CNPs* echogenic glycol chitosan-based NPs, *PFP* perfluoropentane. (Adapted from Min et al. [54]. Copyright © 2015, Ivyspring International Publisher)

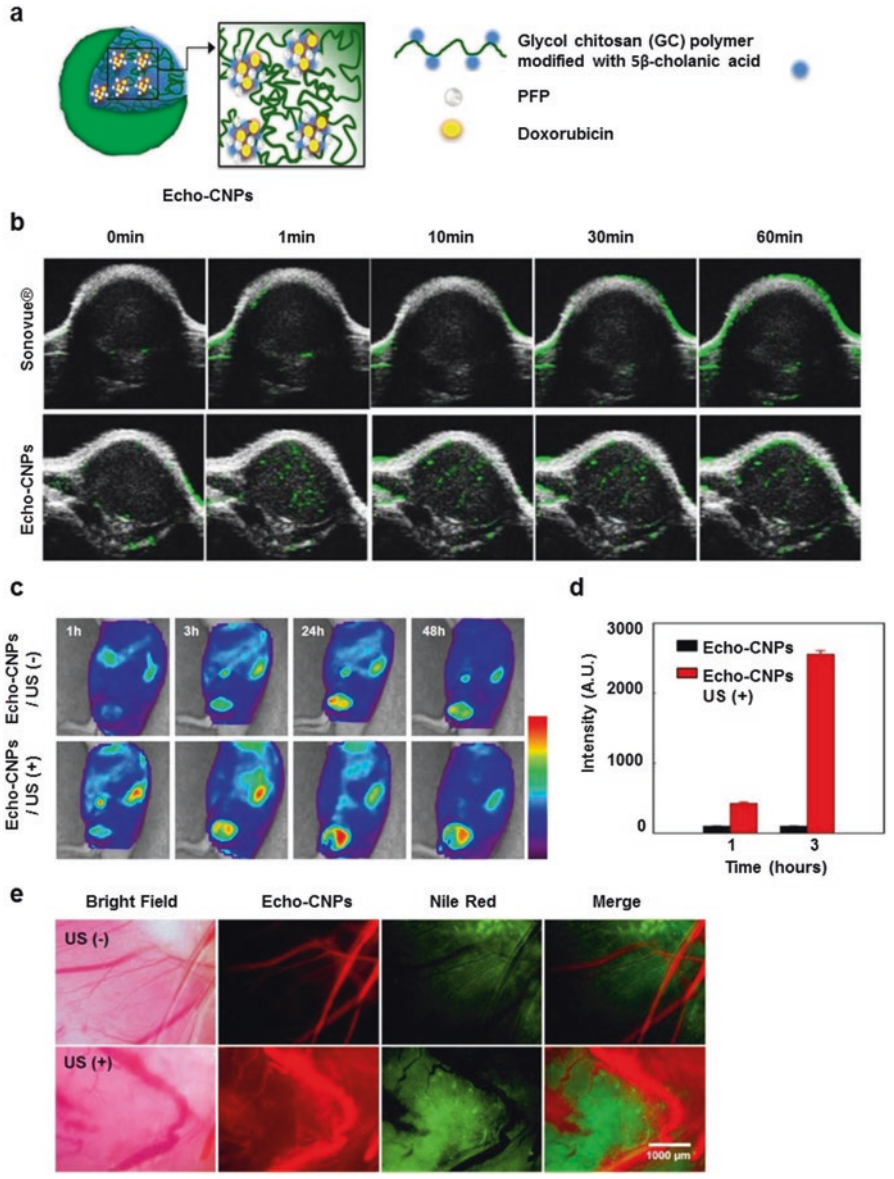

Fig. 15.8 Multimodality IGDD nanoplatform for monitoring tumor targeting and triggering/visualizing drug release. Echo-CNPs for concurrent US/optical imaging and US-triggered chemotherapy of human squamous cell carcinoma in SQ mice xenografts [54]. (**a**) Schematic representation of Echo-CNPs. (**b**) US images of the tumor site following intravenous injection of Echo-CNPs or SonoVue® (diffusible sulfur hexafluoride gas). Due to improved target site accumulation, Echo-CPNs offered a brighter US contrast than SonoVue® in the tumor tissue. (**c**) Biofluorescence images of tumor-bearing mice intravenously injected with Flamma™-labeled Echo-CNPs and irradiated (Echo-CNPs/US+) or not (Echo-CNPs/US−) with US (destruction mode). (**d**) NIRF signal intensity in the tumor site of irradiated and non-irradiated animals at two different time points.

capacity of Echo-CPNs was tested by loading Flamma™-labeled Echo-CPNs with Nile red dye. While only poor distribution was visualized in US non-treated groups, IVM images showed Nile red signal throughout the entire tumor in animals exposed to US treatment. This finding suggested effective US-mediated NP penetration and drug release of NIRF and RFP signals, respectively [54].

15.2.5 Multifunctional IGDD Nanosystems in Cancer Therapy

In order to combine the benefits offered by different imaging techniques, nanotheranostic systems can be designed to incorporate contrast from more than one modality. Herein, we describe representative studies of multimodal IGDD nanoplatforms, highlighting the complimentary role of each imaging method in fulfilling the system with features that can be exploited in varied IGDD applications.

NIR fluorescence is a suitable modality to demonstrate accumulation of nanocarriers in superficial tumors. In vivo biodistribution studies, however, involve the assessment of deeper structures within the body and can be better performed under imaging techniques with high or unlimited penetration depth. Vu-Quang et al. designed PEG-PLGA nanoparticles with NIR and ^{19}F MR imaging capacities utilizing perfluorooctyl bromide (PFOB) and indocyanine green (ICG) as the respective contrast agents. The particles were designed for doxorubicin delivery through passive targeting only or through the addition of an FA ligand for active targeting. Particles were intravenously injected into tumor-bearing mice and followed for tumor targeting and biodistribution. Both in vivo NIRF and ^{19}F MRI successfully demonstrated a clear difference in tumor accumulation between groups injected with folate-targeted and folate-nontargeted particles. The biodistribution of particles within the body could be evaluated in vivo by ^{19}F MRI and ex vivo by NIR fluorescence [19]. In a study by Zhou et al., in vivo NIR fluorescence and MR imaging were, respectively, applied to track the tumor-targeting capacity and the therapeutic effects of IGF1-targeted NIR 830 dye-labeled IONPs. Ex vivo NIR fluorescence also provided an insight in the distribution of the particles throughout the body [56].

Magnetic nanoplatforms can be used to trigger drug release upon exposure to a magnetic field. Hu and collaborators developed multifunctional quantum dot-labeled IONPs with optical and MR imaging capacities, double anticancer drug payload, and designed these to enable magnetic-triggered drug release and chemo-/thermal therapy. Particles were also conjugated to IVO24 peptide to promote active targeting of breast cancer neovasculature. Following systemic delivery to subcutaneous mice models of breast carcinoma, ex vivo biodistribution of the particles was evaluated by quantum dot-mediated fluorescence. In vivo fluorescent imaging showed increased doxorubicin signal at the tumor site following exposure to a magnetic field, suggesting magnetic-mediated triggered drug release. The authors observed that IVO24-mediated targeting combined with dual chemotherapy and exposure to a magnetic field led to tumor growth arrest for the first 15 days after treatment. In addition to triggered drug release, the hyperthermia caused by the

magnetic field might also have contributed to the observed antitumor response [57]. Li et al. applied a molecular imaging contrast agent as both the diagnostic and therapeutic element to build an IGDD nanoplatform. In this study, the combination of chlorin e6 (Ce6), a NIR dye, and iron oxide (IO) nanoclusters enabled dual imaging of the accumulation of particles into the tumor site. IO nanoclusters were also applied for magnetic-mediated tumor targeting, while Ce6 worked as a photosensitizer for photodynamic therapy [58].

Multimodal imaging can also be applied to independently monitor the real-time performance of a DDS nanoplatform itself. Paoli et al. engineered a temperature-sensitive liposome with a dual imaging capacity that enabled separate and independent in vivo evaluation of the lipid bilayer shell and the aqueous core. The lipid shell was labeled with ^{18}F and ^{64}Cu PET probes, while the core was loaded with Alexa Fluor® 750 in a quenchable concentration. PET imaging allowed real-time monitoring of liposome accumulation into the tumor site. NIR fluorescence offered dynamic insight in the heat-mediated cargo release due to the de-quenching of Alexa Fluor® upon liposome disruption [141]. De Smet and collaborators also used temperature-sensitive liposomes for dual imaging modality. Liposomes were labeled with ^{111}In and co-loaded with doxorubicin and a commercially available gadolinium formulation. MRI was used to guide HIFU-mediated hyperthermia and to plan sonication positioning. The biodistribution and tumor accumulation of the ^{111}In-labeled TSLs was monitored using SPECT/CT imaging. Interestingly, the authors found that the HIFU treatment caused the tumor SPECT signal to intensify, which likely happened due to increased blood flow, permeability and extravasation associated with hyperthermia [59].

15.3 Clinical Applications of IGDD in Cancer Therapy

Compared to cancer therapeutics overall, only a very small number of studies have evaluated IGDD nanotheranostic systems at the clinical level. Nanotheranostic systems are still in many ways a nascent technology, with challenges to manufacture and scale up as well as limited knowledge of how varied nanotechnology delivery components impact human physiological systems. Many of the nanomaterials employed as carriers are still under preclinical and clinical investigation to evaluate their potential toxicity, biocompatibility, and efficacy when applied for single purposes, such as therapy or imaging only. Results from trials involving nanotheranostic systems are reported in this section. In 2000, Koukourakis and colleagues conducted a phase I/II clinical trial to evaluate the use of 99mTc-DTPA radiolabeled stealth® liposomal doxorubicin (Caelyx®) in glioblastoma and metastatic brain tumor patients [142]. SPECT/CT revealed that systemic treatment with radiolabeled Caelyx® led to higher drug accumulation in the tumor site than the normal tissue, suggesting that the blood-brain barrier (BBB) was disrupted in these patients and demonstrating that stealth nanotheranostic systems can reach brain tumors [142]. The same authors also demonstrated preferential 99mTc-DTPA-Caelyx®

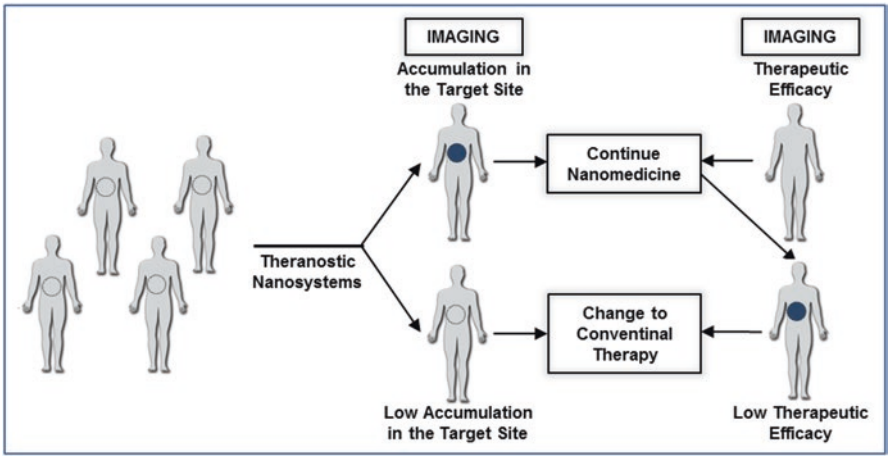

Fig. 15.9 Potential application of nanotheranostic platforms for patient selection in the field of precision medicine

accumulation into sarcoma tumors when compared to the surrounding healthy tissue [143]. Another phase I trial reported the liver targeting capacity of galactosamine-targeted polymeric doxorubicin (PK2). Following intravenous administration in liver cancer patients, the distribution of ^{123}I-labeled PK2 could be assessed with planar scintigraphy and SPECT/CT imaging. Radioactivity distribution confirmed higher liver uptake of PK2 than galactosamine-nontargeted formulations. However, images also revealed that the normal parenchyma, not the tumor, was the main source of ^{123}I radioactivity [144]. Future clinical studies are key to demonstrate the safety of IGDD nanotheranostic systems in cancer patients, the utility of imaging combined with therapy to guide nanomedicine-based patient dosing and targeting, and ultimately validation at enhancing care of patients. IGDD nanotheranostic systems may be a broadly applicable solution to challenges of patient selection, stratified care, and precision medicine (Fig. 15.9).

15.4 Surgical Interventions for Local Drug Delivery under Image Guidance

Imaging techniques can be used to guide local administration of anticancer drugs through minimally invasive procedures. In addition to tumor targeting, this strategy can afford for reduced systemic exposure [145, 146] and has been exploited for the treatment of various cancers including hepatocellular carcinoma (HCC) and brain cancer.

HCC tumors are preferentially supplied by the hepatic artery, unlike healthy hepatic tissues, which are primarily fed by the portal vein [147, 148]. Transarterial

chemoembolization (TACE) takes advantage of this difference to promote selective delivery of chemotherapeutic and embolization agents to tumor-feeding blood vessels (hepatic artery and its branches) [149]. In the clinical setting, anticancer drugs are mixed with contrast media to enable regioselective chemotherapy under image guidance [150]. Preclinical studies have evaluated the TACE technique using theranostic systems. Mouli et al. evaluated the tumor-targeting capacity of locally administered doxorubicin-loaded IONPs when followed or not by embolization therapy. VX2 rabbit models of liver cancer were treated via hepatic artery catheterization or systemic injection (control animals). The authors observed that local delivery could increase tumor uptake of Dox-IONPs, especially when accompanied by administration of an embolization agent [38].

In the case of brain cancer, local drug delivery offers an alternative to bypass the mechanical limitations imposed by the BBB to systemic therapy. Convection-enhanced delivery (CED) enables the direct injection of therapeutic agents into a targeted brain lesion under image guidance [151]. While CED-based therapies are still in clinical trials [152], preclinical studies have already shown the potential of this technique to incorporate theranostic agents. Hadjipanayis et al. engineered IONPs conjugated to anti-EGFRvIII (epidermal growth factor receptor variant III) antibodies to actively target glioma tumors via CED. Effective particle dispersion and tumor penetration could be observed over the course of 7 days through T2-weighted MRI [16]. Poly(beta-amino ester) gene delivery NPs, as a nanotheranostic system, were also shown to successfully non-virally transfect glioma cells in an orthotopic rat model following CED. In this study, fluorescently labeled plasmids, fluorescent protein-encoding plasmids, and HSVtk-encoding plasmids were each delivered, and extended survival was shown with HSVtk/GCV treatment, demonstrating how such a gene therapy approach can enable both imaging and therapy [52]. In another study, Zhou and collaborators used in vivo PET imaging to assess the penetration capacity of [^{18}F]NPB4-labeled polymeric NPs into glioblastoma tumors when injected through CED [46]. Overall, these studies have demonstrated how surgical interventions can be leveraged with IGDD to increase the specificity and potency of nanotheranostics.

15.5 Image-Guided Surgery

Image-guided surgery (IGS), or computer-assisted surgery, uses patients' cross-sectional preoperative scans (CT or MRI) or a combination of pre- and intraoperative images to generate precise three-dimensional (3D) coordinates that guide the surgical procedure [153]. The combined imaging information allows for improvement on surgical accuracy through the creation of unprecedented visual windows to the target area and neighboring structures [154, 155]. Due to the added precision in accessing lesions of the central nervous system [156], IGS has become indispensable in the neurosurgery field [157].

Image-guided neurosurgery was initially developed using external frames fixed to the skull to direct the introduction of instruments to the target site. Preoperative images are used to assign 3D coordinates to the target region and create stereotaxic coordinates within the framespace [158]. This technique strongly relies on a stable fixation of the frame to the patient's head throughout the preoperative imaging and for the duration of the surgery, generating great discomfort and limiting accessibility to deeper brain structures [159]. On the light of these major drawbacks, frame-based surgery gave room to a frameless technique to emerge. First introduced in the late 1980s [160], neuronavigation systems are now designed to not only translate patients' imaging information to stereotaxic coordinates but also to input real-time positioning coordinates from the surgical field to the image space [159, 161]. Rather than fixed frames, neuronavigation employs fiducials markers attached to the patient's head to derive stereotaxic coordinates utilized for reconstruction of 3D anatomic models from preoperative images. In the operating room, these models are then fed with real-time information regarding the positioning of anatomic landmarks and surgical instruments. This is possible with the utilization of a tracking system composed of matching pairs of probes, attached to patient and instrument, and detectors (electromagnetic or, more commonly, optical) [161, 162].

Another possibility is the addition of intraoperative imaging to the system, enabling the navigation dataset to be updated with real-time information [153]. Intraoperative images can be exploited for different purposes and contribute to the improvement of oncological surgery in varied manners. As occurs for nonsurgical techniques, the choice of imaging modality is influenced by its set of strengths and limitations. The implementation of real-time information from CT, MRI, or US scans, for example, provides anatomical insights and can prevent surgical inaccuracies associated with the brain shift that follows skull opening [163] and removal of a brain mass [153, 164]. Optical imaging, on the other hand, allows the detection of cellular processes that are not visible otherwise. Fluorescent probes can afford for better discrimination between diseased and healthy tissues at the tumor margins and, when incorporated into a neuronavigation system, can maximize the extent of tumor removal [165] and, consequently, increase recurrence-free periods and survival of glioblastoma patients [166, 167]. Overall, intraoperative imaging was associated with improved extension of tumor resection in all clinical trials included in a Cochrane Library's systematic literature review from 2014 [168]. Navigation surgery has been applied in biopsies and brain tumor resections [162] and, currently, has been exploited in other medical fields, such as orthopedics [154] and otorhinolaryngology (neurotology) [159].

Complete tumor eradication is not an exclusive goal of neuro-oncology. The presence of diseased cells in the margins of surgical specimens is also associated with local recurrence and poor prognosis in other cancers such as head and neck [169], breast [170], colorectal [171], bladder [172], prostate [173], and lung [174] cancers. The incorporation of photosensitizers (PSs) into fluorescent-guided surgery opens the possibility to not only extend removal of positive tumor margins during the surgical procedure but also to improve even further the elimination of residual cancer cells with post-surgical PDT [175]. 5-Aminolevulinic acid (ALA),

for example, a metabolic precursor of the photosensitizer protoporphyrin IX (PpIX), can be administered as prodrug and induce PpIX accumulation specifically in tumor cells [176, 177]. Employing ALA and Photofrin as PSs, Eljamel et al. demonstrated that adjuvant PDT, i.e., performed after surgical resection, can significantly increase both survival (52.8 versus 24.6 weeks) and time to progression (8.6 versus 4.8 months) when compared to surgery plus conventional radiotherapy for glioblastoma patients [178]. Rigual et al. studied intraoperative PDT for treatment of squamous cell carcinoma of the head and neck. In this phase 1 trial, the authors evaluated 15 patients and found that PDT following IV administration of the chlorin-based photosensitizer 2-(1-hexyloxyethyl)-2-devinylpyropheophorbide-a (HPPH) seems to be a safe option for the treatment of head and neck cancer patients [179]. The choice of HPPH was justified by reduced cutaneous photosensitivity side effects at effective antitumor doses, as shown by previous reports [180]. In another phase 1 clinical trial, Bader et al. demonstrated that transurethral PDT using intravesical hexaminolevulinate (HAL), a ALA derivate, as the photosensitizer is a feasible and safe option for the treatment of non-muscle-invasive intermediate and high-risk bladder cancer following transurethral bladder resection [181]. Previous studies showed that HAL results in higher fluorescence intensity at a more homogeneous distribution in cancerous urothelial cells when compared to ALA [182].

15.6 Image-Guided Radiation Therapy

Image-guided radiation therapy (IGRT) is defined as the utilization of imaging techniques for therapeutic planning and/or real-time guidance of radiation delivery [183, 184]. Among other advantages, IGRT enables the irradiation of a smaller target with higher doses when compared to conventionally external beam radiation, limiting the exposure of normal tissues to harmful ionizing radiation [185] and increasing the balance between cure and toxicity [186]. The delivery method of IGRT can involve surgical interventions, a technique called radiosurgery [183].

Despite the benefits offered by pre- and intra-treatment imaging, IGRT still results in some degree of irradiation to healthy cells. NPs can be utilized to deliver materials with high ionizing capacity (radiosensitizers) selectively to the tumor site, increasing therapeutic target and cell killing effects of radiotherapy [187]. High atomic number materials, such as gadolinium ($Z = 79$) [188], and gold ($Z = 64$) [189, 190] strongly absorb ionizing radiation and, when incorporated into NPs, work both as radiosensitizers and imaging probes [191]. Joh et al. designed long-circulating PEGylated gold (Au) NPs for imaging and radiotherapy of human sarcoma tumors subcutaneously engrafted in mice models [190]. While CT scans from the tumor site confirmed PEG-AuNPs accumulation following systemic injection, CT hyperintensity in serial images of the animal's heart chambers was used to detect and quantify circulating particles. Traces of PEG-AuNPs could be detected even 48 h after their administration. Regarding therapeutic efficacy, the combination of PEG-AuNPs and radiotherapy led to significant tumor regression and increased

survival when compared to all other groups (untreated control, PEG-AuNPs alone, and radiotherapy alone) [190]. Recently, a silica-based NP with dual radiotherapy and dual imaging capacities was synthesized by Detappe et al. [191]. For the NP synthesis, bismuth ($Z = 83$) and gadolinium atoms were entrapped into the particle by grafted DOTA ligands. Silica-based bismuth-gadolinium NPs (SiBiGdNPs) were injected systemically in subcutaneous xenograft models of non-small-cell lung cancer, and the signal was then registered at the tumor site. At the peak of signal (30 min after administration), MRI and CT imaging were performed and the merged images used to delineate the cancer region for irradiation, similarly to what is done in the clinical setting for radiotherapy planning. Following radiotherapy, animals pre-injected with SiBiGdNPs had significantly longer survival than the untreated, radiotherapy only, and SiBiGdNPs only groups. Damage to the cancer cells, measured by breaks in double-strand DNA, was also significantly higher in the group that underwent radiotherapy post SiBiGdNPs than the radiotherapy alone group [191]. The radiosensitization capacity of high atomic number materials is attributed to the emission of secondary electrons (Auger electrons) following absorption of ionizing radiation. These electrons are distributed into the immediate surroundings, depositing energy and increasing radiotherapy efficacy [192]. Alternatively, radiotherapy can also cause damage to biological tissues through the creation of ROS from ionized water molecules [193]. Klein et al. exploited this pathway using IONPs [193], which can generate hydroxyl radicals either through the catalysis of the Haber-Weiss [194] or Fenton reactions [195]. The authors demonstrated that internalized IONPs increased ROS formation and enhanced the radiotherapy effects in cultures of breast cancer cells (MCF-7) [193].

15.7 Pharmacological Modeling in Nanomedicine

Pharmacological modeling offers a set of tools to predict the pharmacokinetic and/or pharmacodynamic behaviors of a drug beyond an existing data. With appropriate mathematical models, results from in vitro studies can be used to simulate the drug's pharmacological behavior in animal models, clinical estimates can be derived from preclinical (in vitro and in vivo) data, and even extrapolation between different patient populations is possible. Physiologically based pharmacokinetics (PBPK) models, for example, can utilize multiple levels of experimental data on drug-specific parameters and combine it with system-specific (e.g., anatomical and physiological characteristics) information to generate pharmacokinetic predictions [196]. Similarly, pharmacokinetic/pharmacodynamic (PK-PD) models assist researchers to understand and predict the time course of effects that follow the administration of a drug in a certain dose regimen [197].

PBPK and PK-PD models have been successfully applied for optimizing the development of traditional drugs and, although somewhat new in the field of nanomedicine, have potential to guide the design of nanoformulations, to improve their pharmacological profiles. In 2008, Lin et al. published a study describing the

utilization of a PBPK model to simulate the distribution of a nanomedicine [198]. Based on in vivo data collected upon administration of Quantum Dot 705 (QD705) to mice, the authors designed a PBPK model that was able to accurately predict the pharmacokinetic profile of these NPs. When compared to measurements obtained from animal tissues, the concentration of QD705 simulated by the model was consistently predicted for all the compartments included (blood, liver, spleen, kidney, and body). These results suggest that this model could be extrapolated to assess the pharmacokinetics of QD705 in humans [198]. Similarly, PK-PD models can help to explain the time-toxicity relationship of different drug formulations (e.g., free or liposomal doxorubicin). Using two-compartment PK-PD transit compartment model for cytotoxicity [199], Soininen et al. were capable of establishing a relationship between time-dependent cell killing and nuclear concentration of doxorubicin. The predicted results reliably estimated the toxicity observed experimentally in rat glioma cells to all doxorubicin formulations tested, indicating that the time course of the toxicity effects is independent of the uptake efficacy of each formulation [200]. For another cancer cell type (melanoma cells), however, Eliaz et al. demonstrated that intracellular doxorubicin concentrations represented a key input parameter to generate the best fit between PK-PD model and observed experimental results. Based on these findings, the authors suggest that the differentiated uptake (higher) of CD44-targeted liposomal doxorubicin might explain its higher toxicity level [201].

15.8 Future Prospects

The potential of IGDD as a preclinical and clinical tool is clear. However, the future of the field relies on multiple factors including the optimization of nanotheranostics platforms, and the engagement of partners on the development and large-scale manufacturing of nanomedicines and evolving clinical paradigms. Even though progress is being achieved, the concept of an IGDD nanotheranostic "magic bullet" is still not a reality. One challenge is that tumor targeting demonstrated in preclinical models, including passive targeting due to the EPR effect, does not necessarily always translate to the clinical setting. Improved, more challenging preclinical models are needed that more closely match the transport and efficacy barriers that are being observed in the clinic. Furthermore, the challenges of the human immune system, leading to rapid clearance of repeat doses of nanoformulations through both innate and adaptive responses, needs to be better accounted for in preclinical models and evaded. In addition, as cancer is a heterogeneous grouping of many different diseases, precision medicine and the precise matching of treatment to a patient's specific disease profile is an important area for future growth. With further contributions from basic scientists, engineers, medical professionals, industrialists, and regulators, IGDD, by improving specificity, efficacy, and safety, has a promising future for the care of oncological patients. In the near future, with the conclusion of more clinical trials investigating NPs as effective and

reliable vectors to deliver imaging or therapeutic agents singly, we anticipate that theranostic nanoplatforms combining both capabilities will move through the clinic and enter the marketplace soon after. In addition, the successful integration of imaging, data processing, 3D reconstruction, and real-time navigation witnessed in the computer-surgery field represents a strong candidate to become part of the IGDD. Such a system could be used to monitor the disease, trigger a drug delivery treatment with spatial and temporal control, and then monitor the response to the treatment, using this feedback to guide any subsequent rounds of treatment.

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Chapter 16
Nanotheranostics-Based Imaging for Cancer Treatment Monitoring

Tianxin Miao, Rachael A. Floreani, Gang Liu, and Xiaoyuan Chen

16.1 Introduction

Approximately 1.7 million new cancer cases were projected in the year 2017, recognized as the one of the primary health threats in the United States [1, 2]. Thus, cancer research is focused on developing effective strategies for cancer diagnosis, treatment, and monitoring treatment response [3]. Despite the progress in the past 20 years, early cancer diagnosis and effective treatment remain critical challenges [2, 4, 5]. Further, early detection of the therapeutic response in a treatment cycle would be beneficial for patients and insightful for physicians to gather the

T. Miao
Bioengineering Program, College of Engineering and Mathematical Sciences, College of Medicine, University of Vermont, Burlington, VT, USA
e-mail: Tianxin.Miao@uvm.edu

R. A. Floreani
Bioengineering Program, College of Engineering and Mathematical Sciences, College of Medicine, University of Vermont, Burlington, VT, USA

Mechanical Engineering Program, College of Engineering and Mathematical Sciences, University of Vermont, Burlington, VT, USA
e-mail: floreani@uvm.edu

G. Liu (✉)
State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics & Center for Molecular Imaging and Translational Medicine, School of Public Health, Xiamen University, Xiamen, China
e-mail: gangliu.cmitm@xmu.edu.cn

X. Chen (✉)
Laboratory of Molecular Imaging and Nanomedicine, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD, USA
e-mail: shawn.chen@nih.gov

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information required to determine if an alternative therapeutic method is needed in cases where the current approach is not working or no longer works.

The field of biomaterials has enabled researchers to develop novel strategies to assist disease diagnosis and monitoring [6, 7], anticancer drug delivery [8, 9], and tissue regeneration [10–12]. The use of nanotechnology in medicine, also referred to as nanomedicine, has been widely applied in the field of biomedicine, especially in cancer therapy, which is an appealing and versatile strategy for selective drug delivery and diagnostics [13]. Nanotechnology refers to the fabrication of materials with dimensions between 1 nm and 100 nm [3, 14], capable of achieving high concentrations in targeted tissue locations [15]. With the development of nanotechnology, nanomedicine holds potential to integrate both diagnostic and therapeutic functionalities into one single material/application, playing a critical role in cancer therapy [14].

Recently, the idea of personal treatment monitoring has gained attention, providing a means to evaluate treatment effectiveness while protecting patients from adverse drug effects [16]. The goal of detecting early signs of response is to predict the possible outcome of treatment in general as well as to identify potential predictive markers of response. The collection of such data will help physicians identify patient groups that are most appropriate for a specific therapy. Markers of response could include, for example, apoptosis or changes in metabolism (glycolysis or amino acid or lipid metabolism) or receptor expression associated with tumor cell death or inhibition of proliferation or inducing cell death, whereas predictors of response may include the expression of, say, hormone receptors, which predict response to anti-hormonal treatment, or microRNAs, or the presence of tumor hypoxia, which can affect the effectiveness of certain treatments like radiation therapy or photodynamic therapy.

The most common technology utilized for treatment monitoring is molecular imaging, defined as "in vivo imaging and characterization of biologic processes at the cellular/molecular level in a noninvasive way," which provides comprehension into both cancer diagnosis and therapeutic response monitoring [2, 17]. The term "theranostics" is now emerging due to the exceptional ability of nanoplatforms to load both imaging and therapeutic cargos, resulting in multifunctional combined nanosystems able to simultaneously detect early signs of cancer, deliver drug, and monitor therapeutic response. Indeed, nanotheranostics (i.e., theranostic nanomedicines) that incorporate imaging and therapeutic functions into a single system (i.e., nanoparticles) provide the ability to monitor drug release and circulation in real time and to predict and certify the effectiveness of cancer therapies [13]. The field of nanotheranostics is growing due to increasing medical needs, including those in the fields of cancer therapy and personalized disease treatments [18, 19]. Furthermore, theranostic nanoparticles have been developed to integrate disease diagnosis, targeted delivery, controlled drug release, and drug monitoring into a unifying platform. In combination with advanced techniques in different imaging instruments, nanoparticles with multimodal imaging capabilities have the potential to offer higher-quality images at multiple length scales and different clinical stages and offer more precise disease diagnosis and monitoring. The advantages of

nanotechnology are that particles can be designed and fabricated with controllable size, shape, and composition, as well as physical properties [19]. The high ratio of surface area to volume for nanomaterials contrary to traditional macroscopic materials also makes it a desirable choice for nanotheranostics [20]. In addition, they can be easily modified through different bioconjugation techniques to enhance the functionalities. An important class of nanoparticles is made of inorganic materials, such as metal [21], metal oxide [22], semiconductors [23], or even rare earth minerals or silica [24]. Organic nanoparticles have also been prepared using various biodegradable polymers such as polylactide-polyglycolide [25] and polycaprolactones [26], as well as proteinaceous materials, such as albumin [27] and collagen [28].

Convincing arguments have been made in the field of cancer therapy monitoring in favor of developing nanoprobe-based, imaging-guided therapy [29–31]. First, revealing the early response of tumors in regard to treatment may provide insights into selecting the most appropriate therapeutic method, offering benefits for patients and for healthcare systems [31]. In a large randomized controlled trial performed by the National Lung Screen Study (NLST), researchers reported that chest radiographs were less efficient in identifying lung cancer among older and former heavy smokers compared to low-dose computed tomography (CT) [32]. This is because in CT, X-ray has a circular movement around the body, allowing a variety of views of the same tissue compared to traditional chest radiographs [33]. This initial large clinical study showed higher efficacy in screening for lung cancer with CT compared to prior trials using sputum cytology and chest radiographs. Now that CT screening has been shown to be effective, more use of this type of screening shall become the next step. Second, imaging techniques allow doctors to monitor the biochemical and cell biology aspects of tumors, providing early indications of whether how and when tumors respond to treatments [31]. Third, with properly labeled cell metabolites or receptor ligands, radionuclide imaging techniques, such as positron-emission tomography (PET) and single-photon emission computed tomography (SPECT), can be applied to monitor tumor metabolism or receptor expression levels of target cancer cells [31]. Fourth, other imaging techniques such as magnetic resonance imaging (MRI) could not only provide high-resolution images of tissue morphology but also cancer cell receptor expression with the assistance of paramagnetic nanomaterials labeled with receptor-targeting ligands [31]. Different imaging techniques have their own advantages in various imaging scenarios. With the advantages of different imaging techniques, we are capable of obtaining images from tissue morphology to cellular metabolites, which have helped to better serve the purpose of treatment monitoring referred to as “nanotheranostics.”

This chapter will focus on the treatment monitoring aspects of nanotheranostics with detailed introduction on application of treatment monitoring in cancer therapy, including MRI [34], PET [35], SPECT [36], and optical imaging [37] since these techniques have been widely applied in the area of treatment monitoring with an extended detection limit for molecular imaging. There is also research on computed tomography (CT) nanoparticles with clinical application of identifying the metastatic lesions on lung cancer [38].

With the development of imaging techniques, clinicians and researchers are not satisfied with visualizing the tumor tissue alone, but are also looking to explore the molecular aspect of the tumor microenvironment. In addition, certain cellular kinase/enzymatic reaction and gene expression will also be invaluable information obtained from molecular imaging. For example, acquiring information on angiogenesis will provide early cancer detection prior to the formation of a solid tumor. The monitoring of metastasis for single circulating cancer cells will enable physicians to initiate a preventive care regimen as early as possible. As biomedical researchers, we are continuously interested in improving the detection and therapeutic monitoring of cancer disease. Therefore, there is a great need to study molecular imaging for therapeutic monitoring in cancer with respect to pathological conditions such as angiogenesis and apoptosis.

16.2 MRI

MRI is a widely used, noninvasive imaging technique offering the possibility of penetrating into soft tissue and great spatial-temporal resolution associated with relative ease of operation procedures [39, 40]. The principle of MRI is similar to that used in chemical nuclear magnetic resonance (NMR) analysis, in which the spins of specific atomic nuclei are visualized within the body [41]. The altered T_1 (longitudinal) and T_2 (transversal) proton relaxation times within various tissues generate autogenous contrast [13]. Generally, increased water content as well as the inflammation/tumor site has a relatively black/dark signal on T_1-weighted images while relative white/bright signal on T_2-weighted images, which identifies tumor tissue over normal healthy region [42]. In addition to its application in disease differentiation, disease diagnosis, and therapy monitoring, MRI is also exploited in nanomedicine research, in order to (1) perform pharmacokinetics and biodistribution analyses, (2) monitor drug release, and (3) enable cell tracking studies. MRI has been integrated into almost every aspect of cancer clinical practice, including diagnosing, assisted-surgery and radio−/chemotherapy monitoring, and so on. In general, MRI assists cancer treatment mainly in the following aspects: first, the high soft tissue resolution of MRI makes it an important tool for delineating, staging, and monitoring treatment efficacy of cancer; secondly, real-time MRI utilizing various physiological parameters including temperature, water content, and pH also provides invaluable insight on anticancer therapy; thirdly, nanoparticles with combined functional groups and targeting moieties are being designed and fabricated to meet the dual purpose of cancer care—diagnosing/monitoring and treatment [43].

Despite being a highly useful and broadly applicable modality for clinical diagnosis and therapy monitoring, several disadvantages are associated with MRI in treatment monitoring, including (1) relatively low contrast agent sensitivity, (2) relatively difficult quantification procedures, and (3) the time and cost involved. To overcome these limitations, nano-based MR contrast agents, as opposed to radionuclides, could offer optimal conditions for assessing drug release due to access to freely diffusing water molecules to generate contrast and, therefore, render

different signals when present within vs outside of nanocarriers [41]. This type of technique has been widely applied in cancer treatment monitoring.

As stated above, by calculating the T_1 longitudinal relaxation and the T_2 transversal proton relaxation time, images are generated by organs within the human body. The two separate types of signals introduce two different categories of MRI contrast agents into T_1 and T_2 [42, 44]. Commercially offered T_1 contrast agents are typically paramagnetic complexes, for instance, gadolinium (Gd)-related compounds including gadoterate and gadodiamide, whereas T_2 contrast agents are usually iron oxides, such as Feridex and Resovist [44]. Due to the different imaging signals of T_1 and T_2, the design of either T_1 or T_2 contrast agents is very different. Cancer tissue is brighter in T_1 and darker in T_2 compared to normal tissue. Therefore, T_1 contrast agents enhance the final signal images of tumor region; T_2-weighted contrast agents actually diminish signal intensity at the same location. The boosting development of nanotechnology saw a number of different nanomaterials generated from basic T_1 and T_2 contrast agents, including polymeric micelles, multifunctional nanoparticles, as well as liposomes often supplemented with basic components of contrast agents, such as iron oxide nanoparticles and paramagnetic metal ions [45–48]. Kaida et al. [49] reported an example of micelle-based MRI contrast agents. By utilizing multifunctional polymeric nanoparticles, anticancer drug platinum and the paramagnetic Gd were incorporated via reversible metal chelation reaction. The dual function system eliminated cancer in an orthotopic animal model of human pancreatic tumor while monitoring the therapeutic efficacy simultaneously [49]. Such polymeric micelles open exciting prospects for improving the management of cancer therapy [13]. Similarly, doxorubicin-loaded thermally cross-linked superparamagnetic iron oxide nanoparticles (Dox@TCL-SPION) is an example for T_2-weighted nanotheranostics. By conjugating doxorubicin on the PEG-coated SPION nanoparticles, this multitasking "rust ball" can answer the question of where a tumor is located, whether drugs are properly accumulated in the tumor area, and how the tumor responds to therapy [50]. In another example, Ng et al. reported the early CRLX101 therapeutic response in a mouse model used to study tumor cell proliferation. CRLX101 contains camptothecin, a DNA topoisomerase I inhibitor with additional polysaccharide (cyclodextrin) coating to form polymeric particles. The drug exhibited excellent early therapeutic effect with good monitoring resolution in a preclinical mouse model of malignant lymphoma using diffusion MRI. All examples above have revealed the potential of combining MRI contrast agents and therapeutics together as nanotheranostics for treatment monitoring in cancer [51]. In the following section, we will focus on utilizing MRI for specific tumor pathophysiology imaging, including angiogenesis, metastasis, and apoptosis.

16.2.1 MRI for Tumor Angiogenesis Imaging

One of the main driving forces for tumor formation is the deregulation of angiogenesis, defined as the development of new blood vessels. This is because the new growth of tissue would require nutrients and oxygen, which are delivered through

the newly grown blood vessels. In 1971, Judah Folkman first proposed the hypothesis of neoangiogenesis [52]. According to this hypothesis, the resting state of the mature endothelial cells can be switched to neovasculature by the activation of chemical signal molecules originated from the tumor cells. Positive regulators of angiogenesis, including vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), placental growth factor (PlGF), transforming growth factor-beta (TGF-β), and angiopoietins (Angs), were discovered to be involved in the transition [53]. Generally, these molecules can be transported outside tumor cells, assembled from the extracellular matrix or recruited by tumor. Up to now, angiogenesis has been recognized as one of the significant pathological features of cancer, enabling better diagnosis and treatment monitoring of metabolic deregulation and tumor metastasis [54].

Several main signal molecules that regulate tumor angiogenesis and cell adhesion include the VEGF/VEGF receptor signaling pathway and integrin $\alpha_v\beta_3$, which can be used as nanomaterial targeting agents that enhance efficacy by homing to the tumor vasculature [55]. Dynamic contrast-enhanced MRI has been used indirectly in measuring vascular permeability to provide insight on tissue perfusion and permeability of tumor vasculature [56, 57]. Researchers have attempted to link MRI parameters with VEGF expression by correlative analysis of tissue morphology, vascular density with CD31 marker, and vascular permeability [58]; however, this was not applicable in some of the reports [59]. Direct evaluation of VEGF/VEGFR expression by MRI without the use of contrast agents has not been achieved. Nevertheless, targeted ultrasound and optical imaging have reported VEGF/VEGFR imaging in the past few decades [60], which will be discussed in a later section.

The integrin signaling pathway plays an important role in tumor angiogenesis, cell migration, survival, and metastasis. Integrin $\alpha_v\beta_3$, in particular, is significantly upregulated on tumor microenvironment blood vessels but not on inactive endothelium [61]. Most imaging studies of $\alpha_v\beta_3$ integrins have used arginine-glycine-aspartic acid (RGD)-based probes to which integrins bind [61, 62]. Targeted contrast agents have incorporated Gd(III) chelates and targeting agents (i.e., RGD) via a direct conjugation reaction. Nevertheless, due to the low sensitivity of MRI and the relatively low concentration of integrin in targeted tissues, such directly conjugated contrast agents with MRI are not as effective as radionuclide imaging. Therefore, different types of carriers, including polymers, dendrimer, liposomes, and micelles, have been designed to deliver sufficient amount of Gd(III) to enhance tumor site MRI signal [63]. Liposomes with paramagnetic Gd^{3+} chelates at sizes of 300–350 nm in diameter were reported to image $\alpha_v\beta_3$ expression with MRI [64]. In the study, Sipkins et al. designed a novel approach to detect angiogenesis using MRI with $\alpha_v\beta_3$ monoclonal antibody in rabbit carcinomas. Significant tumor contrast was found in T_1-weighted images after injection of targeted liposomes but not control liposomes without conjugating targeting moieties [64]. These promising results have led to many other designs of nanocarrier-based MRI contrast agents for angiogenesis [65].

16.2.2 MRI for Evaluating Cancer Metastases

Evaluating the treatment effect in patients with metastatic cancer is very important in daily oncology practice, especially for patients with metastases to the bone, which are often difficult to detect. This difficulty is due to the nature and complexities of fixed bone defects, which range from sclerotic to osteolytic, as well as the low specificity, sensitivity, and spatial resolution of the previously available bone imaging methods, primarily bone scintigraphy. The process by which cancer cells infiltrate the bone marrow can be detected and quantified using morphological imaging with functional approaches, such as MRI or CT [66]. With the injection of cancer cells pre-labeled with iron oxide particles with diameter in micron into the left ventricle of a beating mouse heart, Heyn et al. were able to track exogenous cancer cells after transportation into the murine brain [67]. This approach allowed the imaging of the early delivery and distribution of cells, as well as new tumor tissue growing from a subset of these cells within the whole intact brain. The particle being used here was a commercially available micro-sized iron oxide and highlights the application of MRI to monitor the metastatic process during treatment [67]. More importantly, nanoparticles possess a large surface area for conjugation to several therapeutic and diagnostic agents and are able to pass the blood-brain barrier to reach the target cancer tissue [68].

16.2.3 MRI for Apoptosis

Apoptosis is a mechanism of programmed cell death that is activated during embryonic development, during the normal maintenance of homeostasis, and under pathological circumstances [69]. Successful cancer treatments, such as radiation [70], chemotherapy [71], thermal therapy [72], and photodynamic therapy [73], could induce apoptosis [74]. Imaging apoptosis will give doctors a general idea of when cancer cells start dying and the tumor starts shrinking. Considering the essential role of apoptosis, a robust imaging method is needed to detect and monitor this process. One of the initial biochemical events that occurs during the apoptotic process is the externalization of phosphatidylserine (PS) [75]. PS is normally constrained to the inner membrane layer. However, when PS is externalized, phagocytes recognize the cells initiating the apoptosis process. Apoptotic cells are phagocytosed by macrophages in a manner reliant on externalized PS prior to an increase in plasma membrane permeability. Thus, PS is an important marker for apoptosis and has been used as a potential cancer marker for MRI-based detection of apoptosis [76]. The surface PS can be detected with a human protein named annexin V in a Ca^{2+} dependent, which has been used in the design of molecular imaging probes. Jung et al. reported conjugation of biotinylated annexin V-glutathione onto streptavidin-conjugated SPIO or Gd chelate avidin could elevate T_2- and T_1-weighted MRI signal in detecting apoptotic murine lymphoma cells (Fig. 16.1) [77]. In addition, annexin

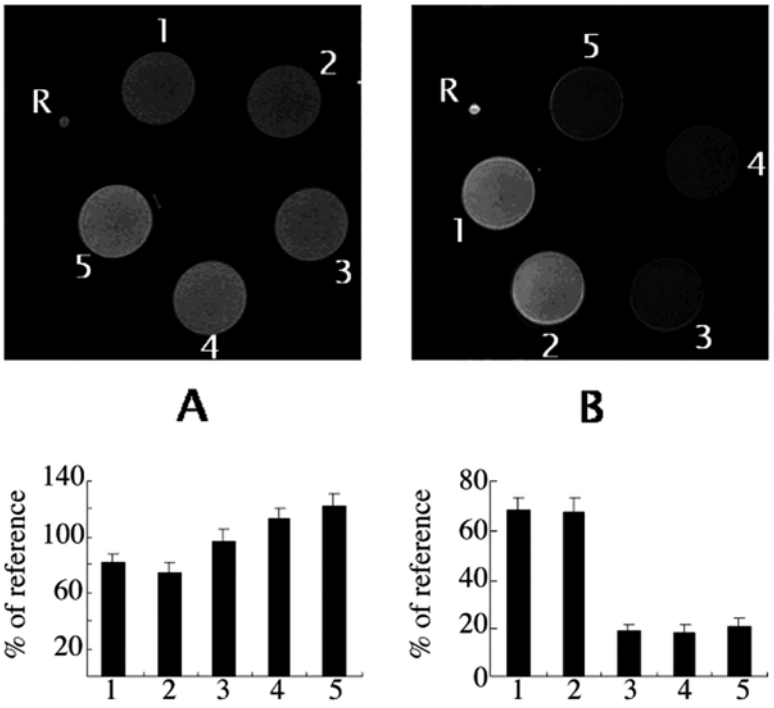

Fig. 16.1 MRI signal of PS-targeted contrast agents. (**a**) T_1- and (**b**) T_2-weighted MR imaging of apoptotic cells after incubation with biotin-annexin V at different concentrations and then subsequently incubated with avidin-GdDTPA or streptavidin-SPIO. (1) Pure apoptotic cells, (2) apoptotic cells with avidin-GdDTPA or streptavidin-SPIO. (3–5) Apoptotic cells with avidin-GdDTPA or streptavidin-SPIO with elevated biotin-annexin V concentration at 1.5 μM, 4.5 μM, and 15 μM. The signal intensity was presented in the bar chart below. Significant MRI signal was observed between the experimental group and the control group, where there were only apoptotic cells or avidin-GdDTPA or streptavidin-SPIO [77]. (Reprint with permission)

V-SPIO conjugates were tail vein injected to characterize the distribution within the tumor region. TUNEL assay of one tissue section from a slice of the tumor indicated the annexin V-SPIO conjugates were localized throughout the tumor region [78].

As one of the most widely applied imaging techniques in both clinical and research applications, MRI can provide useful information regarding cancer metabolic activity evaluation of treatment effects [79]. The hyperpolarized MRI (allows to measure the enzymatic conversion of administrated hyperpolarized molecules) [80] and chemical exchange saturation MRI (external compounds containing exchangeable protons that can be electively saturated and detected implicitly from water signal with better quality of sensitivity) [81] may lead to a future role for cancer treatment monitoring [55]. However, the inherently low sensitivity of MRI limits its application, which can only be remunerated by greater magnetic fields (4.7–14 T) with exogenous contrast agents and longer periods of data acquisition. Functional MRI including dynamic contrast-enhanced MRI (DCE-MRI) is one solution that has been used in the clinic for acquisition of serial MRI

images before, during, and after the administration of an MR contrast agent, allowing the visualization of contrast kinetic changes over long periods of time in addition to the bulk property of the tumor tissue. With the help of pharmacokinetic modeling, color-encoded images can be generated to characterize the tumor masses, identify stage, and even noninvasively monitor therapy [56]. Almost 100 clinical trials have utilized DCE-MRI to evaluate the therapeutic efficacy of antivascular agents [82]. Advanced techniques to enhance image processing, as well as multiparametric analysis, are needed to extend application of DCE-MRI and new purposeful imaging technologies in drug development for cancer therapy [82].

16.2.4 MRI for Detecting Other Markers of Treatment Response

With high spatiotemporal resolution, MRI can also be used to noninvasively detect gene expression in live animals. Mukherjee et al. report of using human water channel aquaporin1 as potential MRI reporters to produce MRI contrast. With 10% aquaporin-expression cells, the cell populations showed enhanced MRI signal. The researchers also explored the efficacy of using this system in a tumor xenograft model. With good contrast ability, biocompatibility, and engineering potential, aquaporin reporter genes could be remarkably applied to molecule imaging of MRI in cancer diagnosis and treatment monitoring.

16.3 Nuclear Imaging

Nuclear imaging is another type of a noninvasive imaging modality that utilizes radioactive isotopes to enable the imaging of biochemical components under normal and diseased conditions in living subjects. Based on the characteristics of the radiotracer, numerous aspects of biological processes can be aimed and visualized by using either PET or SPECT [83]. As discussed above, MRI plays an important role in visualizing the morphology of lesions and locating malignant sites. However, some biochemical processes inside a given tissue are difficult to detect with MRI due to the low sensitivity of the technique [84–86]. Therefore, PET and SPECT have become valuable techniques for monitoring the pharmacokinetics, biodistribution, and target site accumulation of nanomedicine formulations.

PET is an imaging technique that is used to visualize and quantify positron-emitting radionuclides [41], which facilitates four-dimensional (three spatial-dimensions plus temporal) quantitative measurements of the radioactive distribution within the human body in several medical fields including oncology [87]. The principle of dynamic detection by PET is based on annihilation, which occurs when the collision alters the mass of the positron and the electron into electromagnetic radiation after emitting a positron upon decay [83]. SPECT is similar to PET and

also utilizes radioactive isotopes. Nevertheless, SPECT isotopes emit a single photon upon decay, and detection of a single photon involves physical collimators, which shows low geometric competences to reject scattered photons as well as to adjust the vision field. Therefore, this technique is less sensitive than PET, limiting quantitative determinations of tracer accumulation [83, 88].

PET is widely applied to image tumor invasion and the interaction of tumor cells with stroma, including supporting proliferative signaling, avoiding growth suppression, and resisting cell death (apoptosis). The possibility of longitudinal assessment of specific biological processes rather than anatomic changes in tumor size increased the popularity of PET in recent years, providing insights into cellular metabolism for doctors, who are able to then direct further therapy plans for cancer patients. The previous section discussed some applications of MRI for imaging cellular activity, such as apoptosis. However, such markers serve as potential targets for enhancing contrast agent delivery rather than cellular activities that can be monitored by researchers. In contrast with nuclear imaging, monitoring of cellular activity, such as glucose metabolism, could be revolutionized with the use of isotope-labeled substrates [89]. The imaging of these specific types of cellular activity via PET was summarized in a recent review article [83]. For example, PET imaging utilizes ^{18}F, which is the most widely used radionuclide and has become an established clinical tool for whole-body imaging. Sgc8, which is a 41-oligonucleotide that targets protein tyrosine kinase-7 (PTK7), was labeled with F-18 via a two-step chemical synthesis. In the first step, ^{18}F-fluorobenzyl azide reacted with a spirocyclic hypervalent iodine(III) precursor via a one-step radiofluorination. The product was then conjugated with Sgc8-alkyne through copper-mediated “click” chemistry. The synthesized ^{18}F-Sgc8 was able to label aptamers (single-stranded DNA) robustly, allowing the quantification of PTK7 and colon carcinoma kinase-4 (CCK-4) [90]. PTK7 is upregulated in several human carcinomas and plays a major role in canonical Wnt signaling. The level of PTK7 strongly indicates the occurrence of colon carcinoma and thus is worth monitoring for diagnostic and prognostic purposes. An in vivo mouse xenograft study suggested that the 18-F radiolabeling methodology presented here is a powerful technique for tagging aptamers and chemical moieties with similar structures that are suitable for different targets. The quantification of PTK-7 using ^{18}F-Sgs 8 may be a potential strategy for cancer treatment monitoring [90] (Fig. 16.2).

16.3.1 Nuclear Imaging in Monitoring Tumor Growth

PET imaging has become a clinical keystone in cancer staging and restaging, serving as an important parameter in cancer therapeutic monitoring. The most frequently used PET contrast agent is [^{18}F]fluorodeoxyglucose (FDG), which is a glucose equivalent that is electively taken up by malignant cells with a high rate of glucose

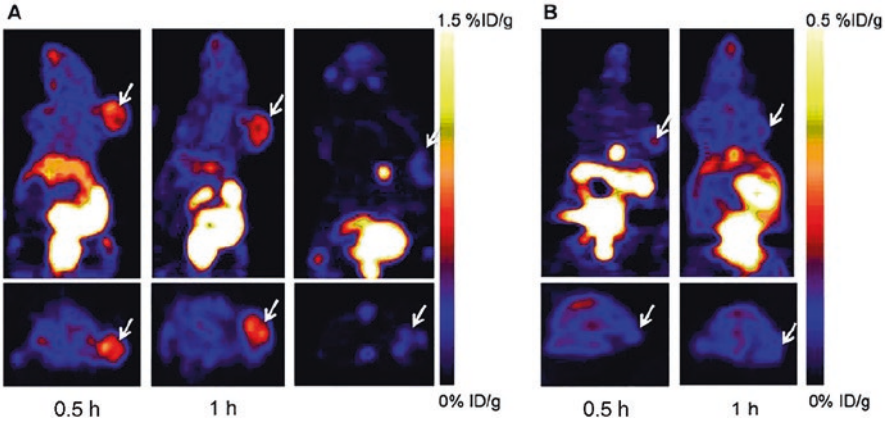

Fig. 16.2 Coronal (upper) and transaxial (lower) PET images of mice with HCT116 (**a**) and U87MG (**b**) xenograft injected with ^{18}F-Tr-Sgc8 at 30 min (0.5 h), 1 h (and 1 h co-injection with an excess amount of unlabeled aptamer (**a**, right panel) [90]. (Reprint with permission)

metabolism [91, 92]. FDG-PET imaging has been widely applied in staging multiple cancer types, including colorectal cancer, esophageal cancer, melanoma, head and neck cancer, non-small cell lung cancers, breast cancer, and lymphoma [91]. For example, FDG-PET was used to monitor patient treatment outcome after the first and third cycle of neoadjuvant chemotherapy in patients with late-stage ovarian cancer [93]. The results suggested sequential FDG-PET was capable of predicting patient therapeutic effects as early as immediately after the first cycle of neoadjuvant chemotherapy and was more accurate than traditional clinical or histopathological response criteria [93].

In addition to the tumor staging agent FDG, PET contrast agents for specifically monitoring the growth and death of tumor cells have also been reported. The essence of this strategy is to develop radiolabeled nucleoside equivalents like thymidine compounds that are able to incorporate into DNA, serving as convenient biomarkers of cell proliferation. Yaghoubi et al. reported PET imaging of thymidine kinase (herpes simplex virus type 1) or mutant HSV1-sr39tk reporter gene expression in mice and humans using 9-4-[^{18}F]fluoro-3-(hydroxymethyl)butyl]guanine ([^{18}F]FHBG) [94]. Another aspect of PET-based cell monitoring is the ability to utilize radiolabeled monoclonal antibodies specific for tumor-associated antigens, including Her2 and carcinoembryonic antigen. However, large protein-based contrast agents are slowly cleared from the blood stream and thus produce a high background signal [92]. Therefore, short-chain, engineered antibody fragments may enhance the signal by improving the signal-to-noise ratio, as they are vacant more speedily [91].

16.3.2 Nuclear Imaging in Monitoring Angiogenesis

As stated above, angiogenesis is a well-established marker for tumor growth, invasion, and metastasis [95]. Integrin $\alpha_v\beta_3$ represents an excellent molecular marker for angiogenesis, as it is significantly upregulated in activated endothelial cells in comparison to quiescent endothelial cells [96]. The Arg-Gly-Asp (RGD) tripeptide sequence is one of the most popular currently available integrin-targeted imaging probes because of its high affinity and specificity for integrin αvβ3 [97]. ^{18}F-Galacto-RGD was the first published RGD peptide conjugates in human subjects [98]. Upon that time, limited RGD containing PET probes have been designed and tested in the clinic. As their structures are different, all of the clinically studied RGD peptides, counting both monomers and dimers, exhibit very analogous in vivo pharmacokinetic properties [99]. The modification of the RGD sequence onto PET probes helps achieve multifunctional probes for accurate tumor metastasis monitoring. Zheng et al. assessed the diagnostic value of ^{68}Ga-NOTA-PRGD2 (NOTA-PEG$_4$-E[c(RGDfK)$_2$]) for PET/CT dual-modality imaging in 91 lung cancer patients (48 men and 43 women). The results of that study suggested an equal diagnostic efficacy for lung cancer but a better effect in assessing lymph node metastasis than ^{18}F-FDG [100] (Fig. 16.3).

16.3.3 Nuclear Imaging for Apoptosis

As stated in the previous section, strategies that enable the visualization and detection of apoptosis would have enormous benefits for treatment monitoring. Utilizing the interaction between Annexin V and PS is one of the most successful and widely applied strategies in apoptosis imaging [74]. This mechanism applies not only to MRI (previous section) but also to nuclear imaging and optical imaging (later section in this chapter). In 1998, Blankenberg et al. reported the preparation of ^{99m}Tc-hydrazinonicotinamide-Annexin V (^{99m}Tc-HYNIC-Annexin V). HYNIC is a nicotinic acid analogue with a bifunctional chelator that is capable of binding proteins on the one hand and sequestering ^{99m}Tc on the other. This molecule was conjugated to human rh-Annexin V and labeled with ^{99m}Tc using tricine as a co-ligand in the presence of stannous ions [101] (Fig. 16.4). A previous study demonstrated that the administered radiolabeled annexin V was able to locate and concentrate for apoptotic cells in vivo [101]. A two- to sixfold increase in the internalization of radiolabeled annexin V at sites of apoptosis was observed in the murine model of Fas-mediated apoptosis and treated murine lymphoma, suggesting that radiolabeled annexin V could be applicable for the detection and monitoring of tissues and organs undergoing programmed cell death [101]. In another study, Kartachova et al. reported the benefits of using ^{99m}Tc-HYNIC-annexin V assisting Pt(IV) chemotherapy in high-staged lung cancer. Significant correlation was observed between the monitoring of annexin V metabolic change via SPECT and the treatment outcome

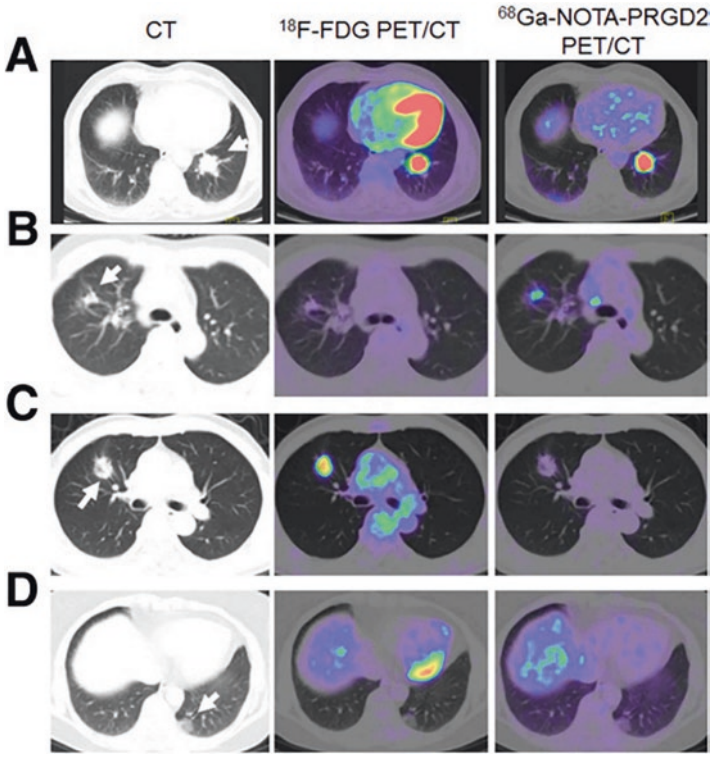

Fig. 16.3 CT, ^{18}F-FDG, and ^{68}Ga-NOTA-PRGD2 PET/CT images of primary lung cancer for four different patients (A, B, C, D). Patient A had an abstemiously differentiated adenocarcinoma in the inferior lobe of the left lung at the age of 76. Patients B and C were female with an exceedingly distinguished adenocarcinoma in the superior lobe of the right lung at the age of 37 and 61, respectively. Patient D was a 61-year-old woman with highly developed adenocarcinoma in the inferior lobe of the left lung. Patient A and B's lesions are strongly visualized on ^{68}Ga-NOTA-PRGD2 PET as the tumor sections show positive integrin $\alpha_v\beta_3$ staining. Arrows point to tumor [100]. (Reprint with permission)

from platinum chemotherapy, indicating ^{99m}Tc-HYNIC-annexin V is a promising therapeutic monitoring agent in human clinical application [102]. In addition to radiotracer ^{99m}Tc in nuclear medicine, many other radioligands are being used to image PS during apoptosis, as summarized in a published review [74].

In addition to radiolabeled protein probes, radiolabeled small-molecule probes are also available for apoptosis imaging via nuclear imaging. Compared with protein and peptide probes, small molecules have their own merits for clinical applications, including docile structural optimization and favorable pharmacokinetics such as organ distribution profiles, rapid diffusion rates, and blood clearance rates. Therefore, it is highly desirable to develop various small-molecule imaging probes with the same target binding affinity as proteins [74]. Zinc dipicolylamine (Zn-DPA) coordination complexes can be alternatives to Annexin V for PS targeting [103].

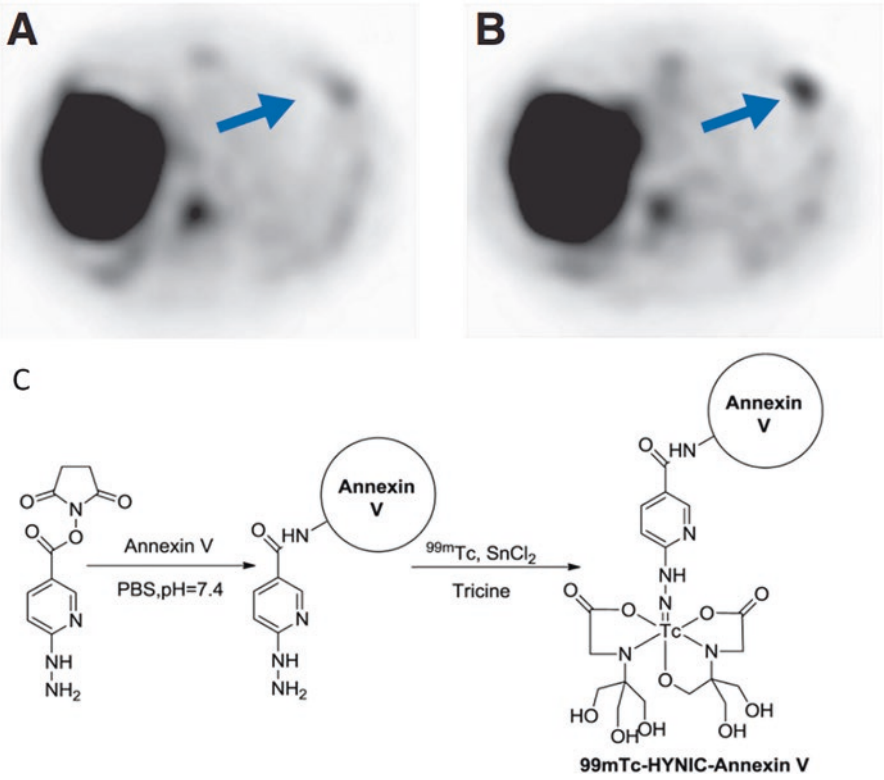

Fig. 16.4 The early ^{99m}Tc-HYNIC-annexin V tumor uptake could be a predictor of response to treatment in cancer patients [93], as demonstrated with a significant correlation between early therapy-induced changes in the probe tumor uptake and tumor response. SPECT images of tumor uptake in the rib region of metastasis before (**a**) and after (**b**) 48 h administration of cisplatin with ^{99m}Tc-HYNIC-annexin V [102]. (**c**) Radiochemistry of ^{99m}Tc-HYNIC-annexin V [74]. (Reprint with permission)

Zn^{2+} ions that mediate the cooperative association of the dipicolylamine ligand and the anionic head group of membrane-bound PS account for the targeting function of Zn-DPA [104]. Based on this information, to further develop this molecular strategy, Oltmanns et al. developed an ^{18}F-labeled zinc-cyclen probe that targets apoptosis [105]. With the ^{18}F label on zinc-cyclen, higher uptake was achieved in an exposed Dunning R3327-AT1 prostate tumor in comparison to the contralateral control tumor in PET imaging. This result suggests the great promise of this probe as a new agent for in vivo applications of treatment monitoring regarding cell death after different types of cancer therapy [105].

Radionuclide-based imaging techniques have been used routinely in clinics since the twenty-first century [55]. The use of molecular-targeted nanoparticles holds many benefits over conventional approaches to cancer treatment monitoring. First, a single nanoparticle can incorporate multiple imaging labels or combinations of

labels from different modalities, dramatically increasing the signal intensity. Second, nanoparticles with different chemical modification and conjugation target moieties are able to bypass biological barriers to improve the treatment monitoring efficacy [55]. Furthermore, recent advances in nuclear imaging systems provide high spatial and temporal resolution for treatment monitoring [106]. With the efforts of researchers in molecule imaging, we foresee the wide application of real-time monitoring with nuclear imaging to be a personalized patient-based treatment approach [107].

16.3.4 Nuclear Imaging for Detecting Other Markers of Treatment Response

Hormone receptors, such as estrogen receptors and progesterone receptors, have been identified as imaging targets in assisting breast cancer therapy staging and therapeutic monitoring. Therefore, many molecular imaging probes have been designed utilize this specific molecular cellular marker. Currin et al. reviewed current progress in predicting breast cancer endocrine responsiveness using 16-α[18F]-flouro-17β-estradiol PET (FES-PET). In general, estrogen-receptor imaging provides accurate measuring tumor response to endocrine therapy in patients [108]. Sun et al. also reported clinical evaluation of FES PEG/CT assisted in making personalized treatment decisions [109] for 33 breast cancer patients who underwent both 18F-FES and 18F-FDG PET/CT. With the three selected lung lesions, FES PET/CT showed one lesion with high update, and the other two lesions were negative, indicating an ER-positive metastasis or secondary primary tumor. Overall, 16 patients received adaptable treatment plans (different than original treatment plan) after FES PET/CT results [109]. These results indicated a good application of PET in assisting personalized adjustable treatment plans, beneficial for cancer treatment monitoring.

16.4 Optical Imaging

As a non-ionizing, noninvasive technique based on the precise optical characteristics of tissue components at different wavelengths, biomedical optical imaging has been developed to deliver quantitative measurements nearly in real time and with a wide range of resolutions, therefore providing high-quality images for the diagnosis and monitoring of treatment efficacy in cancer [110, 111]. However, there are limitations to the therapeutic monitoring of some treatments in patient responders and nonresponders. Researchers have been working to design reporter nanoparticles that can not only deliver chemotherapy or immunotherapy to the tumor but also report

back the efficacy in real time. Kulkarni et al. reported a reporter nanoparticle that monitors its efficacy in real time by presenting a dye activate/quench system inside the particle in addition to antitumor drug. If the experimental mice respond to the therapy, the activation of caspase-3 as part of the cellular apoptosis process will trigger the dye to show fluorescence. However, when the cells develop resistance to this treatment, no apoptosis or activation of caspase-3 will be available, leaving the quenched dye inside particles at the tumor site [112].

Fluorescence reflectance imaging (FRI) is by far the most extensively used optical imaging technique [41]. The ease, versatility, and sensitivity of optical imaging make it possible to image multiple fluorophores in the same animal, which is the most significant benefit of this technique. Kumar et al. reported a mitochondrial-targeting antitumor drug that consists of both 5′-deoxy-5-fluorouridine and apoptotic marker ethidium. By targeting the elevated expression level of H_2O_2 inside mitochondria, 5′-deoxy-5-fluorouridine and ethidium will be released. By monitoring the intrinsic fluorescence changes of ethidium, therapeutic effect would be monitored both in vitro and in vivo [113].

Recent studies focused on addressing the drawbacks of optical imaging, including autofluorescence, poor penetration depth, and limited anatomical information [41]. For example, nanomaterials labeled with fluorescent dyes often tend to use longer wavelengths for excitation (e.g., Alexa Fluor 647, Cy5, or Cy7), which are outside the range of natural autofluorescence [114]. On the other hand, some in vivo studies have validated the potency of quantum dots accumulation in cancer region for optical imaging to enhance the tissue penetration depth [115]. The fluorescent nanoparticles that are currently being used in noninvasive imaging include organic dye-doped nanoparticles, quantum dots, and upconversion nanoparticles. The emergent development of innovative multifunctional nanoparticles can easily be combined with therapeutics to form nanotheranostic materials. For example, NIR dye Cy5.5-labeled chitosan nanoparticles with encapsulated paclitaxel were able to image and assess therapeutic efficacy in mice with SCC7 murine squamous carcinoma tumors [116]. In addition to encapsulating chemotherapeutics, optical imaging-guided photodynamic therapy is a widely exploited technique for cancer treatment [13]. Through the activation of the administered tumor-localizing photosensitizing agents by particulate wavelength photons, the surrounding tumor tissues can be irreversibly photodamaged after a series of biological processes [117]. Luo et al. reported the synthesis of a mitochondria-targeted near-infrared (NIR) photosensitizer for simultaneous cancer photodynamic therapy (PDT) and photothermal therapy. The small-molecule photosensitizer was designed utilizing many synthesized heptamethine cyanine dyes that are able to concentrate in cancer cells via organic-anion transporting polypeptide-mediated active delivery and are retained in mitochondria due to their cationic properties. Furthermore, these photosensitizers for NIR imaging can distinguish the tumor margins from healthy tissue, serving as excellent candidates for precise imaging-guided phototherapy and treatment monitoring [118].

16.4.1 Optical Imaging in Monitoring Angiogenesis

As stated in the previous section, VEGF plays an important role in angiogenesis activity. Here we use one specific example to discuss the use of potential dye-conjugated anti-VEGF to obtain quantitative information about VEGFR expression. Wang et al. developed an Avidin-tagged $VEGF_{121}$ protein, which could form a stable complex with streptavidin-IRDye800 (SA800) after being biotinylated with the bacterial BirA biotin ligase. The dye-associated complex is capable of interacting with VEGFR in vitro at high affinity. In addition, the complex also displayed efficacy for receptor-specific targeting in a 67NR mice xenograft model [119]. Figure 16.6 presents the in vivo imaging of 67NR tumors with IRDye800 conjugates. The $VEGF_{121}$-Avid/SA800 complex may be a potential clinical tool for quantitative and repetitive NIR imaging of VEGFR expression for monitoring cancer treatment (Fig. 16.5) [119].

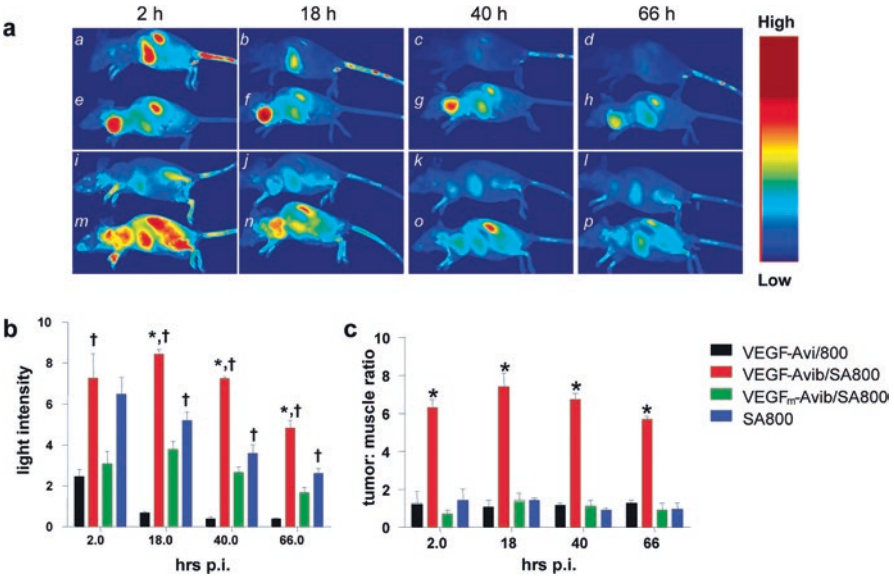

Fig. 16.5 In vivo NIR imaging of 67NR tumor models with IRDye800 conjugates. (**a**) Sagittal images were taken at 2, 18, 40, and 66 h after administration of chemically modified VEGF121-Avi-IRDye800 (VEGF-Avi/800) intravenously (a–d), VEGF121-Avi-biotin/streptavidin-IRDye800 (VEGF-Avib/SA800) (e–h), VEGF mutant-Avi-biotin/streptavidin-IRDye800 (VEGFm-Avib/SA800) (i–l), and streptavidin-IRDye800 (SA800) (m–p). Total fluorescence signals were acquired under the same conditions and normalized by exposure time and ROI area (total signal/ms mm^2). (**b**) Light intensity of tumor and (**c**) tumor to muscle light intensity proportion at multiple time points were presented as bar graphs after administration of certain type of dye complexes [119]. (Reprint with permission)

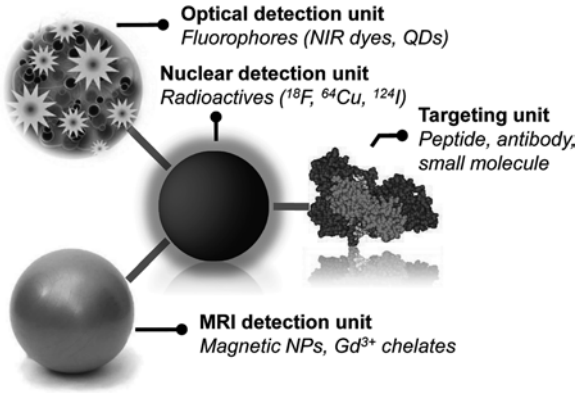

Fig. 16.6 Schematic diagram of multimodality imaging probes. Radioactive isotopes, magnetic particles, fluorophores, and targeting motifs can be integrated into one single system with different combinations. *NIR* near-infrared, *NP* nanoparticles, *QD* quantum dot [120]. (Reprint with permission)

16.4.2 Optical Imaging in Monitoring Apoptosis

In the field of optical imaging, researchers are attempting to monitor apoptosis via advanced probes. The design of optical imaging probes for apoptosis usually falls into two strategies. The first strategy is to conjugate a fluorophore on the probes as a reporter signal. Similar to the strategy described above, utilizing the annexin V-PS binding mechanism, researchers attempted to label annexin-V with the near-infrared fluorophore Cy5.5 [121]. As expected, the conjugation preserves the binding affinity to PS. However, when more than 2 dyes are conjugated, annexin no longer binds to PS [74]. To overcome these limitations, Ntziachristos et al. demonstrated that tumor responses to chemotherapy can be resolved accurately via fluorescence tomography with PS fluorescent probe based on a Cy 5.5 modified annexin V (two types of modification were performed with annexin C. The ratio of Cy 5.5 to annexin V was either 1.1 or 2.4) [122]. A tenfold increase in the fluorescent signal in cyclophosphamide-sensitive tumors and a sevenfold increase in resistant tumors were observed for monitoring apoptosis [122].

The other strategy is to design fluorophore-quenching probes. These activatable probes do not emit a signal continuously and thus allow researchers to control and manipulate the outputs of maximized target signal and minimized background signal by altering the chemical environments. Lee et al. reported an apoptosis nanoprobe that is able to deliver chemically tagged, dual-quenching caspase-3-sensitive fluorogenic peptides into cells, allowing caspase-3-dependent fluorescence intensification to be imaged real-time in apoptotic cells with high resolution [123]. The self-assembled hyaluronic acid nanoparticles were conjugated with a caspase-3-specific substrate to detect apoptosis in cells. The NIR fluorescence quencher BHQ3 and the dye Cy5.5 was conjugated onto hyaluronic acid particle. When interacting with apoptotic cells, the active caspase-3 in apoptotic cells will cleave the bond that connects BHQ3 and Cy 5.5. When Cy 5.5 is cut free from the particle, it would induce strong fluorescent signal of the cells. The system is shown to

effectively identify not only for apoptotic cells in vitro but also in vivo tumor tissue in mice treated after DOX [123].

In contrast to the fluorophore-quenching strategy, aggregation-induced emission properties have also been designed for monitoring drug-induced apoptosis inside single cell. Yuan et al. reported a chemotherapeutic Pt(IV) prodrug with the conjugation of cyclic-RGD peptide as well as caspase-3 enzyme peptide (Asp-Glu-Val-Asp, DEVD) conjugated tetraphenylsilole (TPS) fluorophore (TPS-DEVD). While TPS-DEVD is non-florescent under normal aqueous condition, TPS residue after dissociation with DEVD tends to aggregate to emit fluorescent. The cleavage of DEVD process is controlled by caspase-3, which only happens in response to apoptotic cells. The smart design of nanomaterial could be potentially used as molecular imaging probes for early cancer therapeutic evaluation.

16.4.3 Intraoperative Positioning

In addition to traditional optical imaging examination for cancer diagnosis and prognosis, surgery also plays a key role in cancer treatment. In fact, tumor dissection is the initial treatment for most benign tumors and many malignant tumors. The inherent difficulties in distinguishing tumor and normal tissue make it difficult to perform the procedure. Intraoperative positioning is defined as fluorescent labeling routine that utilizes an imaging system to enable surgeons to distinguish between healthy and malignant tissues that are labeled with a fluorescent detection agent [124]. Despite the wide application of CT, MRI, PET, and SPECT for preoperative tumor diagnosis, such techniques are typically not applicable for intraoperative tumor surgery, and palpation and graphic inspection remain the leading approaches [124, 125]. On the other hand, fluorescence molecular imaging (FMI) has been well known as a dominant tool for guiding accurate intraoperative positioning [126–129]. To facilitate more discriminating tumor detection, fluorescent dyes can be modified with targeting moieties (i.e., peptides, antibodies, or sugars) that are processed systemically and accumulate at lesion sites. Still in the preclinical stage, such fluorescent imaging probes show potential as markers for cancer cells and tumor angiogenesis, making them a desirable surgical guide for imaging tumor microenvironments (Table 16.1), although some of these probes may require a long time for FDA approval [124].

Over the past decade, this technology has enhanced the ability to surgically remove liver metastases[128], breast cancer[129], ovarian cancer[130], melanoma[131], vulvar cancer[132] and cervical cancer[133]. Recently, Kircher et al. reported the use of a gliosarcoma model to explore functional nanoparticles as intraoperative optical probes[134]. Such nanoparticles can be synthesized simply with a strong NIRF signal, enabling real-time imaging for surgical procedures. The intracellular infiltration, extended degradation, and combined optical and magnetic properties of nanoparticles allow radiologists and neurosurgeons to identify the same probe in the same cells, augmenting the precision of surgical resection and the

Table 16.1 Examples of ongoing optical probe clinical trials

Name	Sponsor	Phase	Patient population	ClinicalTrials.gov Identifier	Function
RACPP AVB-620	Avelas Biosciences, Inc.	I	Women with primary, nonrecurrent breast cancer undergoing surgery	NCT02391194	Surgical margins; sentinel lymph node biopsy
LUM105	David Kirsch Lumicell, Inc.	I	Patients with the following conditions: sarcoma, soft tissue sarcoma, breast cancer, colorectal cancer, pancreatic cancer, esophageal cancer	NCT01626066, NCT02438358, NCT02584244	Surgical margins
Tumor Paint (BLZ-100)	Blaze Bioscience Australia Pty Ltd, Blaze Bioscience Inc.,	I	Patients with the following conditions: skin neoplasms, soft tissue sarcoma, central nervous system tumors, glioma, breast cancer	NCT02097875, NCT02464332, NCT02462629, NCT02234297, NCT02496065	Surgical margins
OTL38	On Target Laboratories, LLC	II	Intraoperative imaging of folate receptor α-positive ovarian cancer	NCT02317705	Surgical margins

outlook for many brain cancer patients[134]. Similarly, they also developed nanoparticles containing a gold core with Raman-active layer and a silicone coating with Gd-DOTA to precisely identify the margins of brain tumors in living mice both preoperatively and intraoperatively. The nanoparticles injected intravenously accumulated at the tumor cite whereas none was found in the surrounding healthy tissue, indicating the potential in brain tumor imaging and resection[135].

16.5 Multimodality Imaging for Cancer Treatment Monitoring

In the design of novel clinical diagnostic probes, several parameters are generally considered, including detection sensitivity, spatial resolution, tissue penetration, temporal resolution, signal-to-noise ratio, and quantitative accuracy [136]. Therefore, the design and utilization of multiple modalities simultaneously have become popular in clinical research to overcome the limitations of single imaging techniques [137]. Since the first PET/CT multimodal instrument was introduced in 1997, the sales of monomodal, PET imaging equipment have gradually declined [138–140]. In 2007, the first commercial PET/MRI hybrid prototype human-size

scanner was released, triggering tremendous research in probe design for such dual-imaging techniques [141]. Considering the continuing development of multimodal instrumentation, researchers are currently focused on tracking several molecular targets simultaneously or using different imaging approaches in combination to more precisely identify the localization and expression of certain biochemical markers [141, 142]. A single probe with multimodal detectability is not necessary when designing imaging probes but could help guarantee the same pharmacokinetics and localization of signal from each modality, reducing the stress on the body's blood clearance system. Due to the different sensitivity of each imaging modality (may vary by three orders of magnitude), the concentrations of contrast agents of each modality within a single probe must be carefully considered to meet the requirements for imaging while remaining nontoxic to the human body [137]. Lee et al. summarized the design of multimodality probes for molecular imaging (Fig. 16.6) [120].

An example of an imaging probe for nuclear and MRI combinations was reported by Lee et al. [143]. Polyaspartic acid-coated iron oxide nanoparticles with superficial amino groups were conjugated to cyclic-RGD peptides for integrin $\alpha_v\beta_3$ targeting and macrocyclic 1, 4, 7, 10-tetraazacyclododecane-N, N′, N″, N‴-tetraacetic acid (DOTA) chelators for PET after labeling with ^{64}Cu. The modified iron oxide nanoparticles were further evaluated in vitro and in vivo to demonstrate the efficacy and feasibility of receptor targeting for dual PET/MRI (Fig. 16.7) [143].

In another case, amine-functionalized quantum dots (QD) were modified with RGD peptides and DOTA chelators for integrin $\alpha_v\beta_3$-targeted PET/NIRF imaging

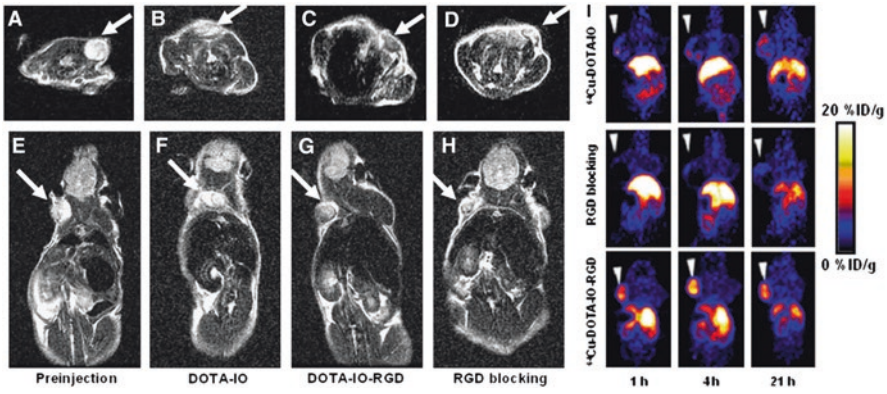

Fig. 16.7 (**a**–**h**) T_2-weighted MR images of mice with U87MG tumor before injection of unmodified iron oxide nanoparticles (**a** and **e**) and at 4 h after tail-vein injection of DOTA-labeled iron oxide nanoparticles (**b** and **f**), DOTA−/RGD-labeled iron oxide nanoparticles (**c** and **g**), and DOTA−/RGD-labeled iron oxide nanoparticle with blocking dose of c(RGDyK) (**d** and **h**). (**i**) Entire body coronal PET images of mouse with human U87MG xenograft at 1, 4, and 21 h after injection of 3.7 MBq of ^{64}Cu-/DOTA-labeled iron oxide nanoparticles, ^{64}Cu-/DOTA−/RGD-labeled iron oxide nanoparticles, or ^{64}Cu-/DOTA−/RGD-labeled iron oxide nanoparticles with c(RGDyK) peptide per kilogram (300 mg of iron equivalent iron oxide nanoparticles per mouse) [143]. (Reprint with permission)

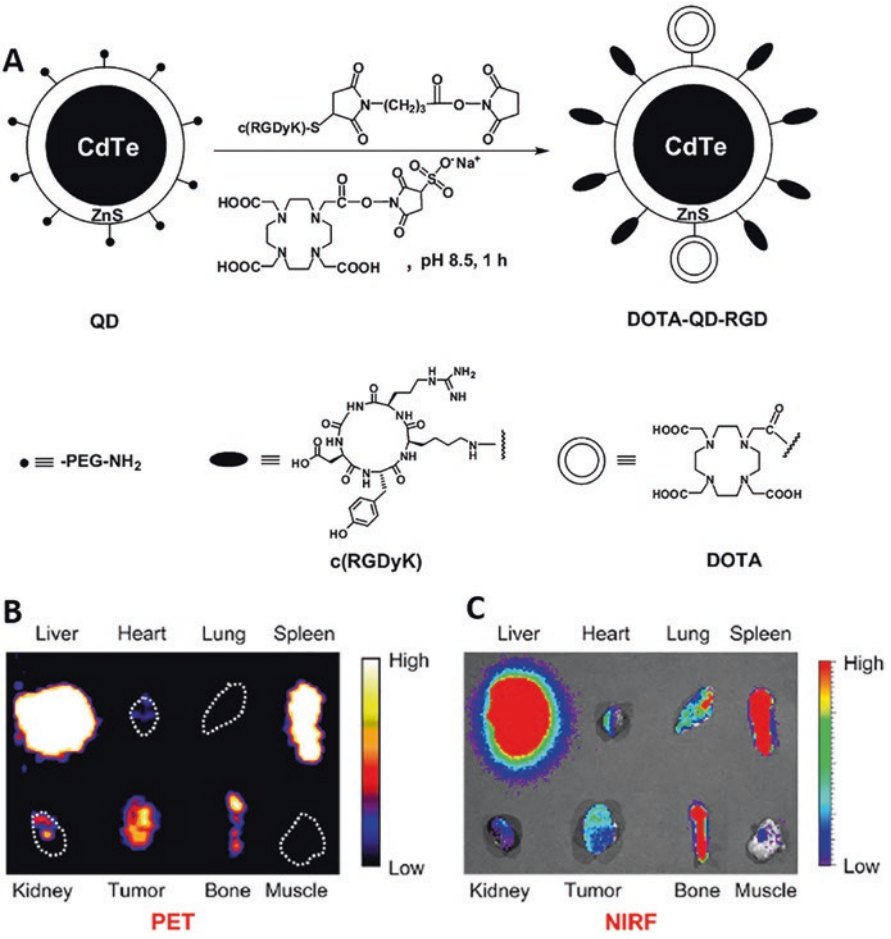

Fig. 16.8 (**a**) Synthesis of dual-function PET/NIRF probe DOTA–QD–RGD. (**b**) PET and (**c**) NIRF image of harvested tissues 5 h after administration of 64Cu-labeled DOTA–QD–RGD [144]

[144]. PET/NIRF imaging, tissue homogenate fluorescence measurements, and immunofluorescence staining were performed with human glioblastoma tumor xenograft-bearing mice to determine the probe uptake amount at the malignancy site and in the major organs (Fig. 16.8) [144]. The liver and spleen exhibited the highest signal intensity for both PET and NIRF. However, the signal at the tumor site was enhanced in comparison to other organs. Cornell dots (C dots) are another categorized optical dye for cancer therapy [145]. The Bradbury group reported the first-in-human clinical trial of using ^{124}I-cRGDY-PEG-C dots in patients with metastatic melanoma. ^{124}I-cRGDY-PEG-C dots were intravenously introduced into patients followed by serial PET and CT assessment to identify the safety pharmacokinetics, clearance profiles, and radiation dosimetry. No adverse effects were observed in metabolic profiles with conventional tests of blood and urine samples

from the patients injected with ^{124}I-cRGDY-PEG-C dots during a 2-week period, indicating the safety of using this PET/optical dual probe for melanoma diagnosis [145].

An easy strategy for designing an optical/MRI dual-functional probe is to fuse MRI contrast agents with QDs through a doping procedure. While doping into bulk semiconductors with transition metals is routine, doping into nanocrystals has been demanding owing to the small size and confined structure. Researchers have attempted to dope manganese into different QDs, such as ZeS [146], ZnS [147], CdSe [148], and InP [146]. In addition, ZnO QDs have also been doped with a number of other transition metals, including Ti, Cr, Co, Ni, Mn, Ru, Pd, Fe, and Ag. However, considering the toxicity of such heavy metals, the clinical applications of transition metal doped QDs are not feasible [137, 149]. In 2012, Bourlinos et al. described Gd(III)-doped carbon dots served as fluorescence-MRI probes for theranostic applications [150]. The obtained Gd(III)-doped carbon dots stably disperse in water, with a size of 3–4 nm in diameter and an even gadolinium distribution on the surface. An ex vivo study suggested that these dots exhibit strong T_1-weighted MRI contrast, bright fluorescence, and low cytotoxicity [150].

Multimodal imaging probes have been designed to visualize apoptosis in vitro and in vivo. In 2004, Schellenberger et al. reported the synthesis of a magneto/optical form of annexin V via the conjugation of Cy5.5 and annexin to an amino-CLIO (cross-linked iron oxide) nanoparticle. The conjugation process preserves the strength of the interaction between annexin V and apoptotic Jurkat T cells while making it possible to detect the particles by using either MRI or NIRF optical methods [105]. Small-molecule multimodal probes are also available, such as the molecular probe LS498, which consists of DOTA for chelating the radionuclide ^{64}Cu, an NIR fluorophore-quencher pair and caspase-3-specific peptide substrates, which is able to trace cellular apoptosis via PET and optical imaging both in vitro and in vivo.

Recently, photoacoustic imaging has gained popularity in the field of multimodal imaging, which has the potential to image animal and human organs with both high-contrast and good spatial resolution [151]. The photoacoustic consequence is the physical basis for photoacoustic imaging and denotes to the creation of acoustic waves by the absorption of electromagnetic energy, including optical or radio-frequency waves [152]. With the current introduction of targeted contrast agents, photoacoustics is capable of molecular imaging in vivo, thus expediting further molecular cellular characterization of cancer in the context of both diagnostic and therapeutic monitoring [153]. Photoacoustic imaging enables the visualization of tumor locations deep within a tissue and provides information about the vasculature [154]. This approach is also able to offer details about hemoglobin oxygen saturation at high resolution with high contrast, without the use of exogenous contrast agents [155], which is superior to other imaging techniques such as blood oxygen level-dependent-MRI and PET [153]. Wang et al. reported the synthesis of ferritin (Fn) nanocages with ultrasmall copper sulfide (CuS) nanoparticles inside the nanocage cavities using a biomimetic synthetic approach. The biological function of Fn is to remove superfluous iron ions in body fluids and accumulate inside its own interior cavity, making it good iron bank as our photoacoustics imaging probes. CuS–Fn

nanocages (CuS–Fn NCs) showed robust near-infrared absorbance and extraordinary photothermal conversion efficiency. Following the guidance of PAI and PET, photothermal therapy with CuS–Fn NCs exhibited great cancer therapeutic efficiency with low toxicity effect both in vitro and in vivo, demonstrating the great potential of bioinspired novel CuS–Fn NCs as clinically translatable cancer theranostics while monitoring tumor/tumor vasculature shrinkage simultaneously. This highly sensitive, noninvasive, and quantitative in vivo guidance method may be suitable for cancer theranostics in applications such as cancer diagnosis, treatment, or drug delivery [156]. Though recent studies are still at the preclinical research stage, movement toward clinical trials is expected for these novel and intricately designed multimodality imaging probes [120]. In another example, Nie et al. reported of synthesizing plasmonic gold nanostars conjugated with cyclic-RGD peptides (RGD-GNS) for photoacoustic imaging to target tumor vasculature environment with elevated $\alpha_v\beta_3$ expression. After injection of the RGD-GNS, tumor-associated blood vessels were clearly visualized, and tumor size was significantly shrink after photoacoustic application [151].

16.6 Conclusion and Challenges

The development of nanotheranostics principles and techniques requires a multidisciplinary approach (including chemistry, physics, material science, drug delivery, and pharmacology) to work toward the common goal of improving the management of cancer. As stated earlier in this chapter, the synchronized delivery of imaging agents and therapeutics will provide the possibility of early diagnosis and feedback on treatment efficacy in real time without the need for traditional endpoints.

However, despite the enthusiasm concerning the use of sophisticated nanotheranostics for cancer applications, many improvements are needed before nanotheranostics can become an effective therapy in clinical practice. Many of the techniques discussed above have only been evaluated in vitro and may not prove to be feasible as imaging and theranostic agents in vivo. Some of the nanotheranostic agents have been investigated in vivo; however, such studies focused on imaging functionality, while the therapeutic effectiveness was largely unknown. Nevertheless, for those nanotheranostics whose imaging and therapeutic efficacy have been investigated, the path for clinical translation is still challenging and strewn with impediments. Drug/imaging agent loading capability, biocompatibility, pharmacokinetic/pharmacodynamics parameters, and risk/advantage estimation must to be investigated. It is worth noting that the dose for nanotheranostics may be different than the dose needed for single therapeutics or imaging probes because the simultaneous therapeutic and diagnostic effect may be altered by several orders of magnitude compared to the effect of a single probe [13, 157]. Additionally, there is a great need for better predictors (biomarkers) of therapeutic response that can be monitored using imaging via nanoparticles early in the treatment cycle, such as hormone receptors discussed in the previous study. Recently, the development of tumor-derived extra-

cellular vesicles could also be used to identify cancer biomarkers, such as ephrin type-A receptor 2 in pancreatic cancer [158]. With further specific biomarkers identified, biomedical engineers can utilize engineering techniques to refine assays for clinical use. Furthermore, multiple in vivo studies and clinical trials would be needed in collaboration with clinicians. Ultimately, the key considerations in the design of an effective therapeutic and an effective imaging agent will be (1) the good biocompatibility and controlled clearance rate for better therapeutic monitoring, (2) the identification and understanding of cancer biomarkers and how molecular imaging agents interact with the biomarkers, (3) the rational design of materials to target cancer tissue environment to better serve the purpose of molecular engineering, and (4) the capability to manufacture the materials in large scale under sterile condition for clinical application [159, 160].

The successful use of noninvasive imaging techniques will improve cancer diagnosis and therapeutic effects. As each of the modalities discussed above has its own advantages and disadvantages, dual- or multimodality theranostic will demonstrate their benefits and synergy in the context of the need to accurately and quantitatively resolve biomedical questions [41]. Ultimately, in theory, theranostic agents can deliver therapeutics to tumors and can use imaging functions to improve the application of diagnosis and therapeutic monitoring.

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Chapter 17
Remotely Triggered Nanotheranostics

Abdul K. Parchur, Jaidip M. Jagtap, Gayatri Sharma, Venkateswara Gogineni, Sarah B. White, and Amit Joshi

17.1 Introduction

Cancer is one of the leading causes of death worldwide. Despite numerous advances in cancer therapy and research, including potent radiotherapy, chemotherapy, immunotherapy, and molecularly guided treatments, overall cancer mortality has not significantly declined. According to the *American Cancer Society*, about 314,290 males and 281,400 females in the USA were expected to die of cancer in 2016 [1]. Further, tumor heterogeneity, both inter-patient and intra-tumoral, has placed significant barriers in the path of "magic bullet" molecular medicine-based therapies. Thus, it is vital to develop therapeutic approaches that can be personalized to individual patients and which are impervious to barriers posed by tumor heterogeneity and de novo and acquired drug-resistance mechanisms. Remotely triggered nanotherapeutics seek to add to the clinician's arsenal of personalized medicine by combining the favorable pharmacokinetics of nanoparticles (NPs) [2], with the spatiotemporal therapy control provided by external field-modulated delivery.

Though NPs have always existed deep into antiquity as pigments and coloring agents, it was not until December 29, 1959 that Richard Feynman, a theoretical physicist, delivered a talk "There's plenty of room at the bottom," where he discussed manipulating atoms and molecules for creating nano-size machines, thus starting the nanotechnology revolution. In 1974, Norio Taniguchi, a professor at Tokyo University, coined the term "nanotechnology." By 2011, the world governments were estimated to spend $10 billion USD annually on research in nanotechnology [3]. The medical profession quickly adopted this technology as well, and the

A. K. Parchur · J. M. Jagtap · G. Sharma · V. Gogineni · S. B. White (✉) · A. Joshi (✉)
Departments of Biomedical Engineering & Radiology, Medical College of Wisconsin, Milwaukee, WI, USA
e-mail: aparchur@mcw.edu; jjagtap@mcw.edu; fgayatri@mcw.edu; vgogineni@mcw.edu; sbwhite@mcw.edu; ajoshi@mcw.edu

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application of nanotechnology in medicine was termed “nanomedicine.” Because NPs are similar in size to biological entities such as subcellular organelles, this technology is advantageous for multiple reasons. For example, NPs have the ability to transit through blood vessel walls and can penetrate into notoriously difficult locations such as the blood-brain barrier, they can avoid rapid filtration by the spleen, and they can traverse the 130–200 nm sieves in the liver composed of Kupffer cells [4]. Another advantage of their size is that they can efficiently interact with the biomolecules expressed on the cell surface. NPs even have the ability to be endocytosed and therefore can be used for intracellular imaging and/or targeted cell death. Besides bypassing barriers to drug transport and clearance, NPs can be modified for signal amplification and multiplexing to derive novel image-guided interventions. These multiplexed nanosystems, which can integrate both imaging and therapeutic modes, are included in an umbrella term “nanotheranostics.” If nanomaterials chosen for nanotheranostics are sensitive to external electromagnetic, sonic, or thermal fields, then complete spatiotemporal control on therapeutic action can be achieved by triggering drug release or nanoparticle-mediated ablation via external image-guided triggers such as light, RF/microwave, magnetic fields, ultrasound, or thermal fields. These remotely triggered nanotheranostics agents comprise one of the most active research directions in nanomedicine.

In the following chapter, we discuss in detail (a) recent advances in nanotheranostics that could be utilized for cancer therapy using external triggering mechanism, (b) mechanisms underlying nanoparticle design for response to external stimuli, and (c) current progress in clinical applications of remotely triggered nanomedicine. Finally, we summarize the current accomplishments, needs, and future directions in remotely triggered nanomedicine research for making a significant clinical impact.

17.1.1 Nanotheranostics

In the USA, the number of cancers diagnosed is sharply increasing with an aging population [5]. Chemotherapy and radiation treatment comprise the core treatments for most cancers, and multiple cycles are usually required [6]. These treatments have potentially life-threatening off-target effects such as anemia, appetite changes, oral mucosa inflammation, kidney dysfunction, dyspnea, cachexia, bleeding or clotting problems, gastric problems, fatigue, dermatological complications, immune dysfunction, sexual dysfunction, infertility, memory and cognitive deficits, and weight gain due to metabolic dysfunction [7]. These problems that primarily result from off-target effects and low therapy efficacy in tumor provide the motivation for developing nanoparticle-mediated therapies. Nanotheranostic platforms include nanostructured materials that combine diagnosis, targeted therapy, and real-time monitoring of therapy response. Structures, which can range from 10 to 100 s of nanometers, offer unique intrinsic properties such as multimodal imaging contrasts and both endogenous and/or exogenous therapeutic modes, which can be exploited for a range of cancer

interventions from early-stage detection to image-guided chemotherapy, radiation, or thermal ablation. According to recent global market statistics, the overall market of nanomaterials with theranostic application will exceed $187 billion in 2017 and may increase at a compound annual growth rate of 10.8%. This analysis includes gold NPs, fullerene C60, carbon nanotubes, quantum dots, antibodies, liposome, and proteins [8]. The specific uptake of NPs within the tumors is essential for effective cancer therapy. Enhanced permeability and retention (EPR) effect, which allows NPs to accumulate into the tumor sites through the aberrant tumor vasculature, is constrained by high interstitial hydrostatic pressure, and as a result, only a fraction of the injected NPs (~1–10%) reaches the tumor [9]. To overcome the delivery challenge, high surface-to-volume ratio of NPs is exploited by tagging targeting or additional therapeutic moieties and by incorporating imaging contrasts to optimize and quantify the tumor delivery. Further, by controlling the surface charge of NPs between −10 mV and +10 mV (neutral), the circulation half-life time can be significantly enhanced to maximize tumor delivery [9]. In spite of these modifications, passive nanocarriers comprise only a miniscule portion of the overall oncology drug market, and smart and targeted approaches to nanomedicine delivery at disease sites are an active area of research. Remote triggering of theranostic nanostructures can amplify and leverage the advantages gained by in vivo imaging, by directing therapy in a spatiotemporally controlled manner.

17.1.2 Remote Triggering Mechanisms

17.1.2.1 Rationale and Advantages of Remote Triggering

Active remote control of nanoparticle biodistribution via electric, magnetic, thermal, or sonic fields provide a way to increase the efficacy of nanomedicine by multiple methods. These include tailoring the biodistribution of NPs via external field guidance or a site-specific drug release to overcome the adverse off-target effects of cytotoxic drugs. While NPs geometry and size modification provide spatial control of drug distribution, remote triggering enables temporal control, which when combined with image guidance can precisely tune the delivery at desired time points and only in the tumor region, sparing normal tissue. Further, remote triggers can be multiplexed for separately enhancing imaging and therapy, e.g., photosensitizer-loaded NPs can be employed both for fluorescence image-guided surgery and for photodynamic therapy, by changing the illumination light wavelength [10]. NPs loaded with drugs and/or contrast agents can be precisely controlled for in vivo biodistribution and disease-responsive drug release in both a spatial and temporal manner using external triggering mechanisms such as ultrasound, X-ray radiation, optical and magnetic stimuli, radiofrequency (RF), or other portions of the electromagnetic spectrum such as microwaves. However, these triggered NPs introduce additional complexity, design constraints, and physics optimizations and need extensive preclinical validation and trials before translation [11]. In the following subsection, we describe the broad classifications for remotely triggered nanotheranostics.

17.1.2.2 Classification of Nanotheranostics by Triggering Modality

Figure 17.1 illustrates the classification of remote triggers by modality. The choice of a remote trigger modality is closely intertwined with the nanomaterial comprising the theranostic structure. The common feature of all triggering modalities is an energy transfer from the triggering energy source to the nanoconstruct. Triggers based on electromagnetic waves at different ends of the energy spectrum ranging from X-rays to microwaves [12] rely on engineering the cross sections of NPs, to preferentially absorb the incident energy and convert it to local thermal or mechanical effects for ablation or drug release. Depending upon the tissue penetration depths at different energies, and the absorption cross sections of nanomaterials, it is often a trade-off between imaging resolution/penetrance and energy transfer for therapeutic action. X-rays enable high-resolution 3D imaging at clinical spatial scales, but the nanoparticle cross sections at X-ray wavelengths are quite low, resulting in significantly lower therapeutic potential [13]. Near-infrared (NIR) optical wavelengths only propagate several centimeters in tissue via multiple scattering leading to low-resolution superficial imaging [14], but numerous nanomaterials have been devised with tunable surface plasmon resonance in the NIR region, leading to dramatic increases in absorption/scattering cross sections and consequently rapid (time scale of seconds) energy transfer for efficient photothermal ablation and drug release [15, 16]. Ultrasound triggering relies on mechanical energy transfer to echogenic theranostic contrast agents, with higher efficacy

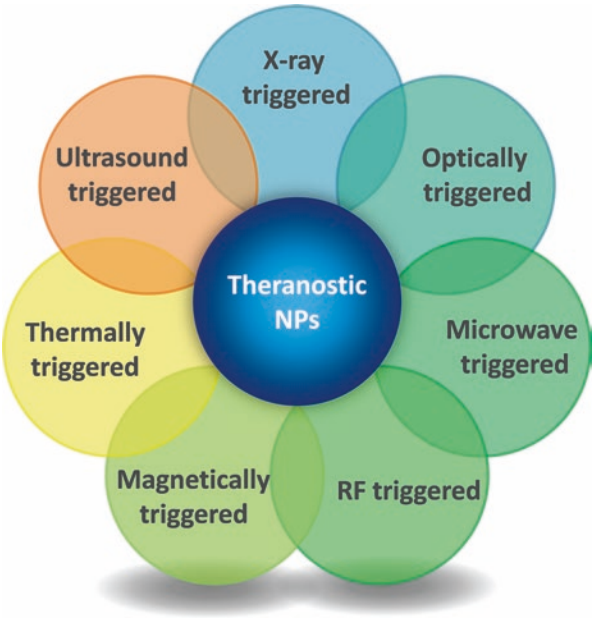

Fig. 17.1 Classification of remote triggers by modality

typically achieved in microbubble geometries, although recently novel nanosized configurations have been devised for cancer imaging and drug release [17]. Thermal triggers are one of the oldest and most well studied. Here the energy transfer is via a relatively slow (time scale of minutes) process of heating the tissue injected with thermally sensitive nanomaterials via conductive, convective, radiative, or a combination of all three heat-transfer modes [12]. Although electromagnetic in principle, RF and microwave-heated NPs also operate via thermal triggering at a fundamental level [18]. Similarly, alternating magnetic field-based heating of ferromagnetic agent containing nanostructures occurs over relatively longer time scales (~15 min), and drug release occurs via thermal triggers to heat-sensitive components of these constructs [19].

17.1.3 Triggerable Theranostic NPs

Remote-triggered theranostic NPs can be efficiently classified both via their triggering mode and their therapeutic applications. Table 17.1 summarizes the leading embodiments of nanoparticle systems triggered by X-rays, NIR light, microwave, alternating magnetic fields, ultrasound, and heat. Table 17.1 also identifies the reported therapeutic agents and the reported or potential imaging contrasts for these constructs and the NP size and charges if reported. A majority of these systems have been validated in small animal models of cancer, with thermally triggered and NIR light-triggered gold nanoshells, having advanced- to early-stage clinical trials. The theranostic nanosystems in Table 17.1 can be classified into three major categories based on their therapeutic mode: (a) the majority of remotely triggered nanosystems encapsulate extant chemotherapeutic agents, and the role of triggered release is site-specific delivery, and minimizing off-target effects associated with systemic chemotherapy, for example, porous/hollow nanostructures (hydrogel-capped Au, NPs, liposomes, etc.) release therapeutic drugs on triggering signal such as ultrasound. Due to their noninvasiveness, and incremental nature of innovation above chemotherapy delivery systems, extensive research has been done in this area in the past few years. However, these agents do not eliminate all off-target effects, as the NPs circulate systemically and their therapeutic action is not completely immune to drug resistance mechanisms evolved by tumors. (b) The second type of NPs has endogenous therapeutic action, which can be triggered to provide absolute spatial and temporal control of therapy delivery. NIR light-resonant nanostructures such as silica-gold or carbon NPs, a few microwave/RF triggered NPs, and alternating magnetic field-sensitive iron-oxide NPs can be employed as photothermally ablative agents, with the therapeutic action constrained at the disease site, and negligible or nonexistent off-target effects. These agents result in 100% tumor remission if sufficient NPs are delivered and are immune to drug resistance mechanisms. Photothermal agents have been extensively studied in small animal systems, with a few advancing to clinical trials. (c) Finally, a small number of triggered nanoparticle-based photodynamic therapy agents have been reported, which have similar efficacy

Table 17.1 Classification of remotely triggered theranostic NPs

Trigger mechanism	Theranostic NPs	Size (nm)	Zeta potential (mV)	Therapeutic agent/diagnostic contrast	Targeted cancer	Citation
X-ray triggered	DNA-coated AuNPs	~16	–	Doxorubicin (DOX)/X-ray	Widely applicable	[20]
	Lanthanide-based micelles	4.6	–	Hypericin/X-ray	Widely applicable	[21]
	Radiosensitive liposomes	100–200	–	Generic payload/generic	Widely applicable	[22]
NIR photothermal triggered	Nanomatryoshka ($Au/SiO_2/Au$)	137	–4.4	Plasmonic NPs/NIR fluorescence	Breast cancer, widely applicable	[23]
	Lipose Au (Liposome@Au)	~99	–	Liposome-gold plasmonic NPs /generic	Breast, fibrosarcoma	[24]
	$CaMoO_4$:Eu@Au hybrid NPs	40–70	+27.6	Plasmonic NPs/NIR fluorescence	Lung cancer	[25]
	$NaYbF_4$:Er/Gd-SiO_2@CuS	75	−10.5	Plasmonic NPs/upconversion, MR imaging, and X-ray	Breast/widely applicable	[26]
NIR photodynamic triggered	HPPH encapsulated liposomes	~100	–	HPPH/NIR fluorescence	Widely applicable	[27]
	Multifunctional core-shell-shell SiO_2 NPs	70	−30	DOX/NIR fluorescence	Widely applicable	[28]
	Purpurin18 peptide conjugates	~30 (dia) x 1000 (length)	–	Photodynamic NPs/ fluorescence	Widely applicable	[29]
	Liposomes	80–150	+3.0	Photodynamic NPs/ fluorescence	Widely applicable	[30]
	Porphyrin-phospholipid-doped liposomes	~100	~1.0	Photodynamic+DOX, gentamycin/fluorescence	Widely applicable	[31]
Microwave triggered	"Abraxane-like" NPs (three components – human serum albumin, paclitaxel, and indocyanine green)	~100	–	Paclitaxel/NIR fluorescence	Lung mets and subcutaneous tumors	[32]
	Fe_3O_4@TiO_2:Er^{3+},Yb^{3+}-glycine-VP16 NPs	51.8	28.4	Etoposide (VP-16)/ upconversion, MRI	Widely applicable	[33]

	IL-DOX-PCM@ZrO2 nanoplatforms	~237	−39.4	DOX/generic fluorescence	Widely applicable	[34]
Magnetic triggered	Fe_3O_4/DOX-loaded polymersomes	194 ± 52	−31.0	DOX/MRI	Widely applicable	[35]
	Dextran-coated iron oxide NPs	50	–	Antisense DNA/MRI	Widely applicable	[36]
	β-Cyclodextrin @Fe_3O_4	8.2	–	Magnetic hyperthermia+generic drug release /MRI	Breast cancer	[37]
	Clustered Fe_3O_4 NPs	225	–	Magnetic hyperthermia/MRI	Lung cancer	[38]
RF triggered	Au-coated magnetoliposomes	208	–	DOX/MRI	Widely applicable	[39]
	Sn^{2+}-doped alginate	100–200	−26	DOX/radiolabeled	Liver cancer	[40]
	Au-titania nanotube	20	−22.5	Indomethacin/X-ray contrast	Widely applicable	[41]
Thermally triggered	Polymer	57	−27.0	DOX/generic	Widely applicable	[42]
	ThermoDox NPs	–	–	DOX/generic	Widely applicable	[43]
	Cisplatin-loaded hydrogel NPs	90	−56.0	Cisplatin/generic	Breast cancer	[44]
Ultrasound triggered	Thermally sensitive liposomes (DOX-TSLs)	116	–	DOX/generic	Widely applicable	[45]
	Polymer-grafted mesoporous SiO_2 NPs	252	−31.6	DOX/ultrasonic	Solid tumors	[46]
	Peptide-hollow mesoporous silica NPs-L-arginine	410	−14	L-arginine (nitric oxide donor)/ ultrasonic	Pancreatic tumors	[47]
	Low-temperature sensitive liposomes	150–200	−22.3	DOX/ultrasonic	Colon tumors	[48]

as NIR photothermal therapy, that is, tumor remission is absolute and constrained only by nanoparticle delivery. These agents have low off-target effects, compared to free photodynamic therapy agents, as the biodistribution and clearance mechanisms for photosensitizers are altered. As indicated in Table 17.1, photodynamic NPs have been proposed for combined photo and chemotherapies as well, where the chemo drug release is controlled by the photodynamic treatment wavelength. In the following section, we discuss representative examples of these three variants of triggered nanotheranostics, grouped by the triggering modality.

17.2 Remote Triggering Mechanisms: Underlying Physics and Nanoparticle Embodiments

17.2.1 Electromagnetically Triggered NPs

Electromagnetic (EM) wave spectrum ranging from X-rays to microwaves is a preferred mode for triggering theranostic nanostructures. Due to their noninvasive nature, ease of use, rapid application, deep tissue penetration, and the availability of a variety of EM wave-generating equipment in clinical settings, EM waves have revolutionized the early-stage detection and treatment of cancer in the clinic. EM-triggered nanoparticles are designed to achieve high absorption or scattering cross sections for the desired wavelength spectra, thus increasing the probability of interaction and energy transfer from the incident excitation to the nanocomplex for increasing imaging contrast and/or drug release.

17.2.1.1 X-Ray Triggered Nanotheranostics

Due to their high penetration depth and ballistic nature of X-ray photon transport, X-rays have become an effective remote triggering tool for the controlled release of drugs from nanotheranostics in a highly controlled manner. Further, X-rays have a long history of use for both preclinical and clinical research for the diagnosis and treatment of cancer. The primary mode for harvesting X-ray energy for triggering is to employ high atomic mass or high Z-number bearing elements, which are biocompatible and functionalized for in vivo delivery. Among nanomaterials gold (Au) NPs have become eminent candidates due to its high absorption cross section for X-rays with energies overlapping with its K-edge electrons, which not only results in enhanced local radiation dose deposition for killing cancer cells via secondary radiation but can also be exploited for drug release. Recently, Antosh et al. functionalized ~1.4 nm Au clusters with maleimide and then conjugated with wild-type pH low insertion peptide (pHLIP) that can tether with cancer cell membranes [49]. An increase in radiation effectiveness was

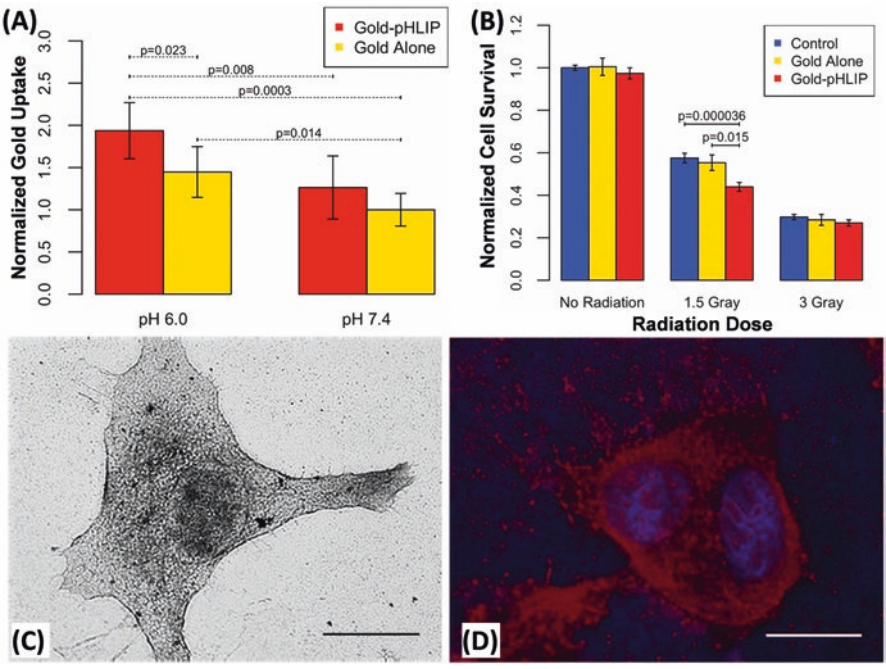

Fig. 17.2 (**a**) Uptake of Au and Au-pHLIP NPs at pH = 6 and 7.4, (**b**) the average cell survival rate at different irradiation doses at pH 6.0; (**c**) bright field and (**d**) fluorescence images of cells treated with Au-pHLIP NPs. (Reprinted with permission from [49])

observed with this construct due to localized radiation dose enhancement. Further the uptake of Au NPs was enhanced at pH < 6 which is a hallmark of many solid tumors (Fig. 17.2) [49]. A limitation of Au NP-based radiation dose enhancement strategies is the need for delivering NPs close to chromatin for the radiation-generated reactive oxygen species (ROS) to be effective in breaking DNA chains. To overcome this limitation, Starkewolf et al. functionalized Au NPs with DOX linked to Au NPs with DNA linkers (DOX-DNA-Au) and incubated them with MCF-7 cells (12 h) followed by radiation treatment. Radiation-generated ROS species broke the DNA tether and released DOX directly in the cancer cells. These NPs had increased toxicity (165% over non-radiated controls) on remote X-ray triggering (10 Gy) [20].

Recently, Ghaemi et al. have reported lanthanide ion (Eu^{3+}/Gd^{3+})-doped ZnO-based NPs as theranostic agents for remote X-ray triggering for the treatment of deep cancerous tumors. These semiconducting NPs also incorporated tumor-specific contrast agents for magnetic resonance (MR)/computed tomography (CT) dual-mode imaging [50]. Figure 17.3a illustrates the schematic representation of the creation of reactive oxygen species (ROS) on remote X-ray triggering, where Eu^{3+}/Gd^{3+} absorb X-ray energy and induce it to ZnO NPs, resulting in the formation of

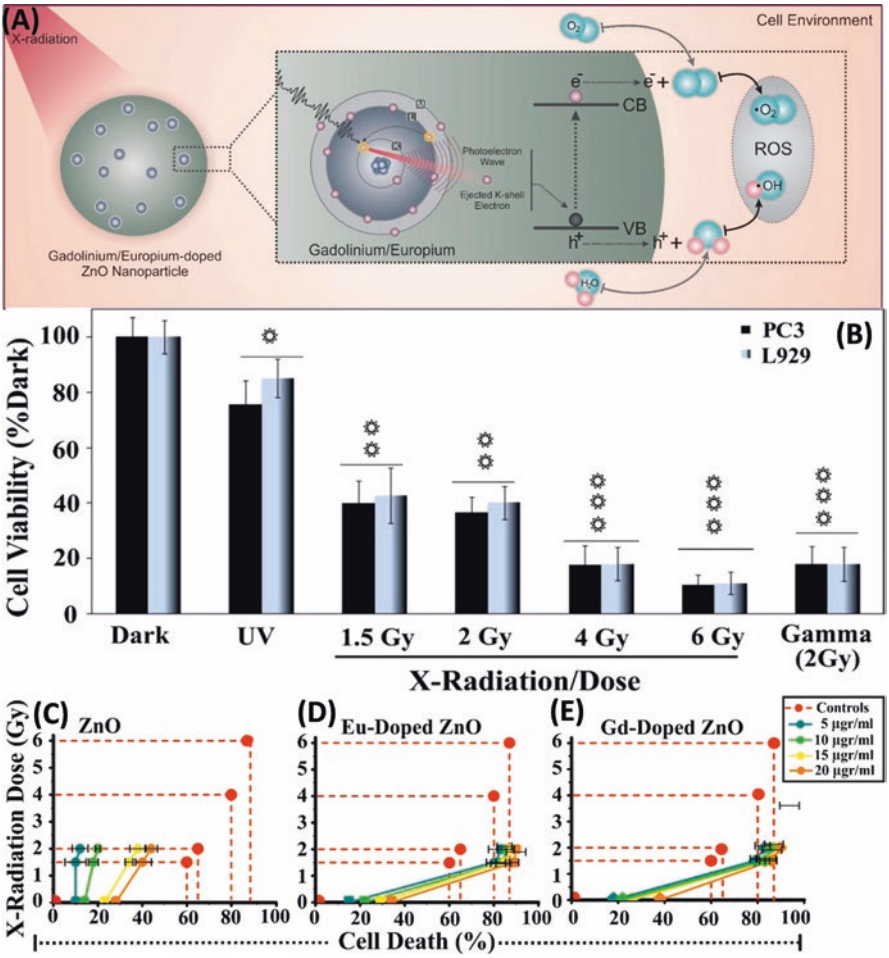

Fig. 17.3 (**a**) A schematic illustration of the "activation mechanism" for Eu/Gd-doped ZnO NPs under X-ray irradiation, (**b**) HeLa and PC3 cell viabilities after exposure to different radiation sources, and (**c**–**e**) effect of different doses of X-ray irradiation on NPs-treated and untreated cells and total cell death (%). $P < 0.001$ (***), $P = 0.001$–0.01 (**), and $P = 0.01$–0.05 (*) were significant. (Reprinted with permission from [50])

excitons in the ZnO host and the creation of additional ROS. These ROS then break DNA strands in tumor cells. They also studied the effect of remote triggering with UV light and gamma radiation. The average cell viability of PC3/L929 prostate cancer cells irradiated with UV and gamma rays (2 Gy) was found to be 24 and 60–90%, respectively, of X-rays (1.5–6 Gy) dosing (Fig. 17.3b). The spatiotemporal guidance of the cell death provided by X-ray triggering confirmed the therapeutic efficiency of Eu^{3+}-/Gd^{3+}-doped ZnO NPs (85%) as compared to bare ZnO NPs (40%) (Fig. 17.3c–e). Overall, an average three times enhancement in the X-ray irradiation effects with NPs was reported [50].

17.2.1.2 Optically Triggered Nanotheranostics

Optical wavelengths, and specifically NIR light in the 700–900 nm range, have unique benefits over X-ray and other high-energy radiation due to their non-ionizing nature, which is safe for tissue, and multiple centimeter propagation via multiple scatterings. Optically triggered nanotheranostics have features smaller than the wavelength of light and with specific geometries and material combinations; NPs with dramatically high absorption and scattering cross sections in the NIR region can be synthesized. In recent years a rapidly expanding selection of noble metal-based NPs with near IR resonances, such as nanorods [51], nanocages [52], and multilayer core-shell structures [53], have been demonstrated with strong absorption at NIR wavelengths. This family of NPs is inert, highly stable, and the best optical-to-thermal energy converters known. NIR absorption by these NPs gives rise to an intense photothermal response that delivers thermal energy to the local environment of the nanoparticle, inducing cell death [54]. Silica-Au shell-based NIR-resonant nanostructures have been extensively studied and proven in preclinical studies over the past decade. A second well studied variant is the Au nanorod, which with a width around 10 nm and the length around 40 nm exhibits strong NIR absorption at 808 nm (due to longitudinal mode of vibration). The surface plasmon band in the NIR region for nanorods can be tuned to higher wavelengths effectively by changing length to width ratio or the so-called aspect ratio of Au nanorods. Nanorods have been demonstrated for both X-ray CT and Raman contrast [55, 56] and studied as an NIR fluorescent contrast agent [57], thus demonstrating a versatile nanotheranostic behavior.

Recently, Ayala-Orozco et al. demonstrated a ~100 nm Au/SiO_2/Au nanostructure called nanomatryoshkas (NM) and compared it with previously reported ~150 nm SiO_2/Au nanoshells (NS). Both these nanostructures had plasmon resonance around 808 nm (Fig. 17.4a, b). The authors found that NM exhibited 24% higher photothermal efficiency than NS on triggering with 808 nm laser (Fig. 17.4c, d) [23]. Further, it was demonstrated that a 50 nm size reduction in nanomatryushka geometry, while keeping nanomaterial composition and surface charge constant, resulted in 4–5X higher nanoparticle accumulation in tumors with corresponding gains in therapy efficacy in mice bearing human breast cancer xenografts (Fig. 17.4e, f).

Optical triggering is not limited to photothermal action. Photodynamic agents, which generate ROS species upon NIR light illumination, can be incorporated in NPs, as photochemical triggers. Recently, Sine et al. incorporated photodynamic therapeutic agent (2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-a (HPPH)) into the core of the liposome and demonstrated liposome cargo release following low levels of laser light illumination in a noninvasive manner [27]. The design of photo-triggerable liposome structure with different drug incorporation capabilities is shown in Fig. 17.5a. The therapeutic agent from the core is released upon selective light irradiation at the same time as photodynamic therapy to surrounding tumor tissue. Proof of concept drug release was demonstrated via an increase in calcein fluorescence upon laser triggering (Fig. 17.5b). The NPs system demonstrated strong therapeutic action in mice bearing breast cancer xenografts (Fig. 17.5c). Similar photodynamically triggered structures have been demonstrated by Carter et al. [31].

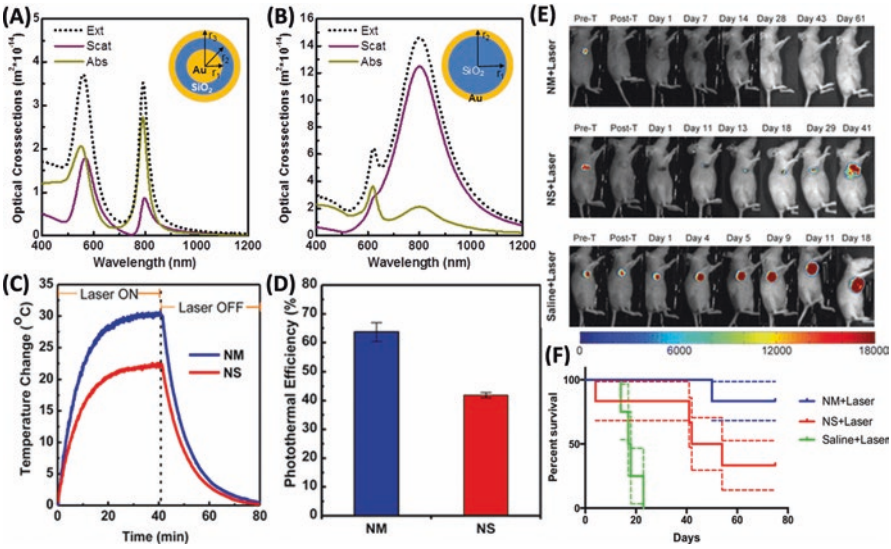

Fig. 17.4 Calculated extinction, scattering, and absorption cross-section spectra of (**a**) nanomatryoshka (NM), (**b**) nanoshell (NS); (**c**) their photothermal transduction and (**d**) photothermal efficiency (λ_{ex} = 810 nm, 2 W/cm^2). (**e**) The evaluation of tumor response to photothermal therapy by bioluminescence imaging in NM+Laser, NS+Laser, and Saline+Laser groups, and (**f**) their survival curves. (Reprinted with permission from [23])

17.2.1.3 Microwave and RF-Triggered Nanotheranostics

Microwaves in the frequency between 0.3 and 300 GHz and radio waves in 0.00003–300GHz electromagnetic spectrum have also been employed as triggers for theranostic NPs. The primary absorber for microwave radiation is water molecules. The dipoles in water molecules interact with oscillating electromagnetic radiation increasing the temperature of water molecules. Due to this phenomenon, microwaves/RF waves have been used as a noninvasive and nonionizing ablative agent. However, microwave-/RF-induced heating is not tumor specific. To improve the tumor specificity of microwave/RF ablation, coupling targeted microwave/RF susceptible materials (e.g., Fe_3O_4, ionic liquid@ZrO_2, NaCl@Na-alginate, etc. [33, 58, 59]) with low bio-toxicity has been proposed for focused energy deposition in tumors. These materials interact with microwave/RF radiation and generate heat above 50 °C in tumor tissue. Recently, Tan et al. synthesized hollow PDA NPs loaded with interleukins (ILs) (ILs/PDA) as microwave triggered agents and tested them in vivo for ablative therapy of solid tumors [60]. The synthesis scheme of ILs/PDA nanocomposites is depicted in Fig. 17.6a, and their TEM image is depicted in Fig. 17.6b. Figure 17.6c depicts the efficacy of microwave ablation in H22 tumor xenografts in mice. The ILs/PDA+MW group almost eliminate H22 tumors by the 14th day posttreatment, while in control groups, significant tumor growth was observed. In another example, Sreekala et al. reported NP-enhanced microwave ablation (10 GHz) for the treatment of a 1 cm tumor placed 20 mm from the surface

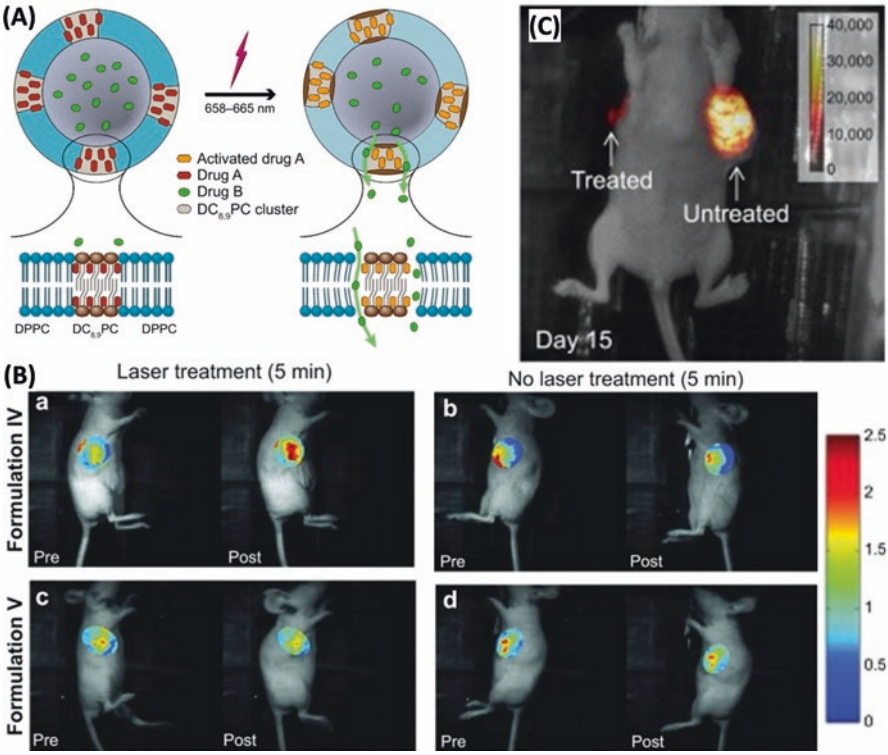

Fig. 17.5 (**a**) The rationale design of dual drug incorporated liposome structure. (**b**) In vivo fluorescence imaging of mice injected with liposomes loaded with calcein dye (**a**, **c**) pre- and post-laser treatment and (**b**, **d**) pre- and post without laser treatment. (**c**) Effect of laser treatments on luciferase expression and tumor regression in liposome-injected mice. (Reprinted with permission from [27])

of the liver. The specific absorption rate (SAR) of 130 W/Kg without NPs was enhanced to 251 W/Kg with NPs-treated tumors [61]. At the same time, the SAR value for normal tissue was found to be 2.5 mW/Kg, lower than the ablation threshold (0.4 W/Kg). NPs-treated tumors exceed 50 °C temperature in 40s of irradiation. These results confirm that the combination of theranostic NPs with microwave-guided ablation is effective in increasing the tumor dose while keeping normal tissue SAR in the safety zone.

Somasundaram et al. have demonstrated similar therapy efficacy gains with radiofrequency (13.6 MHz) ablation with nanomaterials in tumor-bearing Sprague-Dawley (SD) rats [40]. A biodegradable RF nanoprobe based on stannous (Sn^{2+}) cross-linked alginate NPs (100 nm in size) doped with DOX was synthesized. These NPs were demonstrated to have optimum uptake following 40 min exposure to liver cancer cells and demonstrated increased cytotoxicity on irradiation with radiofrequency (15 W) for 1 min. MR image of SD rat confirming the treatment of N1S1 tumor in the liver (Fig. 17.7a) and real-time infrared imaging revealed significantly

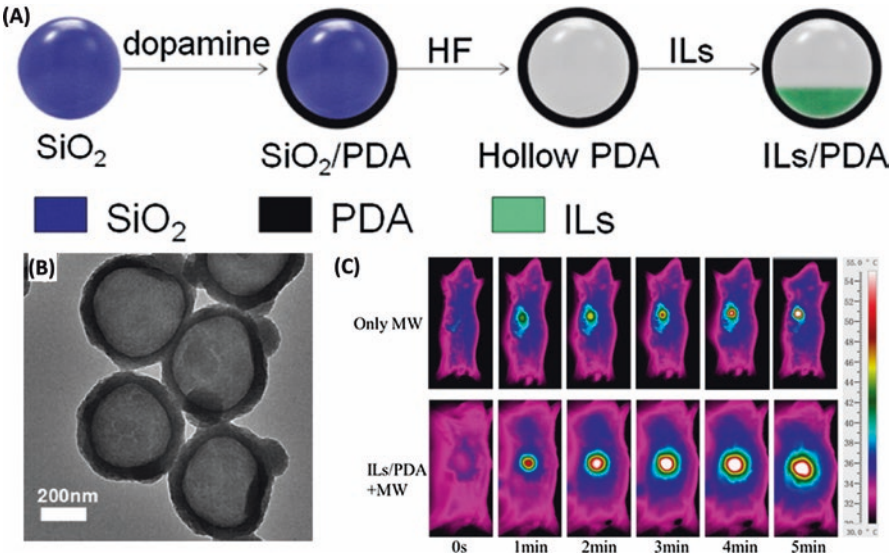

Fig. 17.6 (**a**) Schematic representation of the synthesis of ionic liquids/polydopamine (ILs/PDA) nanocomposites, (**b**) transmission electron microscopy image of ILs/PDA, and (**c**) near-infrared thermal imaging of ICR mice bearing H22 tumors under microwave (MW) treatment for 5 at 1 min intervals. (Reprinted with permission from [60])

higher increase in temperature (delta T of 20 °C) on RF triggering at the tumor site confirmed improved antitumor efficacy over controls. The ablation region in the liver was further confirmed by triphenyl tetrazolium chloride staining to mark metabolically inactive tissue and gross examination. The DOX loaded in the NPs was released in the liver tumor in an RF energy dose-dependent manner (Fig. 17.7b, c). At the same time, the same authors also studied the RF ablation response using biodegradable graphene in hepatocellular carcinoma cells. They reported that >85% of cells were killed upon RF exposure (100 W, 13.5 MHz) for 5 min [62].

17.2.1.4 Magnetically Triggered Nanotheranostics

Unlike optically triggered agents, NPs with magnetic contrasts have an advantage of being imageable via 3D cross-sectional and non-ionizing MR imaging. Magnetic NPs have been demonstrated for the early-stage detection of malignant tumors, metastatic lymph nodes, liver metastases, and inflammatory and degenerative diseases among other conditions [63]. Two variants of magnetic theranostic nanostructures have been reported. Gadolinium-based and iron oxide-based NPs are frequently used as T1 and T2 contrast agents, respectively. In recent years, there has been tremendous progress in designing nanomaterials with dramatically increased T1 and T2 relaxivity by exploiting NP geometries and high surface-to-volume ratio [64], with the objective of improving the sensitivity of clinical MR contrast agents.

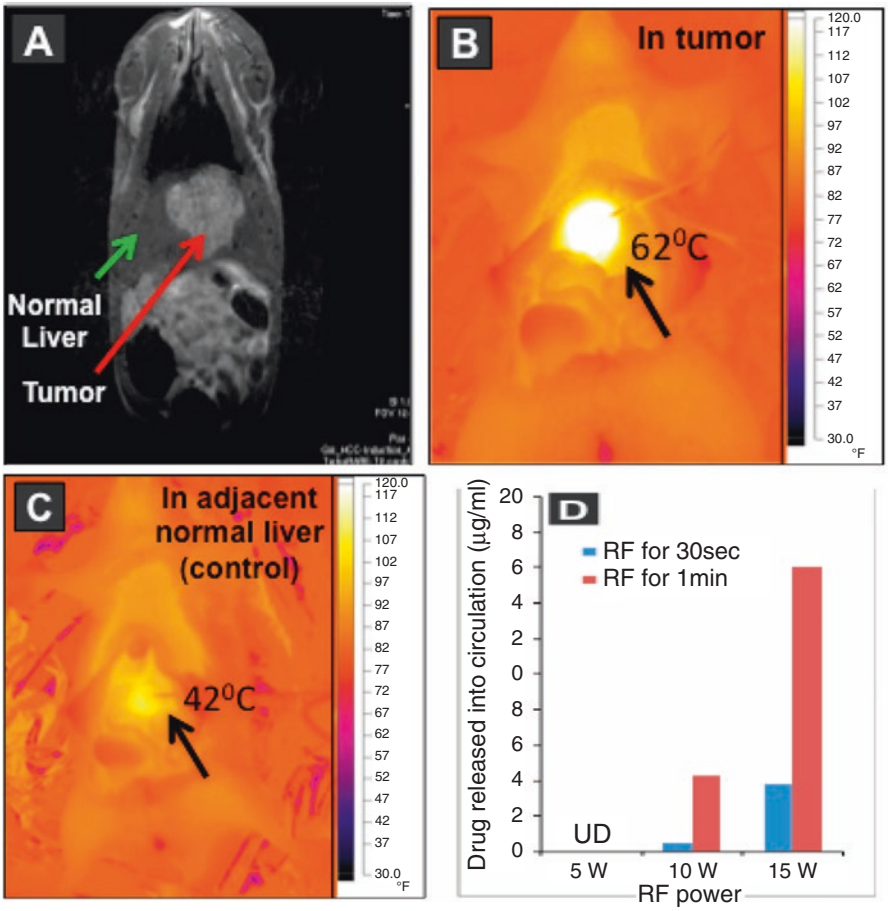

Fig. 17.7 (**a**) MR imaging of N1S1 tumor in the liver of SD rat. (**b**) Infrared imaging performed during the radiofrequency ablation (RFA) procedure showed higher heating ability at the tumor region and (**c**) the normal liver (control). (**d**) Spectroscopic evaluation of doxorubicin (DOX) concentration in circulation after open RFA at different energies and durations. (Reprinted with permission from [40])

Recently, Rotz et al. reported a new class of NPs with Gd(III)-conjugated DNA onto the surface of NIR-resonant gold nanostars (40 nm) that exhibit high relaxivity at both low and high magnetic field strengths. These particles show a remarkably r1 relaxivity up to 25 times higher than clinical contrast agents such as Magnevist [65]. T1 contrast bearing NPs typically have incorporated additional NIR-resonant nanostructures for providing externally triggered photothermal ablation. On the other hand, T2 contrast bearing NPs have a native therapeutic mode via alternating magnetic field-based heating, which can be exploited both for thermal ablation and drug release. Magnetic heating depends on size and the magnetization behavior of NPs [66]. Oscillating magnetic fields (~kHz-MHz) applied to 10–100 nm iron-oxide

NPs has been demonstrated for local tissue heating. A major challenge in magnetic hyperthermia is that inductively coupled magnetic fields require a very high concentration of Fe_3O_4, which can be difficult to achieve in tumors following systemic delivery. Another related problem is the off-target thermal effect, as NPs do not only accumulate in tumors but are present in the liver and spleen in significant amounts, whereas alternating magnetic fields affect the whole body [67]. These limitations have motivated the development of NPs that do not rely solely on thermal ablation as a therapeutic mechanism and utilize the thermal trigger for drug release with lower energy inputs. As an example, Hayashi et al. [19] have reported smart NPs comprised of iron oxide and DOX (Fe_3O_4/DOX/PPy-PEG-FA) that generate heat in response to an alternating-current magnetic field (ACMF) and sequentially release DOX directly at tumor location. The experimental setup is depicted in Fig. 17.8a. Excellent in vivo therapeutic efficacy was observed for combined magnetic hyperthermia and chemotherapy in mice with xenograft tumors (Fig. 17.8b). Chen et al. recently demonstrated use of poly(lactide-co-glycolide) microspheres (~13 μm in size) for the delivery of sorafenib/Fe_3O_4 NPs to the liver in a rabbit model under T2 MR image guidance using a catheter via hepatic artery. Significant enhancement in T_2^* and decrease in anti-CD31 microvessel density results confirm the delivery of NPs and release of drug to liver tumor (Fig. 17.8c, d) [68]. Furthermore, controlled release of drug/NPs may be possible on external triggering.

17.2.2 Thermally Triggered Nanotheranostics

Thermally sensitive liposomes (TSL) are the oldest and most studied theranostic constructs, and they have received much attention due to their safe drug delivery applications [69, 70]. Nanotheranostics that can respond to thermal triggering are of great interest in treating cancer in clinical cancer therapy. However, the major challenge is to develop efficient nanotheranostics with stability and high drug loading at the normal physiological temperature of 37 °C and that can release loaded anticancer drugs on application of a precise thermal trigger. Dewhirst and his co-workers have extensively demonstrated thermally sensitive liposomes (TSL) for chemotherapy delivery, and their cancer therapy efficiency has been evaluated in animal models [45]. These constructs release chemotherapy drugs (DOX (doxorubicin)) by disruption of the liposome's temperature-sensitive lipid bilayer membrane at 40–42 °C. Histologic assessment confirmed drug penetration for free DOX+HT and DOX-TSL+HT treatments groups from blood vessels in a flank tumor model. Also, the vascular pharmacokinetics and extravascular accumulation of DOX in tumor tissue over time were studied for free DOX and DOX-loaded liposomes with and without heat. Furthermore, Manzoor et al. [45] confirmed that the thermal triggering significantly enhances the drug uptake up to ~30 times higher than the free drug. Variants of thermosensitive liposomes loaded with MR imaging or optical contrast or drug contrast agents have been reported for experimental diagnostic imaging of the brain, liver, spleen, cardiovascular system, tumors, inflammation, and infections [45, 71]. While in a subject of

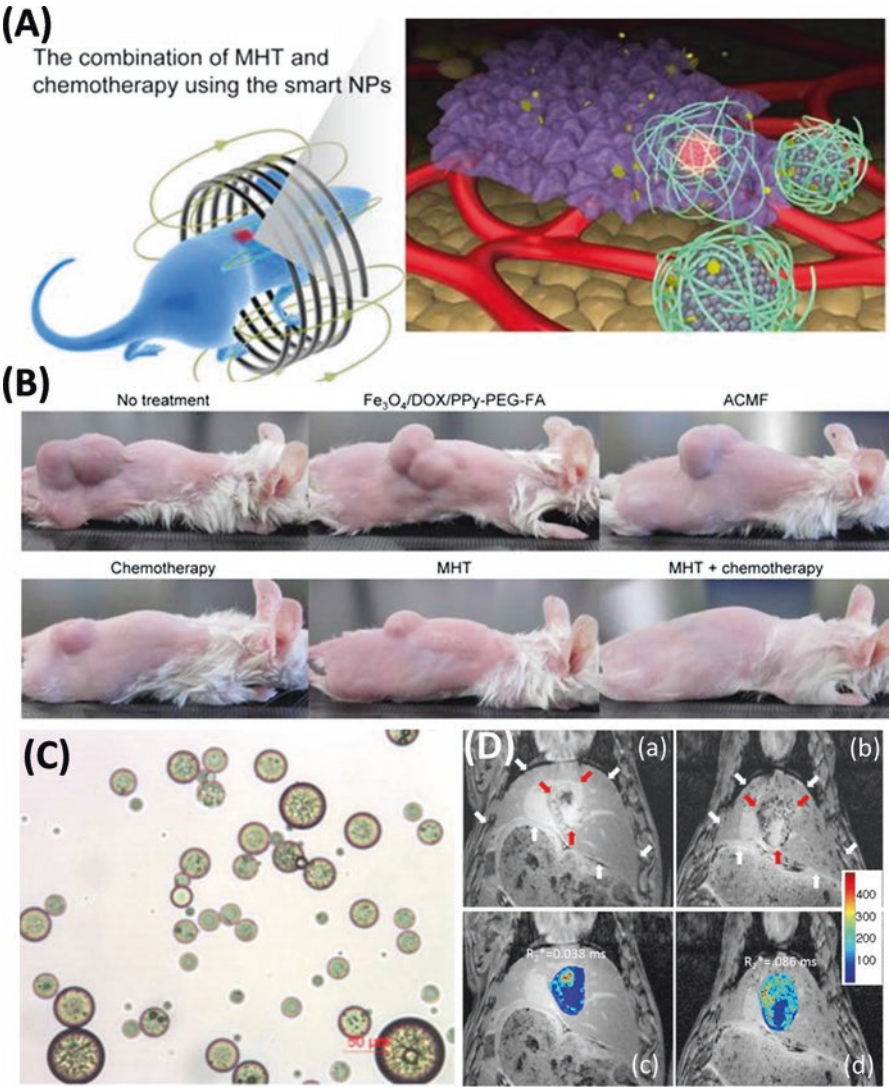

Fig. 17.8 (**a**) An experimental setup used for cancer treatment with magnetic hyperthermia treatment and chemotherapy using NPs and (**b**) digital photograph of the mice after different therapeutic treatments. (**c**) Confocal microscopic image of Poly(lactide-co-glycolide) (PLG) sorafenib iron oxide microspheres (scale bare = 50 μm). (**d**) T^*2-weighted MR image of the liver, (**a**) pre- and (**b**) post-procedural and superimposition of reconstructed R^*2 map of tumor upon preprocedural T^*2-weighted images (**c**, **d**). (Reprinted with permission from [19, 68])

numerous clinical trials and extensive preclinical validation, thermally sensitive liposomes are challenged by the constraints of delivering sufficient heat flux to deep tissue. Unlike, electromagnetic triggers, conductive heat transfer cannot assess deep tissue locations following surface application, without damaging normal surface tissue, thus limiting the available temperature stimulus.

17.2.3 Ultrasound-Triggered Nanotheranostics

Ultrasound is analogous to optical triggers in its non-ionizing nature, and as a thermally ablative modality via beam focusing, with the advantage of deeper tissue penetration, real-time imaging ability, high-resolution 3D imaging ability, and relatively low instrumentation cost [69, 72]. In addition to its excellent point of care diagnostic imaging capabilities, ultrasound waves are used as a therapeutic modality in combination with drugs and/or using focused ultrasound energy for local cancer treatment [73]. Focused ultrasound waves produce thermal effects, acoustic cavitation, mechanical effects, and other helpful modifications in tissue that have been exploited for ablation and drug delivery [74, 75]. For treatment, high-intensity ultrasound waves are focused at the center of tumor to increase the temperature to above hyperthermia regime to kill cancer cells. High-intensity focused ultrasound-based tumor ablation has been tested for prostate, kidney, and uterine tumors, but current applications are constrained by challenges of accounting for patient motion and heat deposition [76]. Focused ultrasound can also change the blood vessel and cell membrane permeability at insonation sites, thus enabling enhanced local penetration of drugs or nanoparticles both in tumor regions and intracellularly [77]. In recent years, multiple ultrasound-triggered theranostic NPs comprising of air or low diffusivity gas-filled bubbles with stable gas-liquid interfaces have been proposed and designed for simultaneous tumor imaging and therapy [76, 78]. These micro-/nanobubble contrast agents are typically loaded with an anticancer drug that releases on external ultrasound triggering. In a recent work, Min et al. designed a new approach, where glycol chitosan NPs were encapsulated by anticancer drug [79]. A schematic of echogenic chitosan-based NPs (Echo-CNPs) efficiently loaded with a chemo drug is illustrated in Fig. 17.9a. The formation of gas bubbling and the drug-releasing behavior from the Echo-CNPs and passive tumor targeting by the EPR effect and ultrasound-triggered drug delivery mechanism is depicted in this fig [79]. These methods have also been demonstrated for plasmid-based gene therapy [80]. Wong et al. used DOX-loaded magnetic NPs to treat breast cancer tumors in a mouse model using focused ultrasound therapy. Results confirm that the uptake of NPs/drug increased by 50-fold, resulting in the complete cure of cancer [81].

The clinical relevance for ultrasound-triggered NPs is high due to the low cost, ease of use, strong penetration, and integration of ultrasound as a point-of-care device in clinical practice. The limitation of ultrasound-sensitive NPs has been the decreased sensitivity compared to optically resonant NPs. However, with the recent improvements in nanobubbles, US triggers have gained clinical significance.

17.3 Clinical Applications of Remotely Triggered Theranostics

For deploying remotely triggered nanotheranostics effectively in clinical oncology, the hallmarks of cancer must be understood. There are six main hallmarks: (i) limitless replicative potential, (ii) tissue invasion and metastasis, (iii) insensitivity to

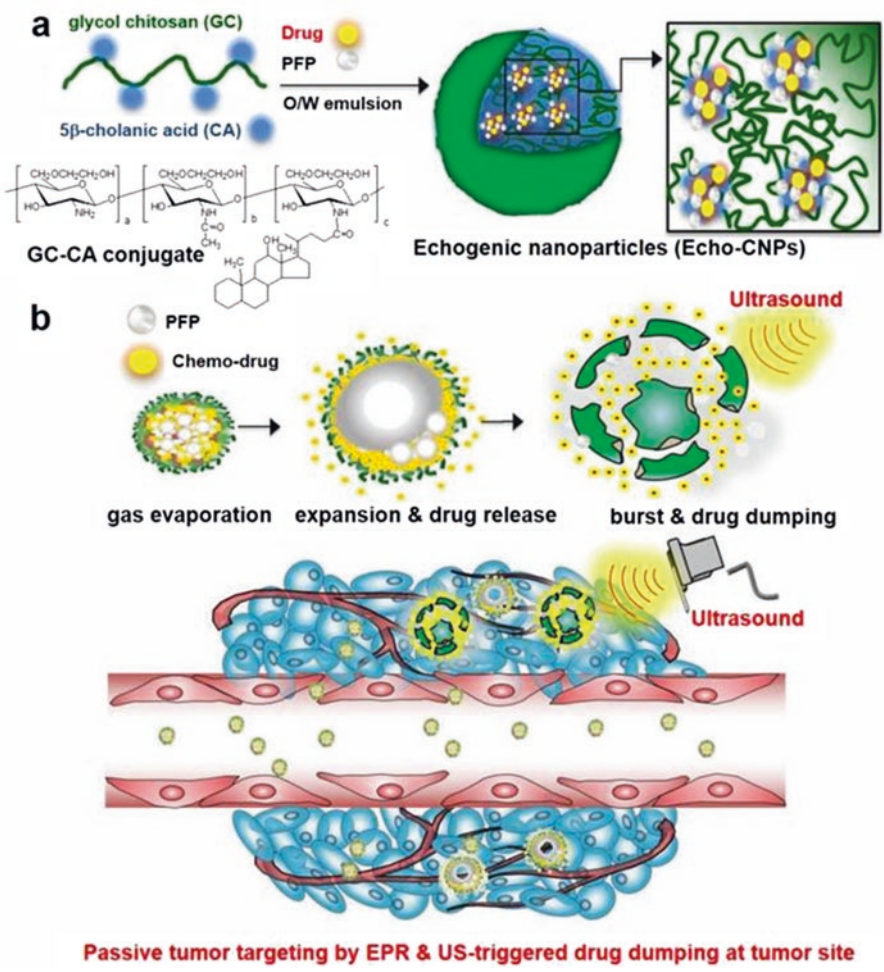

Fig. 17.9 (**a**) A schematic of the echogenic glycol chitosan-based nanoparticles (Echo-CNPs). Chemo drug was loaded into CNPs. (**b**) Gas bubbling and drug-releasing behaviors of perfluoropentane (PFP/chemo drug) co-loaded Echo-CNPs and passive tumor targeting by enhanced permeation and retention (EPR) and ultrasound (US)-triggered drug delivery. (Reprinted with permission from [79])

anti-growth signals, (iv) self-sufficiency in growth signals, (v) evasion of apoptosis, and (vi) sustained angiogenesis. In addition to these, two additional hallmarks and two enabling characteristics were recently added, which are (vii) deregulating cellular energetics, (viii) avoiding immune destruction, (ix) genome instability and mutations, and (x) tumor-promoting inflammation. Most cells acquire these properties during their development into cancer cells, which are usually the result of mutations in relevant genes [82, 83]. This results in large differences between tumor cells and the tumor microenvironment and the microenvironment found in normal tissue. Tumor vessels are typically abnormal and are notoriously "leaky" owing to the

rapid proliferation of endothelial cells and decreased number of pericytes, resulting in large pores ranging in size from 100 nm to several hundreds of nm in diameter versus normal vessels whose pores are between 5 and 10 nm [84]. In addition, tumors also have poor lymphatic drainage, which results in the retention of NPs (due to EPR effect).

At present, when standard intravenous conventional chemotherapeutics are given to patients, the drugs are distributed throughout the body, where they affect both cancer cells and normal cells. However, NPs preferentially accumulate in tumors by the EPR effect. The differential biodistribution helps nanoparticle-coupled drugs to achieve a higher drug concentration within tumors while demonstrating lower concentrations of drug in normal tissues [85, 86]. This also translates to a higher therapeutic efficacy, with lower systemic toxicities. In addition, if engineered appropriately, these probes can be seen on imaging which can help to confirm local delivery and in certain cases quantify the amount of drug within the tumor [87]. There are, however, several limitations to broad clinical applications of NPs. Upon systemic administration of NPs, sequestration into the spleen and clearing by the reticuloendothelial system limit their delivery to target tumors leading to off-target delivery to healthy tissue. Furthermore, several groups have shown that although tumors do demonstrate an EPR effect, it is very heterogeneous for large tumors and metastatic lesions, limiting overall NPs uptake. Ligand-based targeting strategies designed to circumvent these issues have been largely ineffective at increasing total tumor uptake due to tumor heterogeneity [88–92]. Nonetheless, drug-loaded and site-directed liposomes that rely on antibody-mediated targeting are currently undergoing phase I/II clinical trials (NCT00470613, NCT00964080, and [93]). While nanoformulation trial with CALAA-01 was terminated (NCT00689065), another polymer-based second-line therapy trials with BIND-014 are actively underway in different cancers (NCT01792479, NCT01812746). Although the trials on dextran polymer-based formulation of camptothecin (CRLX101) has proven to be safe in patients [94], most of the ongoing trials with CRLX 101 were designed to use combination therapeutic strategies (NCT02648711, NCT02010567, NCT02389985). Taken together, most of the FDA-approved nanoformulations are liposome based and have physical interactions as a similarity [95]. To date, there have been over 16 clinical trials evaluating the safety and efficacy of NPs in clinical practice (see Table 17.2).

The field of tunable or stimuli-responsive NPs using organic and inorganic systems for imaging and therapeutic applications is under intense investigation with enormous preclinical success as well [96]. Clinical trials to evaluate some of these platforms have also started yielding results. A trial evaluating laser-triggered gold nanoshell therapy (AuroLase therapy) in 22 prostate cancer patients has shown no adverse effects and good safety profiles during the 6-month follow-up (NCT00848042 and [97]), correlating well with animal studies [98]. However, AuroLase therapy in head and neck cancers was shown not to be as safe, with 4 of the 11 patients developing adverse events (NCT00848042). Additionally, phase I clinical trials are underway utilizing MRI-/US-guided laser irradiation with gold NS (AuroShell) for the treatment of prostate cancer (NCT02680535).

Table 17.2 Remotely triggered NPs in clinical trials[a]

Theranostic NPs	Therapeutic agent	Trigger mechanism	Cancer targeted	Study phase	Sponsor	Clinical trials.gov identifier no.
Liposomes	DOX	High temp (~39 °C) causes release	Liver (hepatocellular)	Phase III	Celsion	NCT00617981
	DOX	Focused ultrasound	Liver	Phase I	University of Oxford	NCT02181075
	DOX	Hyperthermia	Breast	Phase I, Terminated (funding)	Duke University	NCT00346229
	5-fluorouracil/ interferon-a/liposomal DOX	Hyperthermia	Breast Endometrial Cervix, Ovarian Neoplasms	Phase II	University of Texas Health Center	NCT00178802
	Fluorouracil & pegylated liposomal DOX hydrochloride	Hyperthermia	Breast Endometrial Cervical Ovarian	Phase I/II	University of Texas Health Center	NCT00003135
	DOX HCl liposomal injection	Hyperthermia	Rhabdomyosarcoma Neuroblastoma Sarcoma Ewing sarcoma Osteosarcoma	Phase I	Theodore Laetsch	NCT02557854
	Pegylated liposomal DOX hydrochloride	Hyperthermia/microwave therapy	Prostate	Phase I	Celsion	NCT00061867

(continued)

Table 17.2 (continued)

Theranostic NPs	Therapeutic agent	Trigger mechanism	Cancer targeted	Study phase	Sponsor	Clinical trials.gov identifier no.
	Thermodox	Hyperthermia/microwave therapy	Breast	Phase I/II	Celsion	NCT00826085
	Doxil	Heat treatment	Breast cancer	Phase I/II	National Center for Research Resources	NCT00006433
	Vincristine, DOXIL (DOX HCl liposomal injection), and dexamethasone	Hyperthermia	Multiple myeloma Myeloma M-protein Myeloma proteins	Phase III	Johnson & Johnson Pharmaceutical Research & Development	NCT00344422
Polymeric NPs	siRNA	pH drop (<6.0) in endosomes	Cancer, solid tumor	Phase I	Calando Pharmaceuticals	NCT00689065
Fe_3O_4 NPs	No drug; thermal ablation	Magnetic (apply magnetic field, NPs heat up, and ablate the tumor)	Prostate	Phase 0	University College London Hospitals	NCT02033447
Gold shell, silica core NPs	No drug; thermal ablation	NIR	Prostate	Phase I	Nanospectra Biosciences	NCT02680535

[a]Data in the table has been adapted from https://clinicaltrials.gov/

Because systemic infusion of nanomaterial is not optimal due to the EPR effect [98], a safety phase 0 trial of "Magnablate I" is underway, whereby iron oxide NPs are directly injected into the prostates of men prior to cystoprostatectomy. Ex vivo evaluation is being performed to determine the retention of NPs (NCT02033447). Although the ultimate goal was to use magnetic fields to stimulate NPs for thermal ablation, the status of recruitment and outcomes of this trial is still unknown (NCT02033447). ThermaDox, a temperature-sensitive liposomal DOX formulation and a successor of Doxil, was given together with hyperthermia in the treatment of women with locally recurrent breast cancer in two different phase I trials (NCT00346229, NCT00826085). It was found that microwave-triggered release of DOX at the highest tested concentrations was safe and overall local response was 48% [99]. Although the partial success and recurrence in the untreated areas were attributed to advanced stages of cancer in the patients, technological improvements and active targeting were warranted. The results of a ThermoDox® phase III trial (HEAT trial, NCT00441376) of RF ablation of primary and metastatic tumors of the liver did not provide sufficient evidence for the clinical effectiveness of ThermoDox® as measured by the primary endpoint of trial. However, the findings from the HEAT trial led to an ongoing standardized RFA for the treatment of hepatocellular carcinoma (HCC) (OPTIMA trial) (NCT02112656) and targeted chemotherapy with ultrasound (TARDOX) trial (NCT02181075) [100].

Radiation-sensitive hafnium oxide NPs (NBTXR3) have, however, shown cancer cell death in vitro [97] and antitumor effects with intratumoral injections in preclinical animal models [101] and is now in a phase I clinical trial (NCT01433068, [102]) NBTXR3. The results suggested a good safety profile in humans, and its initial success has led to an ongoing phase II clinical trial in soft tissue sarcoma (NCT02379845). Multiple clinical trials with NBTXR3 and combination treatment regimen in unresectable rectal cancer (NCT02465593) and different routes of infusion in squamous cell carcinoma (NCT01946867) and HCC (NCT02721056) are also underway. Site-selective delivery may improve safety and efficacy. However, there is no clinical data available yet. Exploitation of tumor-specific phospholipase A2 as a biological triggering mechanism for the selective release of cisplatin from liposomes (LiPlacis) has shown superior pharmacokinetic parameters compared to free cisplatin in mouse models [103]. A phase I clinical trial to assess adverse events and safety and tolerability of LiPlacis is underway (NCT01861496). Besides relying on EPR for tumor-specific accumulation, varied expression of phospholipase A2 in solid tumors may pose additional challenges for this approach.

Despite having several theranostic NPs in the product pipeline and preclinical evaluation [104], currently, very few of the diagnostic NPs have made it to clinical trials with enormous impact on the imaging of human disease. [^{18}F]FAC-based PET imaging is a classic example wherein tumor models [105] and biodistribution trials on healthy volunteers indicated safety [106] opening widespread possibilities for noninvasive imaging in autoimmune diseases and cancers. Similarly, MR imaging with superparamagnetic NPs for diagnosis and staging of cancers was shown in some clinical trials [107], and several therapeutic strategies are under development.

17.4 Future Outlook and Translation Challenges

Even two decades after initial FDA clearance of nanoparticles as drug delivery vehicles, only liposomes have penetrated the oncology market. However, both diagnostic and therapeutic functionalities are not available in any of the currently approved delivery systems, e.g., Doxil or Abraxane. It is quite encouraging from a technology development point of view that most of the existing generation of therapeutic nanoparticle systems in current clinical trials (Table 17.2) either use selective targeting to enhance bioavailability or stimuli-responsive mechanisms to control the drug release in specific tissues, reflecting the increased realization in the clinical community of the importance of targeting. This enthusiasm is tempered by the fact that theranostic agents have not yet made it into clinical practice. Furthermore, the clinical challenges of nanomaterials both in terms of safety concerns, cost-effective strategy, and therapeutic efficacy are often very intriguing and require combination approaches, which still needs to be investigated comprehensively. Nanotheranostic agent development and clinical translation is hampered by multiple factors: (i) at the bench, scale challenges range from technological issues like good manufacturing practices for scaling up the synthesis of nanomaterials with multimodal and hard to standardize components. Most preclinical work is conducted with rodent models ranging from 20 to 100 g body weight. Scale up of multimodal nanoparticle synthesis for a 70 Kg human patient, while maintaining consistency of elemental composition, size, polydispersity, porosity, charge, contrast intensity, remote trigger sensitivity, drug release rates, etc., is challenging, especially in the absence of investments from major pharmaceutical companies. Different imaging/trigger modalities require further careful characterization of second-order physical properties such as absorption/scattering cross sections for optical triggers, and thermoelastic behavior for ultrasound triggers, and magnetic susceptibility and relaxivity for MR imaging and magnetic triggering. (ii) Theranostic nanoconstructs bearing a multiplicity of components that can disintegrate in vivo are likely to face additional scrutiny especially as certain imaging agents such as Gd^{3+} for T1 MR imaging are known to be toxic in free form [108]. (iii) A third concern for nanoparticle translation arises from the nanoscale geometry itself. The high surface-to-volume ratio of nanoparticles provides for hitherto unknown in vivo reactivity and off-target effects [109, 110]. (iv) Translation of combined diagnostic and therapeutic agents is further stressed by the complexities of clinical trial design and patient. Imaging agents are typically microdosed and the FDA approval process is simpler, as the toxicity and safety concerns are limited or nonexistent at microdose levels [111]; however, therapeutic doses are typically much higher, especially for nanoparticles bearing chemotherapy payloads. Thus, even for imaging applications, a theranostic construct can face extensive preclinical validation and safety/toxicity studies. The nanotechnology characterization laboratory of NCI (http://ncl.cancer.gov) has developed safety assays and characterization protocols for nanomedicine agents in collaboration with the FDA, and these developments should accelerate preclinical and translational developments of remote-triggered nanotheranostics as well.

In spite of the above concerns, there is a strong incentive for government and industry to invest in disease-targeted theranostic nanomedicine, as the potential benefits in reducing off-target effects, and higher therapeutic efficacy dwarf the challenges in development. This has resulted in tremendous growth of the "nanomedicine market" in North America and the world. According to Grand View Research Inc., the global nanomedicine market is foreseen to reach $344 billion by 2024 [112].

To accelerate clinical acceptance and translation, it is imperative that the development of hybrid/multifunctionalized and triggered nano-platforms be explored in conjunction with combination strategies to synergize with existing approved chemo and radiation therapies. With the rapid advance of bio-nanotechnology and the initiation and completion of further phase I trials, the investments by medical technology and pharmaceutical industry are expected to increase, and triggered nanotheranostics should exhibit rapidly increasing clinical use over the next decades.

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Chapter 18
Concluding Remarks and the Future of Nanotheranostics

Janel L. Kydd, Praveena Velpurisiva, Stephanie A. Morris, and Prakash Rai

The year 2018 is predicted to have more than 1.7 million cases of cancer diagnosed with approximately 609,640 mortalities resulting in equivocating 1700 deaths per day [1]. Of these cancers diagnosed, the most fatal and prevalent are lung, prostate, and colorectal in men and breast, lung, and colorectal cancer in women [1]. Prostate cancer will account for 20% of the oncologic disease incidence in men, while breast cancer will account for 63,960 cases in women [1]. The daily diagnoses of 4700 affected individuals render this type of pathology prominent and in dire need of finding more ways to effectively diagnose and treat patients, thus reducing the mortality associated with these various types of cancer [1]. A summary hereto to elaborate and reflect upon these efforts to improve the lives of patients and the rationale behind such attempts to further improve on detection and diagnosis and effectively treat cancer pathologies are discussed as we conclude this book.

The concept of personalized, targeted, multifunctional, and cohesive therapy paradigms involving nanomedicine has been focused upon throughout the chapters of this comprehensive discussion. The simultaneous integration of several

J. L. Kydd · P. Velpurisiva
Department of Biomedical Engineering and Biotechnology, University of Massachusetts Lowell, Lowell, MA, USA

S. A. Morris (✉)
National Cancer Institute, NIH, Nanodelivery Systems and Devices Branch, Cancer Imaging Program, Division of Cancer Treatment and Diagnosis, Rockville, MD, USA
e-mail: morriss2@mail.nih.gov

P. Rai (✉)
Department of Biomedical Engineering and Biotechnology, University of Massachusetts Lowell, Lowell, MA, USA

Department of Chemical Engineering, University of Massachusetts Lowell, Lowell, MA, USA
e-mail: prakash_rai@uml.edu

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functionalities into a single platform for diagnostic imaging and therapeutic applications provides a potent new paradigm for advancing treatments against cancers and other diseases. The dual functionality that can be implored in designing nanoparticles that co-encapsulate chemotherapeutics and imaging agents provides real-time monitoring of such treatments and guided delivery, creating a more concrete foundation upon which clinicians, researchers, and insurance providers can join efforts and deem these interventions necessary and worthwhile for coverage and part of commonplace patient disease monitoring and treatment methods [2–7]. The defining characteristics, obstacles, attempts to resolve, and future of medical interventions utilizing nanomedicine with a focus on nanotheranostics have been discussed at length in the chapters entailing this book.

The growing field of theranostics is quickly enabling the move from the conventional, "trial and error" medicine to a more personalized approach that holds considerable potential for superior clinical outcomes. Theranostics provides a useful tool for classifying, stratifying, and choosing patients with a specific molecular phenotype predictive of affirmative response to a specific drug. Theranostics has the ability to help improve drug potency by helping a physician understand which patients will receive the greatest benefit from a particular drug. The specific targeting virtues of a theranostic will help to enhance the drug safety profile, reducing off-target toxicities to healthy tissues, a common problem with chemotherapies. Also, from an economic standpoint, theranostics could result in cheaper and effective drug programs, directing preclinical drug development or clinical trial design to assist in amplifying the possibility of positive results.

The combination of molecular targeting drugs and imaging agents in a nanoplatform facilitates screening of patients and helps us to assign them to a set treatment modality and monitor the tumor progression after the treatment, with the help of contrast agents. Since it helps us to identify the proteins (markers) expressed on the surface of certain types of tumor cells, metabolomics and nanotheranostics enable a personalized treatment to such patients instead of a brute-force method of administering the drugs to all patients suffering from that cancer type. Patients can be carefully selected in a multistep process using a combination of companion diagnostics, image-guided therapy, and theranostics as described below: a companion diagnostic will predict if nanoparticles are likely to accumulate in the tumor due to EPR effect, and imaging will confirm if the nanotheranostics accumulated in the targeted site due to the EPR effect, helping the clinicians to sort the patients with low and high accumulation, followed by conventional treatments to those emitting low signal while administering personalized nanomedicine to those emitting high signal observed from imaging. The second step will involve evaluating patient's response to the intervention and, if required, initiating a secondary treatment.

Nanoparticles have unique properties such as surface modification with peptides or ligands for increased targeting to tumor tissues; size distribution, which can be customized to the required application like crossing of the blood-brain barrier (BBB); as well as surface charge modification and biocompatibility. Such features were discussed in the preceding chapters in reference to Doxil®, the FDA-approved liposomal form of doxorubicin [8–12]. The leaky tumor vasculature and poor

lymphatic drainage can be exploited for enhanced permeability and retention of nanocarriers which, upon avoidance of recognition by the reticuloendothelial system, can deliver drug payload to the tumor directly by passive diffusion [13, 14]. Nanoparticles between 100 and 200 nm have shown ideal avoidance of both renal clearance and RES recognition that provide longer circulation time in the blood [5–7, 15]. Surface modifications such as pegylation have also shown promise in extending the blood circulation time of nanoparticles by a similar method of avoiding macrophage uptake and minimizing accumulation in MPS organs [16, 17]. Systemic toxicity is reduced using nanoparticles whereby a higher payload of drug therapy can be contained within nanocarriers, delivered to the site of interest, and spare healthy tissue and organs the deleterious side effects associated with cancer therapies [17, 18]. The ideal nanoconstruct will encompass multiple modalities that include therapy, imaging, and targeted drug delivery, in addition to controlled, sustained release to provide optimal efficacy and outcomes in patient overall survival. This ability of nanomedicines to serve as both therapy and diagnostic or monitoring system, i.e., as a nanotheranostic, is being honed by medical insurance providers, whereby the previous descriptor of drug value was placed on progression-free survival and overall response [19].

The ultimate goal of theranostic nanomedicine research is to effectively use nanodiagnostic, nanotherapeutic, and nanotheranostic platforms in patient care. Several nanodiagnostics and nanomedicines have successfully evolved from the bench to bedside as discussed throughout this book [20]. The design of new cancer therapeutics and diagnostics, like nanotheranostics, must be based on the greater understanding of cancer biology, the tumor microenvironment, and new mechanisms of drug resistance. The major challenges in the success of nanotheranostics have been centered upon the characteristic tumor microenvironment which is described as acidic, heterogeneous, and hypoxic with high interstitial fluid pressure (IFP), leading to enhanced cell mutations and adaptive metabolic rerouting. This has rendered many treatments futile over time due to the evolving, caustic, and multidrug-resistant population of cancer cells [17]. Cell intravasation, tumor tissue stroma, immune system recognition and subsequent macrophage uptake and lysosomal degradation, inadequate tumor uptake with slow diffusion, and difficulty with homogeneous drug distribution at the tumor site are among the prevalent concerns of scientists developing contingent nanotherapies [17]. The importance of the surface chemistry of nanoparticles must be emphasized as opsonization systemically and cell uptake at the cellular level are highly affected by ligand-binding and surface charge characteristics. Adsorption of serum proteins can cause RES sequestration and render nanotherapy null; therefore methods such as pegylation can certainly increase the stealth capabilities of nanocarriers when polyethylene glycol (PEG) chain length and grafting density are strategically placed. If the physicochemical composition of nanoparticles is optimized and carefully considered, the beauty of nanotheranostics can be revealed through the multitude of implementations of noninvasive biophysical imaging and tracking methods such as MRI, optical fluorescence, computed topography (CT), and positron emission tomography (PET). Ultrasound (US) imaging can be combined with medicinal

treatment in nanotherapy for drug release observation, biodistribution, and therapeutic efficacy. Alternative treatments that include precise control of drug activity by localized, remotely triggered, targeted treatments, photothermal therapy (PTT), and photodynamic therapy (PDT), for example, may be utilized in combination with nanoparticle drug administration [21, 22]. These remarkable capabilities will shape personalized cancer care in the future.

What is the necessity for an individual approach? No two people are alike. Our genetic diversity comprises our intrinsic unique makeup. This basic underlying principle is lacking in the therapies designed to treat disease processes, including cancer. In order to effectively and safely cure disease or control its progression, biomarkers must be identified for each person's type of pathology using microfluidic detection and serological analysis via a solid genomic understanding of the type of cancer under consideration. This method of considering tumor heterogeneity and patient stratification is followed by tumor characterization which must be paired with an ideal drug combination match based on predictive mathematical modeling and statistical analysis software development for nano-human body interactions. Better simulation models using software such as Simulink® will help us simulate the in vivo tumor environment and their interaction with the normal cells. A collaborative effort between theoretical and experimental scientists will help us design new experiments and techniques that aid in a better understanding of the disease and thereby design effective strategies to combat the disease. The clinical relevance of nanotheranostics must be improved using reproducibility in scaled-up synthesis of nanoparticles, regulatory protocols which ensure sterility and safety in human administration, drug to imaging loading capability, well-understood pharmacokinetics and pharmacodynamics, as well as ideal risk to advance estimation and cost-benefit analysis. The following paragraphs highlight some unmet needs and how to address these as we move to advance the highly multidisciplinary field of nanotheranostics.

There is a growing need for employing multidisciplinary approaches in optimizing the design and synthesis of nanosystems. Through collaboration, researchers from different disciplines such as engineering, sciences, pharmaceutical industry, and medicine can accomplish more by teaming together. There is a specific need for integrated research and clinical teams to effectively pair nanotechnologies with the most critical needs in cancer medicine and implement them into clinical care. There is also a continuous demand for fundamental research to understand mechanisms of how nanotechnology works at the cellular and tissue levels for effective translation of novel theranostic platforms into the clinic. Multidisciplinary research should try to move beyond basic collaboration to pooling together useful nanoparticle data, protocols, assessments, and principles from several disciplines to strengthen basic understanding, which in turn helps to solve actual biomedical problems. Interdisciplinary research necessitates that a researcher obtains a depth of information in multiple disciplines and be fluent in their jargons and procedures or more often that multidisciplinary groups bring together and create a shared research language and structure for constant innovation and discovery. Such multidisciplinary research teams that conduct fundamental, translational, and clinical research need to

continue to come together to ensure success in cancer theranostics. A multidisciplinary approach is key to providing the right drug delivery systems while researching diseases such as cancer [23]. Material scientists will provide means to improve the stability of the nanoparticles and offer various organic and inorganic materials that are biocompatible and biodegradable. Chemists design and synthesize drug delivery vehicles that are stable by functionalizing the surface of nanoparticles and enable the sustained release of the cargo. Biologists play an important role in elaborating the underlying molecular mechanisms and identifying the targets that are crucial for cancer cell survival. Thus, based on the biologist's work, chemists discover or synthesize compounds and screen them for their specificity and selectivity to the molecular targets. Multidisciplinary efforts are therefore vital for the success of nanomedicine.

There continues to be a great need for interdisciplinary training programs that are focused on translational nanomedicine research and clinical use. Training students who can overcome the hurdles that happen among the different disciplines necessitates improvements in teaching methods and student learning. In the universities, most of the existing training programs mainly center on thorough training in a discipline or a set of interrelated sub-disciplines. To develop the collection of researchers who are well prepared for multidisciplinary nanotheranostics research, there is a need for undergraduate training plans that provide in-depth knowledge in the major discipline(s) and also support students to take part in interdisciplinary lab-based courses and research experiences that crossover their major discipline. In a 2014 talk given at a symposium, well-known nanomedicine scholar Dr. Ferrari said, "Between 5%–10% of all cancer drugs (in the clinic) are nanodrugs. Less than 5–10% of cancer doctors know that" [24]. This opinion is also held by Dr. Moore, who cautioned: "Currently, nanomedicine rarely features in mainstream medical training or in continuing professional development. (…) Yet, by the time the current intake of medical students graduate as doctors and subsequently complete their two-year residency, many new nanomedical products are likely to be appearing" [25]. These comments underline the point that modern medicine is now progressively dynamic and that swift advancements in nanotherapeutic research and allied technologies are expected to strongly influence forthcoming medical practice. With the speed at which such developments are happening, the relative lack of knowledge demonstrated by several current and future doctors regarding nanomedicine implies a progressively crucial requirement for training and education in nanomedicine. Contemporary medical, pharmaceutical, and engineering education therefore must continue to acclimate and evolve if it is to sufficiently prepare the next generation of engineers, pharmacists, and physicians to develop new approaches and practice medicine over the next few decades [25].

Notwithstanding the enormous resources presently devoted to biomedical research and development (R&D), the frequency of approval of novel medicines into the clinic is declining. The process of taking a major new drug to the clinic, from discovery to marketing, takes more than a decade and is often very expensive [26–28]. To overcome this time- and cost-prohibitive process, pharmaceutical companies have established advanced computer models that can lower the risk and

improbability intrinsic in the drug development route. In these mathematics-based models, a researcher begins with a computer model of the structure of a target or a receptor and a drug molecule. The purpose is to envisage by simulation the mechanism by which drug will bind with a target or the way the receptor will fold. Drug design based on mathematical models will become an arduous undertaking with nanomedicine. While nanotechnology offers great visions of enhanced, individualized treatment of disease, it also makes the problem of choosing the candidates for biological assessment a lot more complicated. The new notion of "design maps" for multifunctional nanoparticles, comparable to the notion of the periodic table for chemical elements, could offer assistance for the design and development of optimized multifunctional nanoparticles through mathematical modeling. Also, the current system of clinical trials puts too many roadblocks in the drug development process, especially in oncology. The widespread format of clinical trials was developed for cancer drugs, a long time ago, mostly to test low molecular weight drugs. Although the format has been revised to some protein therapeutics, it is undoubtedly incompetent for the more complex, novel treatments like nanomedicines. Therefore for these new modalities to impact patient care, clinical trials have to evolve and become more state-of-the-art and significantly streamlined. To assuage this concern, some researchers have brought up the notion of information technology-guided clinical trials or "e-trials" that may help expand the patient base. Another idea is that of virtual R&D, using computer simulations of the human body with the intention to replace the arduous testing in human patients and lower the possibility of failure. Such approaches could improve the success rates in the clinical approvals for nanoparticle-based drug products [26–28].

As the market for targeted drug therapies expands and the niche for nanotheranostics becomes more prominent, it is simultaneously squelched by intricate drug development guidelines implemented by FDA approval and exhaustive costs that render research economically weighted by a 20-year process of FDA Investigational New Drug (IND) applications for drug discovery to market approval that involve an estimated 2.6 billion USD [29–32]. This further requires vital research collaborations between industry, academia, and medical hospitals in an effort to cut these costs and time to approval. FDA's Emerging Technology Program (ETP) embraces novel approaches in the design and production of pharmaceuticals. Unlike the scrutiny of the IND application post submission, this program offers constant feedback from regulatory boards early on to channelize product development in a regulated fashion. Thus academic researchers or industries developing nanotherapeutics and diagnostics can work closely with the regulatory bodies to avoid potential problems. Several opportunities for investigators to interact with the FDA are for the development of innovative manufacturing processes concurrent with new products like nanotherapeutics. The FDA also provides their guidance in adopting novel approaches to pharmaceutical product design and manufacturing. The FDA also encourages pharmaceutical industry representatives to meet with the agency to discuss, identify, and resolve potential problems of novel tech prior to filing a regulatory submission. Further, in 2007 the FDA's Nanotechnology Task Force report called for academia, industry, and medical doctors to participate in its collaborative

efforts, to further understand biological interactions of nanomaterials, and to investigate opportunities to facilitate innovation using nanomaterials to develop nontoxic and effective drugs and devices under its regulatory authority [29–32]. Allied with the need to encourage and partake in such collaborations whereby investors can leverage aptitude and assets to encourage innovation in nanomedicine, FDA has also initiated several public-private partnerships (PPPs) as a part of its public health mission. Such PPPs help to address the fragmentation of research efforts between the private and public sector as well as across borders. Secondly, they are designed to help increase both public and private investment in nanomedicine-based research to push more translational research into the clinic without all the financial burden borne by the private sector. PPPs have already helped eradicate several terrible diseases across various developing countries and could certainly help in the global fight against cancer [29–32].

The global market for nanomedicine is predicted to be 350.8 billion USD by the year 2025 according to Grand View Research statistics [33]. Early detection of cancer using nanodiagnostics could provide the most benefit since the majority of cancer deaths result from metastasis, which is usually detected only via surgical biopsy. In some types of cancers, such as lung cancer, where the tissue biopsy is difficult to obtain, liquid biopsy analyzed by nanodiagnostics provides information about the circulating tumor cells (often referred to as CTCs). Diagnostics have offered a helping hand in guiding medicine toward more educated treatments in various diseases. Recently academic researchers and pharmaceutical companies have fast-tracked the quest for novel biomarkers for disease subtypes, further development or enhancement of well-designed biomaterials, and more individualized treatments, signaling the potential of this field for clinical translation. This book points to a budding paradigm shift in medicine toward integrating diagnostics and therapeutics in a single platform rather than developing and using them separately. In this steady progress toward more efficient and personalized medicines, companion diagnostics are an intermediate step. More research should explore the use and design of diagnostics and companion diagnostics to better guide the development of nanotherapeutics and their selection as a treatment option in the clinic. Nanotheranostics is the next viable step and involves single nano-sized chemical agents to obtain disease diagnosis and deliver therapy concurrently. This approach is being used in cancer and is now emerging as an option for various other diseases, where its viability has attracted the interest of researchers from academia and industry.

Pharmaceutical chemists have yet to fully understand the subtleties of nanotheranostic action and have therefore not yet come up with unanimously certified approaches for developing clinical theranostic applications for diseases. However, given the growing signs of the possibly immense benefits that nanotheranostics could bring to our combat against all the diseases plaguing mankind, further exhaustive fundamental and translational research is necessary. Advances in medicinal chemistry, molecular biology, and nano-engineering have enabled the design, synthesis, development, and application of complex and integrated diagnostic and treatment modalities for clinical patient care. Theranostics is increasingly being used to

investigate differences in treatment responses in patients to various drugs, vaccines, or lifestyle alterations suggested by doctors. Notwithstanding its immense potential to push the evolution of precise diagnostics and drug delivery at the cellular and molecular level, theranostics still faces various challenges, with a major one being the difficult biological barriers they must overcome such as the host's unique immune system. A more thorough understanding of these biological barriers will guide the development of more advanced and targeted theranostics that are, for example, able to effectively navigate to their intended diseased areas with minimal toxicities to healthy areas [20]. Subdisciplines of targeted nanotheranostics are emerging as time passes, all in the ultimate race for individualized, optimized, safe, valuable cancer diagnosis, monitoring, and treatment. Clinicians should be more open to the adoption of novel technologies and apply it in patients for improved outcomes. The pharmaceutical industry and regulatory agencies should also work together in quickly adopting these transformative technologies. It is anticipated that in the coming decades, the actual potential of multifunctional nanomedicines will be fulfilled, on par with the significant progress that has been observed from the use of monoclonal antibodies. Antibodies are now becoming more widely used in the clinic, more than 40 years since their discovery. Nanotheranostics may take a while longer to become the standard of care, but their impacts are already being felt, and these will only continue to grow over the next few years.

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