Part One
Entry to the Nanopharmacy Revolution

1
History: Potential, Challenges, and Future Development in Nanopharmaceutical Research and Industry

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Since the advent in 1906 of Dr. P. Erlich's magic bullets that would lookfor specific disease-causing agents in the human body, many therapies have been developed to increase the delivery of drugs to the target site. Nanoparticle-based delivery systems provide new opportunities to overcome the limitations associated with traditional drug therapy and aim to achieve both therapeutic and diagnostic functions in the same platform. These nanocarriers allow targeting of the medication to the site of action and release the drug in a controllable manner. Other features linked to nanopharmaceuticals are increased drug loading, increased bioavailability, enhanced efficacy, and increased safety. The nanocarriers are designed to be biocompatible and biodegradable.

A wide range of therapies are nowadays on the market or in late-stage development for the treatment of serious conditions such as cancer and infectious diseases [1,2]. Therapies include carriers of nanopharmaceuticals such as liposomes, lipid-based formulations such as solid lipid nanoparticles, nanocrystals, polymer-based nanoformulations, protein–drug conjugate nanoparticles, surfactant-based nanoformulations, metal-based nanoparticles such as iron oxide or gold nanoparticles, dendrimers, virosomes, and modified viruses.

The first product on the market employing nanotechnology was Doxil® that received US-FDA approval in 1995 for the treatment of AIDS-related Kaposi's sarcoma [3]. Doxil® are stealth liposomes encapsulating about 10 000 doxorubicin molecules [3,4]. Encapsulation minimizes side effects, such as cardiotoxicity, neutropenia, vomiting, myelosuppression, and alopecia, which are associated with high doses of free doxorubicin [5]. The incorporation of lipid and specifically cholesterol increases the bilayer cohesiveness and reduces leakage. The liposomes are designed by their size of approximately 100 nm and their pegylated surface to target to solid tumors via EPR effect (enhanced permeability and retention effect) and reduce toxicity to healthy tissues.

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1.1 Nanopharmaceuticals in Cancer Therapy

Since, nanopharmaceuticals have become valuable arsenals in cancer therapy with enhancement of drug efficacy and decreased side effects. The efficiency of drug or gene delivery to a tumor site is dependent on the physicochemical properties of the delivery platform and a range of physiologically imposed design constraints, including clearance by the mononuclear phagocyte system and extravasation from circulation at the tumor site by the EPR effect.

The nanofeature of the pharmaceuticals contributes to enhanced solubility and chemical stability of the compounds along with potential protection from degradation by encapsulation into nanocarriers or coupling to synthetic polymers. Nanoparticle biodistribution and uptake by the reticuloendothelial system warranted the design of nanoparticles to evade rapid uptake such as lipid liposomes, albumin carriers, and PEGylation. PEGylation and conjugation to albumin respectively resulted in prolonged circulation and enhanced biodistribution of compounds, while the small size of nanopharmaceuticals led to improved tumor tissue accumulation [6].

Nanopharmaceuticals have improved biodistribution and targeting features as well as the potential of stimuli-sensitive microenvironments payload release. These collective features have led to the development of nanoparticle therapeutics of large antibody–drug conjugates (brentuximab vedotin and trastuzumab emtansine [6]) and small-molecule platforms such as liposomes (Doxil, DaunoXome, DepoCyt, Marqibo, Mepact, Myocet, Lipoplatin), polymeric nanoparticles (Eligard, Genexol, Opaxio, Zinostatin stimalamer), albumin nanoparticles (Abraxane), and metal-based nanoformulations (NanoTherm) [1,7].

Brentuximab vedotin and trastuzumab emtansine are antibody–drug conjugates (ADCs) with an anticancer drug conjugated to a targeting molecule. Brentuximab targets the protein CD30, a glycosylated phosphoprotein expressed by B cells, including B-cell lymphomas, some leukemias, and melanoma cancer stem cells [8–10]. Trastuzumab targets the human epidermal growth factor receptor 2 (HER2) overexpressed in HER2-positive breast cancer [11,12]. Monomethyl auristan E (MMAE) (brentuximab vedotin) and mertansine (trastuzumab emtansine) are too toxic to be used alone and hence coupling to a targeting antibody reduces toxic side effects. Several drug molecules are conjugated to each antibody via a valine–citrulline cleavable linker (brentuximab vedotin) or covalent linkage (trastuzumab emtansine) that is enzymatically degraded in endosomes following uptake. The relatively small number of approved ADCs highlights the difficulty in the development of nanotherapeutics to the clinic.

Since the introduction of Doxil on the market, many other liposomal formulations are developed [13]. More advanced liposomal carriers are designed to release their drug triggered by internal stimulus such as changes of pH or oxygen level or external stimulation such as local heating. Thermosensitive liposomes, such as ThermoDox, can release their payload in the tumor region with

local heating owing to the gel-to-liquid crystalline phase change of the lipids at about 42 °C, a temperature that can be reached by local hyperthermia [13].

Another nanotherapeutic using temperature to induce tumor cell destruction or sensitization is NanoTherm, 15-nm-sized superparamagnetic iron oxide nanoparticles (SPION) coated with aminosilane. These nanoparticles are introduced directly in solid brain tumors and are exposed to a magnetic field that changes its polarity up to 100 000 times per second generating a local increase in temperature. Depending on the duration of exposure to the alternating magnetic field, the tumor cells may be destroyed or sensitized for further chemotherapy. Through the aminosilane coating, the nanoparticles remain localized, which allows repeated treatments [14].

Nanoparticle albumin-bound (nab™) technology is a nanotechnology-based drug delivery platform that exploits the natural properties of albumin to achieve a safe, solvent-free, efficient, and targeted drug delivery. Abraxane, or nab-paclitaxel, is a cremophor-free, albumin-bound 130-nm particle form of paclitaxel. The paclitaxel and albumin are not covalently linked but rather associated through hydrophobic interactions [15]. The particles of paclitaxel are in a noncrystalline, amorphous, readily bioavailable state, allowing for rapid drug release from the particles following intravenous administration. The albumin is thought to facilitate endothelial transcytosis and to play a role in preferential intratumoral accumulation of paclitaxel through its binding to SPARC (secreted protein acid and rich cysteine), a glycoprotein overexpressed in many tumors [15].

Nanopharmaceuticals have benefited from the concept of site-specific delivery. Active targeting of a nanoparticle is a way to minimize uptake in normal tissue and increase accumulation in a tumor. Active targeting can be achieved by linking, to the surface of the nanopharmaceuticals, molecules that bind specifically to surface membrane proteins that are upregulated in cancer cells [16]. These so-called targeting molecules are typically antibodies [17], antibody fragments [18], aptamers [19], or small molecules. Monoclonal IgG antibodies are widely used for protein recognition and targeting, since they have two epitope binding sites, high selectivity, and high binding affinity. Fab2 fragments of the antibodies retain both antigen-binding sites. Aptamers are folded single-strand oligonucleotides, usually 25–100 nucleotides in length (8–25 kDa) that bind to molecular targets [19]. Small molecules for targeting include peptides, growth factors, carbohydrates, ureas, and receptor ligands [20]. The interested reader is referred to Chapter 16 “Drug Targeting in Nanomedicine and Nanopharmacy: A Systems Approach” to read more on this topic.

1.2
Nanoparticles Actively Using the Host Machinery

Apart from cancer therapy, nanoparticles have become very valuable in vaccine therapy. Vaccine nanotechnology *avant la lettre* are the aluminum particles

traditionally used as adjuvants, since most proteins are poorly immunogenic when administered alone. Strong adaptive immune responses to protein antigens typically require the antigen to be administered together with an adjuvant. Adsorption of the antigen on aluminum particles, either aluminum phosphate or aluminum hydroxide, transforms soluble antigens into particulate material. This form delays the release of the antigen and enhances the immune response by specifically activating macrophages and, additionally makes the antigen more prone to uptake by these antigen-presenting cells. Although used successfully since the 1930s, this approach does not work for every antigen, for example, tuberculosis and malaria. There is also some public concern regarding the ability of aluminum to translocate to the brain.

A more innovative approach to enhance the immune response is to integrate the antigen in liposomes. These virosomes consist of unilamellar phospholipid membrane nanovesicles incorporating virus-derived glycoproteins, 100–150 nm in diameter. The first commercial product based on this technology has been InflexalV®. The principle was to deliver influenza antigens within a nanostructure resembling a native influenza virus, but deprived of its genetic material and therefore of its *in vivo* replication capability. The preparation of virosomes [21,22] is based on virus dissolution, followed by a detergent removal procedure leading to the reconstitution of virus-like particles containing only the main virus antigens embedded in a lipid bilayer. Neither viral DNA nor core proteins are present in the final product. Properly formed virosomes retain the cell binding and membrane fusion properties of the native virus, mimicking the natural infection mechanism. This property, together with the particulate nature of virosomes, gives these structures the capability of triggering a broad immune response, involving both major histocompatibility complexes, MHC-I and MHC-II, while preventing the response against the structures themselves [23–25]. These nanoparticles are therefore considered an efficient delivery system, obsoleting the need for an extra adjuvant. Importantly, virosomes have a solid safety track record, as demonstrated during almost two decades of use. Due to versatility of this technology, flu vaccination was not the only application of this technology. The advantage of these reconstituted influenza virosomes is that the influenza glycoproteins embedded in the lipid bilayer activate macrophages and mediate membrane fusion and endocytosis. This leads to an accelerated cellular and humoral response [26,27]. The adjuvanting effect of influenza-derived virosomes was also exploited for the delivery of hepatitis A antigens (Epaxal®). Extensive clinical and postmarketing monitoring showed that Epaxal has an improved safety profile compared to an aluminum-adsorbed vaccine, while inducing a similar immune response. The difficulties with the stability of such virosomal structures have been recently overcome optimizing the formulation, allowing frozen as well as 4 °C long-term storage [28].

In contrast to these human-designed nanoparticles, Nature has provided us with surprisingly elegant nanopharmaceuticals, but only recently do we have the technology to unlock its potential. Millions of years of evolution have perfected

viruses in their ability to infect host cells and express their viral genes. Although the viral replication materializes at the expense of the host cell, not all viruses are as pathogenic. For example, wild-type (wt) adenovirus typically causes only mild symptoms and is cleared by the immune system in healthy individuals. The relatively large icosahedral particles (about 80 nm) are proficient in transferring their linear double-stranded DNA to host cells. Scientists have taken advantage of this feature by deleting part of the viral genome (the E1 gene) and replacing it by a gene of choice. The resultant replication-deficient virus is a very efficient delivery device for any gene of interest, expressing high levels of the encoded protein. Originally explored for gene therapy purposes, it became soon apparent that E1-deleted adenoviruses coexpress low levels of viral proteins after cell transduction. This leads to an unexpectedly broad immune response, including local chemokine and cytokine responses. Transduced cells are rapidly cleared rendering the transgenic protein production transient. Although less suitable for gene therapy, this holds great promise for vaccination purposes. As opposed to one bolus injection of antigen in classical vaccines, recombinant adenoviruses use the cell machinery to produce the antigens *in vivo* over a period of a few weeks. This substantial but transient *in vivo* production supported by the immunogenic properties of adenoviruses lead to a robust and more balanced T- and B-cell immune response. Some infectious diseases that are unresponsive to traditional vaccines, such as Ebola or HIV, may finally be overcome using this technology [29–31]. In addition to prophylactic use, adenoviruses are currently also investigated for therapeutic use, for example in HPV infection, a major cause of cervical cancer. There are, however, two drawbacks that need to be addressed to use this next generation of vaccines to improve global health. First, one advantage of adenoviruses is also a potential problem. Its immunogenic character will lead to neutralizing antibodies against the vector requiring alternate vectors that show less preexisting immunity [32–34]. The second challenge is viral stability. Adenoviruses are sensitive to degradation during storage due to physical and chemical instability. Therefore, most adenoviruses are formulated as lyophilized products or as liquid formulations to be stored at −80 °C. However, recent advances have shown that with new and tailored formulations, it is possible to stabilize these complex biological structures to provide 2–3 year stability upon storage at 4 °C [35].

In recent years, significant progress has also been made in the field of viral gene therapy. After a few vector-related adverse events in the past, more suitable vectors have been further optimized for gene transfer applications. A wide range of target tissue is now in scope (e.g., muscle, liver, eye, salivary glands, and joint) and almost 1900 clinical trials [36] are ongoing in fields like cancer (e.g., gynecological and lung), neurological disorders (e.g., Alzheimer's), inflammatory diseases (e.g., rheumatoid arthritis), monogenic disorders (e.g., hemophilia A and B), ocular disorders (e.g., macular degeneration), and diabetes. A vector that appears to be particularly promising is adeno-associated virus (AAV). The first gene therapy product approved by the EMA in 2012 for lipoprotein lipase

deficiency used this vector [37]. Originally discovered as a contaminant in adenovirus batches, AAV is now widely acknowledged for application in gene therapy. AAV is small compared to adenovirus, also nonenveloped but a single-stranded DNA virus from the family of parvoviruses. They are nontoxic and nonpathogenic in humans. The key advantage of recombinant AAV, replication incompetent, is that the absence of viral gene expression minimizes host immune responses. This allows stable gene transfer and long-term transgene expression. Using specific AAV serotypes allows tailoring of the tropism to the target organ. Further optimization can be done using tissue-specific promoters and codon-optimized transgenes. Difficulties with cumbersome AAV production and purification techniques have been overcome [38–40], but a point of concern remains the physically small transgene packaging capacity in AAVs limiting its use to relatively small genes. Recently, also RNA molecules and oligonucleotides based on RNA interference have proven their potential in gene therapy applications [41]. Liposomes incorporating nucleic acids or virus vector-mediated gene therapy can be used to downregulate specific cellular protein expression through RNA interference or microRNA production.

1.3 Nanopharmaceuticals for Oral Administration and Long-Acting Injectable Therapy

Although most research and commercial nanoparticles are administered parenterally, nanoparticles also have proven to enable oral drug delivery by increasing oral bioavailability. In recent years, advances in drug discovery and combinatorial chemistry have led to many potential drug candidates that can be characterized by poor aqueous solubility and hence low bioavailability [42]. Because of the large increase in surface area at smaller particle sizes, the dissolution rate and solubility of nanoparticulate drugs are significantly increased as described by respectively the Noyes–Whitney and Ostwald–Freundlich equations [42,43].

Particle size reduction techniques for small molecule APIs (active pharmaceutical ingredients) can be classified as “top-down” or “bottom-up” processes. Top-down processes are characterized by the milling of coarse particles into smaller particles, usually to a size range of 200–500 nm. Bottom-up nanoparticles are generated by controlled crystallization of a supersaturated solution. The most widely used top-down technique is wet media milling [44]. In this technique, drug crystals are reduced in particle size through the combination of high-energy shear and impact forces of the milling beads on the coarse drug particles. The nanoparticles that are generated are stabilized in the liquid phase by polymers or surfactants. The technology is proven to be scalable from discovery scale using as little as 10 mg API up to commercial manufacture [45]. Another technique that is frequently used to generate nanoparticles is high-pressure homogenization [46]. The nanoparticle suspensions that are formed can easily be further processed using techniques such as spray-drying, freeze-drying, or granulation to

form solid oral tablets or fill into capsules [44]. The interested reader can learn more about particle size reduction techniques in Chapter 9 "Overview of Techniques and Description of Established Processes" and Chapter 12 "Scale-Up and cGMP Manufacturing of Nanodrug Delivery Systems for Clinical Investigations".

Multiple products, such as rapamycin, fenofibrate, aprepitant, and megasterol acetate, are on the market using the NanoCrystal® technology or the DissoCube® technology. The benefit of these nanoproducts over their conventional counterparts is that the dose can be significantly reduced and/or the food effect is much less pronounced [47]. The interested reader can learn more about oral applications of nanodrugs in Chapter 24 "Nanodrugs in Medicine and Healthcare: Oral Delivery."

Nanosuspension generated by particle-size reduction can also be used for parenteral applications to promote long-acting injectable therapies and enhance the amount of drug that can be administered. This approach is already frequently used in preclinical studies but commercial and clinical applications remain limited [42,48]. The only commercial application currently is paliperidone palmitate injectable formulation (Invega Sustenna) that is a slow-release intramuscular injectable formulation for the treatment of patients with schizophrenia [49]. This therapy offers the opportunity of improved adherence and simplified medication regimen over the oral therapy that needs to be dosed daily. Although no longer nanoparticles, Invega Trinza uses the same technology to obtain particles showing a 3 month sustained release profile, further improving patient's quality of life. A number of other nanoparticles are currently under clinical evaluation such as thymectacin (Theralux, Celmed) and a combination therapy of cabotegravir (GlaxoSmithKline) and Rilpivirine (Janssen). The combination therapy of the two long-acting HIV therapies that are dosed every 4 or 8 weeks has shown to be comparable in maintaining viral suppression rates to a three-drug oral therapy of cabotegravir and two nucleoside reverse transcriptase inhibitors (ViiV Healthcare) [50]. This therapy offers great opportunities in developing countries where distribution and procurement of drugs is precarious and where adherence to daily oral therapy remains strikingly low for many reasons such as stigma against HIV. Data from studies in nonhuman primates have also shown great potential of the long-acting therapy in the prevention of HIV and may present a useful alternative for HIV preexposure prophylaxis (PrEP) [51].

Long-acting injectable formulations do not only lead to improved patient comfort but also have the potential to increase therapeutic compliance and efficacy even when patients cannot autonomously or reliably take their medication (e.g., due to disability/morbidity) or have limited access to medication such as in developing countries. The controlled release rate translates into lower variability in plasma drug concentrations, often reducing adverse effects, which in turn contributes to better clinical outcome. This is especially relevant for the treatment of chronic viral or bacterial infections in developing countries such as HIV, tuberculosis, malaria, and dengue. The failure to maintain minimal inhibitory concentrations could rapidly lead to incomplete viral suppression and ultimately drug resistance [52].

1.4 Bridging Future Nanomedicines to Commercialization

A lot of nanotherapeutics have already gained market access worldwide. Although for nanopharmacy to show its full potential in healthcare industry, a number of challenges remain to be addressed:

- Stabilization of nanotherapeutics remains challenging. Further understanding on the principles governing physical stability of these colloidal drugs and prevention of specific degradation routes, for example, by formulation, remains to be elucidated.
- The scale-up and manufacturing of nanotherapeutics remains variable. Process control is challenging and concerns related to the unpredictable impact of small variations to chemistry, manufacturing, and control (CMC) on the *in vivo* faith of the nanodrugs require tight controls of physicochemical properties of individual drug batches. Well-defined analytical tools that allow the full characterization of the nanoparticles' safety, efficacy, and quality need further development. Existing *in vitro* techniques have limitations in a way that *in vitro in vivo* correlation (IVIVC) cannot be performed easily and extensive bioequivalence testing is still needed. It would be great if novel *in vitro* methods become available that are straightforward to use and are predictive of the *in vivo* behavior. The interested reader can learn more about developments in this field in Chapter 12 "Scale-Up and cGMP Manufacturing of Nanodrug Delivery Systems for Clinical Investigations," Chapter 17 "Nanoparticle Toxicity: General Overview and Insights Into Immunological Compatibility," and Chapter 15 "Computational Predictive Models for Nanomedicine."
- Toxicity related to nanotherapeutics remains to be further unraveled. Nanoparticles are developed in many cases to reduce toxicity of the drug by enhanced targeting or shielding from healthy tissue. New materials such as dendrimers may be employed as carriers. Although knowledge about the potential toxicity of these, more recent nanocarriers themselves remain to be elucidated as well as the potential new toxicities that may develop because of the small size of the dosage form. Existing toxicological assessment is developed for conventional dosage forms; appropriate tests to fully understand the toxicity of nanoparticles need further development [53]. Therefore, regulatory hurdles to establish long-term safety of nanotherapeutics remain high with the need for accountability for biodistribution, mass balance, long-term residence, and ultimate elimination. The interested reader can learn more about this topic in Chapter 17 "Nanoparticle Toxicity: General Overview and Insights Into Immunological Compatibility" and Chapter 18 "An Overview of Nanoparticle BioBiocompatibility for Their Use in Nanomedicine."
- Sophisticated methods for the detection and more exact quantitation and biocompatibility of the nanoparticles in tissues need further development and validation to assess short- and long-term potential toxicities.

In order to bridge these knowledge gaps, long-term collaborations between industry and universities are very important. Such collaborations allow the

scientific experts at universities to unravel the knowledge gaps that restrain industry in their ability to develop robust nanopharmaceuticals with predictable physicochemical and biological properties that ensure its quality, safety, and efficacy that are required to gain regulatory approval. Governmental policies providing a predictable and stable environment of funding and regulations nourishing such long-term partnerships have shown to be crucial for the success of these interactions. Indeed, successful collaborations are built around a common vision, strong professional relationships and trust, and shared benefits that allow bridging the cultural differences between the academic and industrial world [54]. It will also contribute to the translation of great innovative ideas with a lot of promise for the society to robust products that can be commercialized. Indeed, currently a great culture of promise exists around nanopharmacy in academia and small start-ups but they face a lot of challenges to manufacture first batches of innovative nanotherapeutics according to good manufacturing practices that can be evaluated in clinical trials and translated into commercial products beneficial for the society.

A close interaction between different stakeholders, such as researchers, patient groups, healthcare providers, regulators, and society, will also help in advancing the impact of nanotherapeutics on global public health. Current studies suggest that there is skepticism about the safety of nanotechnology and its impact to the environment, while others see great potential for the treatment and prevention of diseases such as cancer [55]. More research driven by innovators across different disciplines is needed to continuously address knowledge gaps regarding potential health and safety implications of nanopharmaceuticals in a way more information becomes available regarding its impact on health outcomes and overall health care cost. The interested reader is referred to Chapter 21 "Social Studies of Nanopharmacy" to read more on this topic.

A better understanding of the physicochemical and biological parameters affecting the quality, safety, and efficacy of the nanopharmaceuticals will also enable "follow-on" nanotherapeutics to gain easier access to the market. Currently, nanotherapeutic product applications are assessed under conventional regulatory framework and it is extremely challenging to develop nanosimilars that could be approved after the innovator patents expiration. Therefore, the regulatory authorities have established an *ad hoc* expert group that gives guidance and drafted a number of reflection papers that give scientific and regulatory guidance for the development of follow-on nanotherapeutics and emerging next-generation nanotherapeutics [56]. In addition, innovators developing nanotherapeutics are encouraged to seek regulatory agency's advice on the appropriate tests and studies to be performed during the development of a novel nanotherapeutic. The interested reader can read more in Chapter 20 "Regulatory Issues in Nanomedicines."

1.5 Future Outlook

The use of nanotechnology in the pharmaceutical industry has already shown great potential in increasing bioavailability, reducing toxicity, and side effects of

existing drugs and in improving overall health, adherence, and comfort of patients. The technology is set to spread rapidly. Many more new nanotherapeutics using GRAS (Generally Recognized as Safe) or new materials are currently in various stages of development going from early discovery to late-stage clinical trials. Although nanotherapeutics are usually administered intravenously, recent clinical studies have also shown great potential if dosed subcutaneously, dermally, or as aerosols [13,57]. While in the past nanotherapies were mainly used as vaccine or cancer therapy, current advancements encompass all major disease areas, such as Alzheimer's disease, rheumatoid arthritis, diabetes, gene therapy, infectious diseases, and many others. The future technologies exploit further cell-specific targeting enabled by advancements made in the understanding of how the molecular buildup of diseased organs differentiate from healthy tissue and how nanotherapeutics interact in the body. In addition, developments in manufacturing technologies will enable more efficient loading of drugs in the nanoparticles and even enable loading of two or more therapeutic agents into one nanocarrier as, for example, in nanoparticles prepared by the layer-by-layer technology [58]. Although the biggest leap forward in patient care that is trending more and more toward personalized medicine, and the detection, diagnosis, and prevention of diseases can be expected by the combination of nanotherapeutics with other nanotechnologies, including nanobots, cell therapy, nanodevices, sensory feedback, enhanced imaging, and others. Such combinations will undoubtedly revolutionize medical care and provide enormous benefit to the patient and society in the management of health, disease, and potentially aging. However, strong research efforts in an intensive cooperation of industry and universities are required to develop such revolutionizing combinations.

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