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Roadmap on Nanomedicine

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Abstract

Since the launch of the Alliance for Nanotechnology in Cancer by the National Cancer Institute in the late 2004, several similar initiatives were promoted all over the globe with the intent to advance the diagnosis, treatment and prevention of cancer in the wake of Nanoscience and Nanotechnology. All this has encouraged scientists with diverse backgrounds to team up with one another, learn from each other, and generate new knowledge at the interface between engineering, physics, chemistry and biomedical sciences. Importantly, this new knowledge has been wisely channeled towards the development of novel diagnostic, imaging and therapeutic nano-systems, many of which are currently at different stages of clinical development.

This roadmap collects eight brief articles elaborating on the interaction of nanomedicines with human biology; the biomedical and clinical applications of nanomedicines; and the importance of patient stratification in the development of future nanomedicines. The first article reports on the role of geometry and mechanical properties in nanomedicine rational design; while the second articulates on the interaction of nanomedicines with cells of the immune system and the third deals with exploiting endogenous molecules, such as albumin, to carry therapeutic agents. The second group of articles highlights the successful application of nanomedicines in the treatment of cancer with the optimal delivery of nucleic acids, diabetes with the sustained and controlled release of insulin, stroke by using thrombolytic particles and atherosclerosis with the development of targeted nanoparticles. Finally, the last contribution comments on how nanomedicine and theranostics could play a pivotal role in the development of personalized medicines.

As this roadmap cannot cover the massive development of nanomedicine over the past 15 years, only a few major achievements are highlighted as the field progressively matures from the initial hype to the consolidation phase.

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Introduction

Nanomedicine is defined as the medical application of nanotechnology. Nanomedicine ranges from the medical applications of nanomaterials and biological devices, to nanoelectronics and biosensors, and even possibly future applications of molecular nanotechnology such as nanorobotics, cyborg machines that implement biology with technology and Nano-surgery using sophisticated tiny blades.

The development of nanomedicines for the diagnosis, treatment and management of various diseases is driven by principles in many fields of science. The various sizes, geometries, materials, and targeting moieties of nano-scale platforms introduce the possibility of targeting organs, tissues, and individual cells. [1,2] Fundamental steps have been made in the last 15 years in the field of nanomedicine with more nanodrugs on the market. [3]

This roadmap focuses on the emerging implications of nanomedicines. The vibrant activity in this field is demonstrated by the large number of studies using various delivery platforms and the promising results from clinical trials. Nano-scale carriers are already having a profound impact on medicine and will likely play an increasing role in the treatment of diseases in the near future. [4]

We start this roadmap with the vision of Paolo Decuzzi and colleagues on “Modulating Size, Shape, Surface properties and Mechanical Stiffness to boost the efficacy of drug delivery systems”. Decuzzi and colleagues provide conceptual understanding on the importance of size, shape, surface and mechanical properties to lift the therapeutic potential of drug nanocarriers. We then shed light on Nanomaterials and the complement challenge by S. Moein Moghimi and Z. Shadi Farhangrazi. This roadmap provides a fresh look at the role of the complement system and how we can overcome some of the challenges related to this fundamental system.

We continue to the future outlook of Multifunctional Biomolecular Drug Designs by Kenneth Howard, which point for new directions in the design of carriers. We then move to showcase the development in several diseases starting with the contribution of Daniel Rosenblum and Dan Peer on Cancer Nanomedicine and the current developments there with some future outlook.

Then the contribution of Zhen Gu and colleagues on Nanotechnology in Diabetes. We move to the contribution of Netanel Korin on Nanomedicines for thrombolysis and we finalize this part with the contribution of D. Letourneur, C. Chauvierre on Nanomedicine for Atherosclerosis.

The roadmap is finalized with the work of Twan Lammers and colleagues on Patient Stratification to Improve Nanomedicine Performance and Clinical Translation.

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1. Modulating Size, Shape, Surface properties and Mechanical Stiffness to boost the efficacy of drug delivery systems.

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Status

Over the last 50 years, powerful molecules have been developed for the treatment of different malignancies. However, limited aqueous solubility, lack of specificity and high toxicity have often impaired their actual potential. Nanomedicines can overcome these typical drawbacks of the small chemotherapeutic molecules[1]. By exploiting the enhanced permeability and retention (EPR) effect, resulting from the combination of a fenestrated tumor vasculature with poor lymphatic drainage, nanomedicines have been documented to passively accumulate within the tumor parenchyma at doses which are 1 to 2 orders of magnitude above chemotherapeutic drugs. This passive deposition can be further enhanced by tagging specific recognition moieties on the surface of nanomedicines. Moreover, the core and surface properties of nanomedicines can be designed to control drug release, from diffusion-limited to stimulus-triggered, thus ameliorating therapeutic efficacy and off-target toxicity[2]. Over the last two decades, various nanomedicines have been developed, including assemblies of protein-drug conjugates, dendrimers, micelles, liposomes, and polymeric, hydrogel-based and inorganic nanoparticles. In addition to synthetic molecules, nanomedicines can also deliver biologicals, such as siRNA, miRNA, peptides, proteins, and activatable molecules. The first generation of clinically approved nanomedicines, involving doxorubicin-loaded liposomes (Doxil®), paclitaxel-loaded micelles (Genexol®-PM) and albumin-bound paclitaxel (Abraxane®), significantly extended the circulation half-life, improved the bioavailability and reduced the toxicity of the corresponding free drug. Furthermore, nanomedicines can be simultaneously loaded with different active molecules enabling combinatorial therapies[3] (co-delivery of two or more therapeutic agents) and theranostics[4] (co-delivery of therapeutic and imaging agents). In the latter case, nanomedicines can support diagnostic and follow up imaging together with therapy[4]. Although the majority of nanomedicines are realized via a bottom-up approach relying on self-assembly and colloidal interactions, fabrication strategies based on top-down approaches are recently emerging[5-6]. These allow a precise control on the size, shape, surface properties and mechanical stiffness of nanomedicines – the 4S design parameters. The ability to control geometrical, physico-chemical and mechanical features of nanomedicines together with their multiple payloads and functionalities is key to improve homing at biological targets and boost therapeutic performance. This is expected to extend the range of application for nanomedicine to several other diseases in addition to cancer.

Current and Future Challenges

The clinical integration of nanomedicines is hampered by a number of challenges, including specific accumulation at biological targets; development of patient-specific interventions; and production scaling-up. In oncological applications, it is well accepted that nanomedicines accumulate in most tumors at higher doses than conventional drugs. Systemically injected, untargeted nanoparticles can typically reach intratumor concentrations in the order of 1% ID/g tissue (injected dose per gram)[1]. However, some nanoparticles have demonstrated tumor accumulation values as high as 20% ID/g[8]. The decoration of the nanoparticle surface with ligands, recognizing specific receptors on malignant cells, has been extensively used to improve intratumor accumulation. However, although some encouraging results were

documented preclinically, with intratumor accumulations up to ~ 10% ID/g tissue, this approach has not had yet any clinical impact. On one hand, this strategy complicates the nanoparticle architecture and industrial development. On the other hand, the identification of a truly specific cell receptor is difficult. Also, upon systemic injection, nanoparticles tend to be coated by blood proteins, which alter the actual ligand-receptor affinity. The biophysical attributes of tumors typically vary with time and from one patient to another. Focusing solely on the EPR effect, some patients may have a hyper-permeable tumor vasculature whereas others might have a malignant mass with very low perfusion. These scenarios may also occur within the same patient, at different stages of the disease. Thus, depending on the tumor biophysical attributes, different types of nanomedicines should be used to successfully deliver the therapeutic cargo, as one type does not fit all tumors! Here, the challenge is to develop minimally invasive imaging tools that could help to personalize and fine-tune the therapeutic intervention throughout the disease development. Finally, the composition and fabrication protocols vary dramatically across the full nanoparticle spectrum. Indeed, this is needed to protect the intellectual property associated with the technology but it also complicates significantly the industrial development. Scaling-up the production of nanoparticles form preclinical experiments in small rodents (typical subject weight of 20 g) to clinical studies and practice (average subject weight of 70 kg) represents a major technological and economical challenge. Often, the latter dictates the success of a nanomedicine over conventional, small molecule-based therapeutic approaches.

Advances in Science and Technology to Meet Challenges

Tailoring the geometry (Size and Shape) and physico-chemical attributes (Surface properties and mechanical Stiffness) of nanomedicines for the specific disease can help to address these challenges.

Non-spherical particles, mostly discoidal and cylindrical, were shown to efficiently adhere to the diseased vasculature because of the larger area exposed to endothelial cells[9]. Over this area, proper multivalent interactions can be established by simultaneously engaging multiple ligand-receptor bonds and realizing combinations of different ligands to improve molecular recognition. Deformable particles, mimicking blood cells, were shown to circulate longer in the blood pool and effectively evade the sequestration by hepatic and splenic macrophages, as compared to their rigid counterparts[10]. This resulted in higher deposition rates within the tumor vasculature, achieving accumulation levels as high as 20% ID/gram tissue, without any molecular targeting (Figure 1)[2,3]. Also, deformable particles can more finely integrate themselves within the surrounding biological environment improving drug deposition[4]. Exogenous energy sources, such as magnetic fields, have been also exploited to improve tissue accumulation. Microdosing of radio-labelled nanoparticles can be safely administered to patients to quantify organ-specific accumulation. Technologies are available to image simultaneously and separately multiple radionuclides[5]. As such, the intratumor distribution of 2, or more, nanoparticles, labelled with different radionuclides, could be assessed to identify the best delivery strategy for that specific patient, at that specific stage of the disease. The alliance between Nanomedicine and Nuclear Imaging can boost the development of truly patient-specific interventions. The production of most spherical nanomedicines is based on colloidal interactions and self-assembly. This can be readily scaled-up by using microfluidic-based systems that optimize mass mixing and nanoprecipitation on large working volumes (several litres)[6]. For the synthesis of particles with more complex attributes, flow lithography systems have been also demonstrated. However, these are mostly limited to polymeric hydrogels with characteristic sizes larger than 10 µm, which can only be used as implantable systems rather than nanomedicines[7]. For the 500 nm - 10 µm size regime, template strategies have been mostly used so far[8,9]. These offer a precise control on particle morphology but have limited manufacturing rates. More efforts are currently needed to develop flow-based, high-throughput fabrication strategies for the production of sub-micron and micron-sized particles with complex morphologies.

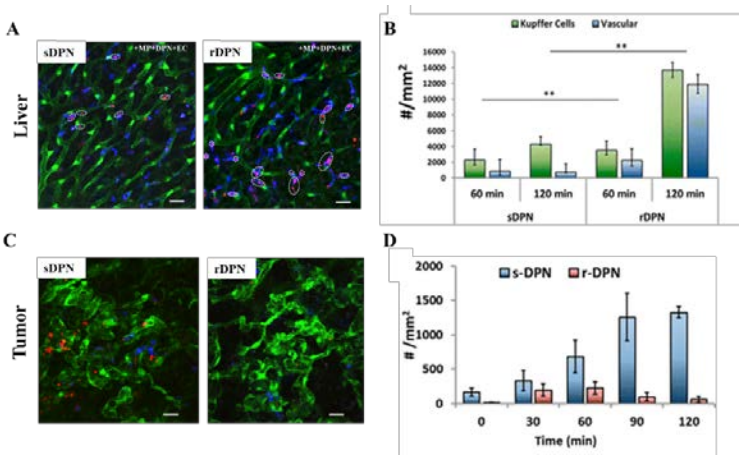

Figure 1. Discoidal Polymeric Nanoconstructs in Cancer. (A) Representative intravital microscopy images of 1 µm soft and rigid DPNs (red dots) in the liver of Tie2 mice (green fluorescence – endothelium; blue fluorescence – immune cells). (**B**) Quantification for the soft and rigid DPN accumulation in the liver vasculature and in Kupffer cells. (**C**) Representative intravital microscopy images of 1 µm soft and rigid DPNs (red dots) in the tumor vasculature of Tie2 mice (green fluorescence – endothelium; blue fluorescence – immune cells). Scale bars: 20 µm. **(D)** Quantification for the soft and rigid DPN accumulation in the tumor vasculature. (Reprinted (adapted) with permission from Key, J., et al., Soft Discoidal Polymeric Nanoconstructs Resist Macrophage Uptake and Enhance Vascular Targeting in Tumors. ACS Nano, 2015. 9(12): p. 11628-11641. Copyright (2015) American Chemical Society).

Concluding Remarks

Modular manufacturing, the combination of imaging and therapy, and the development of more general and effective scaling-up procedures are key to the success of nanomedicines and their full integration into clinical practice. Modular manufacturing allows the realization of nanomedicines with different attributes, where the size can range from a few hundreds of nanometers to a few microns; the shape can be discoidal, cylindrical, cubical and spherical; the particle surface can be decorated with different molecules; and the mechanical stiffness can range from soft (1 kPa) to rigid (10 MPa). Multi-radionuclide imaging can pave the way towards personalized interventions via patient stratification and the identification of the best nanomedicines for that specific disease stage. Finally, the implementation of artificial intelligence algorithms will help to select, within this highly complex scenario, specific particle parameters to boost biological performance for that specific patient, at that specific stage of the disease.

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2. Nanomaterials and the complement challenge

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Status

The complement system comprises a series of plasma and cell membrane proteins that contributes to non-specific host defence, the inflammatory response and disease progression, tissue regeneration and maintenance of homeostasis [1]. Thus, complement not only acts as a functional bridge between innate and adaptive arms of the immune system, but also links the immune system with the coagulation (contact) system.

Nanoparticles (including nanomedicines) have long been known to trigger complement activation and induce variable responses [1]. The mechanisms by which nanoparticles, including "stealth" nanosystems, bind to complement proteins and trigger complement responses are complex, multifaceted and poorly understood [1]. Nevertheless, nanoparticle-mediated complement activation has ramifications in product stability (e.g., liposomes and a wide range of non-lamellar liquid crystalline dispersions, exosomes), pharmacokinetics (opsonisation and immune cell clearance) and therapeutic performance [1]. Notwithstanding, there is sporadic reporting and inconsistencies in complement activation by engineered nanoparticles and nanomedicines mostly arising from inappropriate plasma (or sera) handling, assay procedures, design sophistication and in some cases from contaminants (e.g., residual solvents, endotoxins) (discussed in [1]). There are also disparities in immune responses to nanomaterials among species and simply assessing complement activation/responses in animals or in their sera/plasma may not necessarily represent human responses [1, 2]. Furthermore, possible in vitro-in vivo differences in complement activation pathways, mechanisms and responses are imposing more difficulties in development of immune-safe nanomedicines [1]. Considering these, a better understanding of nanomaterial characteristics and integrated bio-interfacial interactions that modulate complement activation and responses in human-derived materials (and validated preclinical models) is therefore needed and could lead to improved and safer design initiatives in therapeutic medicine and biomedical engineering. Here we outline important challenges at the interface of nanomedicine-complement research and suggest a complement-motivated roadmap to improve nanomedicine design and performance in the future.

Current and Future Challenges

Opsonisation. Key steps in the assembly of complement convertases and mass deposition of the third complement protein (C3) on nanoparticles (referred to as opsonisation) are variable and complex. Recent evidence suggests that these steps may be dependent on deposition of non-specific blood proteins on nanoparticles [3]. Some of these proteins generate necessary antigenic epitopes for docking of natural antibodies, which subsequently serve as target for nascent C3b attack and formation of C3bBb-properdin convertases [3, 4]. Adsorbed proteins could further provide abundant reactive moieties for covalent binding to C3b, thereby inadvertently promoting complement opsonisation. Furthermore, C3 opsonisation is continuous and changeable in vivo [4]. Collectively, the dynamics of non-specific blood protein binding might explain species disparities in complement activation pathways, opsonisation efficacy and kinetics. Thus, to address C3 opsonisation challenge, improved strategies are needed either to overcome (or dramatically suppress) non-specific protein deposition or render bound IgG (or C3b)

functionally inactive.

Disease progression/regression. C5a is a potent anaphylatoxin, which is liberated on cleavage of the fifth complement protein (C5) [1]. C5a gradient helps with recruitment of immunosuppressive cells [5]. This is of concern with current use and future developments of anti-cancer nanomedicines, since intratumoural elevation of C5a (i.e., through intratumoural complement activation by extravasated nanomedicines) may promote integrated immunosuppressive activities and accelerate tumour growth in due course [5]. In parallel, the possibility of nanoparticle-mediated intracellular complement (complosome) activation may modulate cell physiology and metabolism and initiate resistance to immunotherapies [1, 6]. Therefore, complement and compolosome inhibition strategies must be sought to address these challenges. On the other hand, the therapeutic efficacy of extravasated nanoparticles in certain inflammatory conditions (e.g., atherosclerosis) might be enhanced through local and controlled nanoparticle-mediated complement activation processes, since locally liberated anaplyatoxins may promote recruitment of immunosuppressive cells.

Infusion-related reactions. Prediction of infusion reactions to nanomedicines has proven unsuccessful with available allergy tests [7]. However, there are sporadic reports claiming a causal role for complement anaphylatoxins in adverse reactions to nanomedicines [8], but prior complement/cytokine profiling in human blood has not convincingly and reliably identified individuals at risk of adverse infusion reactions to nanomedicines [1, 7]. Furthermore, there are attempts that have tried to establish correlation between nanoparticle-mediated complement activation in human serum and cardiopulmonary responses in pigs [8], but these empirical approaches have not satisfactorily established a role for complement activation in infusion reactions [1]. Contrary to these, compelling evidence indicate a direct role for some macrophage sub-populations (and other immune cells) in infusion-related reactions independent of complement activation, which questions the use of the porcine model (and other cloven-hoof animals) in nanomedicine safety assessment [1, 7, 9]. Considering disparities in complement activation between preclinical animal models and predicting hypersensitivity (including complement knockout murine models), the role of complement (or complosome) activation in hypersensitivity reactions to nanomedicines in human subjects, if any, still remain to be deciphered.

Advances in Science and Technology to Meet Challenges

We provide a complement-motivated roadmap to address the abovementioned challenges through improved surface engineering initiatives and the use of complement inhibitors **(Figure 1)**.

It is widely accepted that nanoparticle surface functionalization with long chain poly(ethylene glycol)s (PEGs) and related polymers can reduce protein deposition, but this approach has not prevented complement activation [1]. However, PEG conformation and spacing can modulate these events and for optimal protein exclusion PEG chains need to be 1 nm apart [1]. A possible approach to achieve such desired topological characteristics is through surface pairing of long PEG chains with their shorter counterparts. Thus, void filling with shorter PEG chains may not only reduce protein binding to the pristine surface, but could also modulate configuration of adjacent longer PEG chains [10].

There are also continuous efforts in designing alternative biocompatible polymers to replace PEG. Poly(oxazoline)s are at the forefront of such efforts. In the murine model, poly(oxazoline)-coated nanoparticles avoid complement activation and circulate for prolonged periods of time. However, in human blood these nanoparticles rapidly trigger complement activation through C1q binding and undergo C3b opsonisation, which makes them prone to rapid ingestion by human macrophages [2]. Thus, future efforts in polymer engineering should focus on design of biocompatible superhydrophobic species that confer universal protein repelling properties. Other alternative approaches may take initiative from those microbial surface strategies that promote IgG deposition through its Fc moiety, which consequently suppresses or halt complement activation [1]. Another approach would be to functionalise surfaces with phage-derived peptides displaying high factor H-capturing activity [1].

Future development of a broader library of species-specific complement inhibitors could also address many of the shortfalls in translational nanomedicine [1]. Different complement inhibitors may be used either for surface functionalization of nanoparticles or alternatively employed in combination therapies with nanomedicines. Broader availability of species-specific complement inhibitors as well as developments in C3 knockout pigs and identification of spontaneous complement deficient large animals (e.g., dogs) may further resolve the role of complement and complement receptors, if any, in nanoparticle-mediated infusion reactions [1, 11, 12]. Despite these suggestions, arguably, C3 deficiency (or inactivity) in humans may increase susceptibility to multiple bacterial infections and limit repeated administration of C3 inhibitors. However, prolonged treatment of non-human primates with the C3 inhibitor compstatin, which has resulted in complete saturation of plasma C3, showed no weakened immune system or susceptibility to infections [13]. More significantly, recently a compstatin-based intervention (AMY-101) has successfully and safely treated a Coronavirus disease 2019 patient [14]. These observations at least confirm safety profile after prolonged C3 inhibition and strengthen the case for the use of complement inhibitors in nanomedicine initiatives.

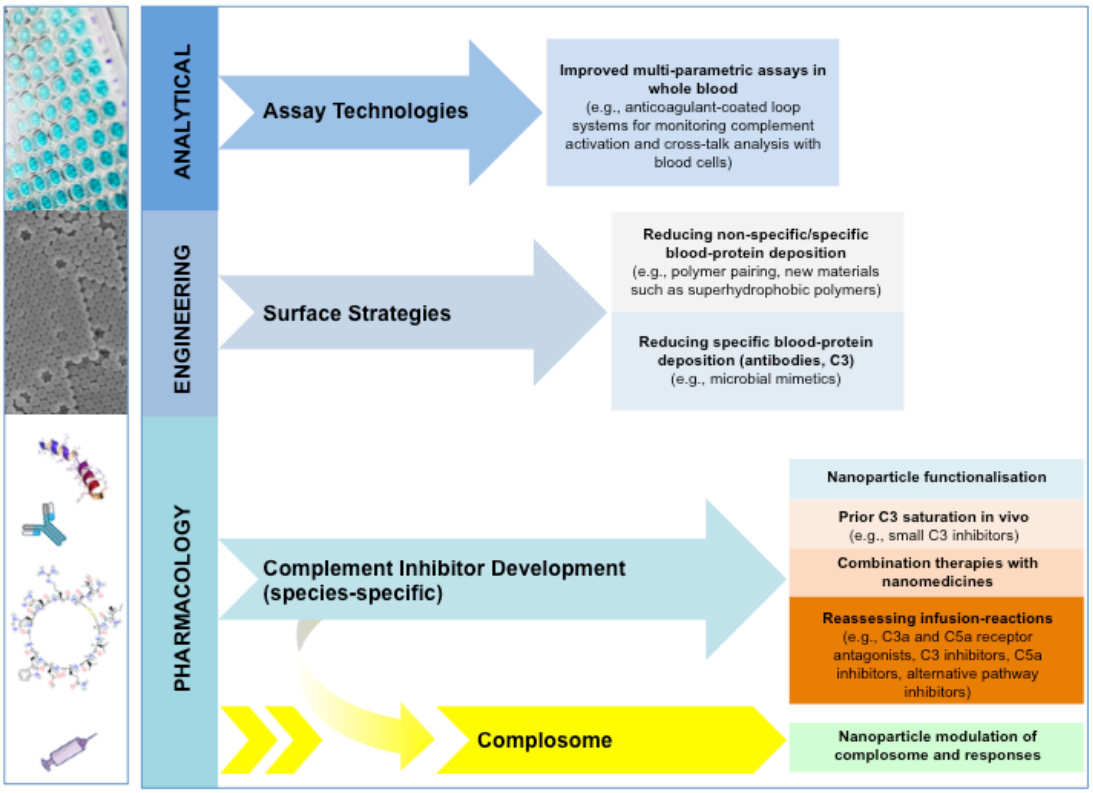

Figure 1. An explorative complement roadmap to guide future nanomedicine research and development.

Concluding Remarks

Complement testing has long been included in the criteria for assessment of haemocompatibility of biomaterials defined in ISO 10993–4. However, there are technical difficulties and limitations in nanomedicine engineering for generating complement-inert products. An outstanding issue is non-specific protein adsorption and its role in complement activation either directly and/or through contact system. Developments in multiparametric complement assay procedures in whole human blood **(Figure 1)** as well as concerted "nanomedicine structure-function" profiling are expected to thrive and improve

our understanding of integrated biological pathways and material features that trigger complement. This will pave the way toward development of "immune safe-by-design" initiatives from a disease and "patient-centric" perspectives. Within this context, future development of species- and pathway-specific complement inhibitors could open up new avenues for assessing the role of complement and complosome in nanomedicine performance and safety. On the other hand, controlled nanomedicine-directed complement activation could be beneficial in vaccination strategies (due to complement adjuvanticity [1]) and pathological conditions that will benefit from influx of immunosuppressive cells.

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3. Multifunctional Biomolecular Drug Designs

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Status

Drug delivery technologies aim to optimise drug efficacy and reduce side effects by controlling temporal and spatial accumulation at the target site. Marketed controlled release technologies have had immense impact in healthcare, for example, sustained release oral capsules, enteric coatings, and implants that control the rate, timing and amount of drug release. Furthermore, half-life extension technologies e.g. PEGylation or Fc fusions built into the drug design, increase blood residency to facilitate longer-lasting drug effects. Whilst, temporal controlled delivery has had a major impact, site-specific drug delivery remains a challenge in the field. Antibody drug conjugates (ADCs) available in the clinic facilitate active targeting, but have a low drug payload capacity and the number of identified disease-specific targets are limited.

Nanocarrier-based nanomedicines have gained great attention due to nanoscale physical and physiological properties such as a high surface to volume ratio that amplifies surface engineered functions e.g. steric coats and ligand-mediated targeting, and nanoscale dimensions, to allow migration through the vasculature, extravasation into tissue, and cellular entry. This, in combination with high drug loading, promotes them as an attractive drug delivery platform. Present-marketed nanocarriers are limited to non-targeted systems such as the anti-cancer liposomes Doxil® and DaunoXome® that rely on passive accumulation at the disease site. Furthermore, multicomponent nanocarriers that combine multiple modalities such as drug and imaging that could, for example, allow theranostics approaches have not reached the clinic.

Technological solutions that allow multifunctional designs with precise display of functional molecules and site-selective targeting would fulfil an unmet need and take the nanomedicine field forward. The application of a modular design to enable "*of-the-shelf*" selection of functional group combinations would work towards personalised medicines.

Current and Future Challenges

The surface of a nanocarrier determines its biological interaction with blood and cellular components. This includes not only the disease target, but also phagocytic cells and proteins of the mononuclear phagocyte system (MPS) involved in nanoparticle clearance. Controlling this *"nano-bio"* interface through particle surface engineering is crucial in order to achieve the desired biological effect e.g. non-detection, or "stealth", to MPS recognition, and active engagement with the disease target. Furthermore, the number, spacing, orientation and conformational state of the binding ligands strongly influence interaction with the target. Recent advances has seen controlled display of monoclonal antibodies on lipid nanoparticles[10]. There is, however, an absence of generic methods to control the surface density and display of a wider panel of functional molecules on different nanocarriers. This is even more problematic with multimodal nanocarriers combining therapeutic, imaging and targeting functionalities that are susceptible to inter-molecule steric hindrance, if not precisely positioned. Moreover, advanced surface analysis techniques such as x-ray photoelectron spectroscopy used to measure the surface elemental composition is a rarely used technique, with too much reliance on hydrodynamic diameter, morphology and surface charge for characterisation of coatings that has contributed to a lack of standardisation and optimisation in the nanocarrier field[11]. There is still a dependency on the use of poly (ethylene glycol) (PEG) as a steric coat to reduce MPS-mediated particle clearance. Possible immunogenicity, its non-

degradability, and limitation to precisely-tune the pharmacokinetics by PEGylation calls for other stealth technologies.

An alternative platform to nanocarriers is required that meets the criteria of well-defined, reproducible control of functional molecule display in order to maximise functionality.

Advances in Science and Technology to Meet Challenges

Modular biomolecular drug designs based on protein-nucleic acid constructs could provide the solution. Albumin is utilised in marketed drug delivery products[12] due to its natural transport properties facilitated by multiple ligand binding sites and long circulatory half-life due to engagement with the cellular recycling neonatal Fc receptor (FcRn)[13]. A recent advance is recombinant human albumins engineered with different FcRn affinities by single-point amino acid substitutions in albumin domain III for fine-tuning of pharmacokinetics[14]. The availability of a single free thiol at position 34 (cys34) can allow site-selective attachment of an oligonucleotide (ODN) "handle" distant from main the FcRn-binding interface. Base-driven annealing can then be used to direct the incorporation of complementary ODN strands (or modules) bearing functional molecules into the construct (Figure 1A)[15]. The number and the inter-molecule distance of the functional molecules, therefore, precisely defined and controlled by ODN strand length and position of the modified nucleotide in the ODN sequence. Possible steric effects between closely packed functional molecules is a challenge when utilising simple double ODN stranded designs.

Utilisation of nucleic acid nanotechnology to direct the controlled assembly of more complex nanostructures could expand the repertoire, display pattern and number of functional molecules, and importantly, drug loading capacity and synergistic combinations. Nucleic acid origami[16], or self-assembled nucleic acid nanoscaffolds[17] could provide the solution. Assembly of functionalised precursor strands allows programmable display of molecules (Figure 1B). This would allow display of low affinity ligands in polyvalent formats that may discriminate between disease and normal tissue by selected engagement in disease areas exhibiting overexpressed receptor clusters. This may be used as a strategy to circumvent the low number of identified cancer specific targets.

Nucleic acid modifications such as Locked Nucleic Acids incorporated into the nucleic acid design are needed for stability in serum, and stable thiol linker chemistries such as monobromomaleimide[18] to allow stable attachment to albumin. Alternatively, "click" chemistry can be utilised for nucleic acid conjugation to albumin or functional groups. Stimuli-responsive chemistries used in nanocarrier [19] and ADCs designs could be incorporated into the biomolecular constructs for triggered release of drug at the target site if required for its action. Human albumin is a well-tolerated, non-immunogenic serum protein. Whilst, recombinant human variants displaying described mutations for tunable FcRn engagement show no increase in immunogenicity in an EpiScreen™ time course T cell assay. Nucleic acids can elicit innate immune reactions by engagement with, for example, cellular Toll-like receptors but can be mitigated by strand length, sequence and design, and chemical modifications.

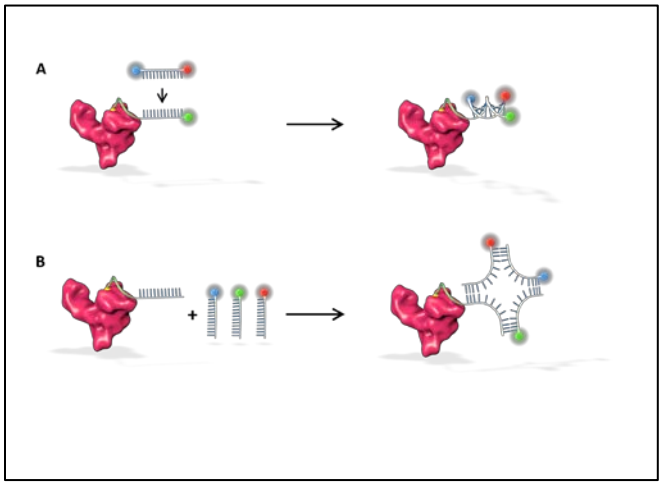

Figure 1. Schematic representation of multifunctional biomolecular drug designs composed of albumin (red) and functional molecule (blue, red and green spheres) bearing oligonucleotide modules based on double stranded (A) or nanoscaffold (B) assemblies.

Concluding Remarks

Albumin-nucleic acid biomolecular constructs offering programmable display of functional molecules in combination with tunable pharmacokinetics is a paradigm shift in the nanomedicine field. Furthermore, modular designs based on a library of functionalised ODN strands works towards *"of-the-shelf"* selection personalised to the patient.

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4. Nanomedicines for cancer therapy: Challenges and Future Prospective

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Status

Nanocarriers (NCs), the essence of nanomedicines are being implemented for more than three decades to overcome several limitations of conventional chemotherapy. These limitations include nonspecific biodistribution, poor water solubility of most common chemotherapeutic drugs (i.e. Paclitaxel, cis-platin, Doxetaxel etc.), low therapeutic indices, and multidrug tumor resistance. Furthermore, NCs enable encapsulation of large therapeutic molecules (e.g., RNAi, mRNA, CRISPR etc.), to improve their solubility and bioavailability, alter their bio-distribution, and can also facilitate entry into the target cell. Most of NCs based delivery systems for cancer therapy exploits the enhanced permeability and retention (EPR) effect, unique characteristics of solid tumors (i.e., leaky vasculature and defective lymphatic drainage), which allow NCs to preferentially accumulate in the tumor ("passive targeting") (Figure 1)[20]. NCs can also be decorated by targeting moieties (e.g, folate, monoclonal antibodies, peptides etc.) to achieve active cellular targeting, improve cellular entry and tumor retention [21,22]. Active targeted NCs must first reach the target to take advantage of this increased affinity and avidity. Therefore, efficient passive targeting is a requirement for NCs designed to systemically target solid tumors or its microenvironment. However, only a small percentage of systemically administrated NCs accumulate even in high-EPR xenografted tumors. A recent meta-analysis of preclinical data on NC-based delivery platforms for tumors published over the past 10 years suggested that a median of about 0.7% of the injected dose (ID) of NCs reaches the target tumors [23]. In absolute terms, this number seems small, raising serious concerns about the relevance of EPR based therapies. However, in relative terms, a delivery efficiency of 0.7% for NCs is substantially higher than the delivery efficiency for most of the conventional formulations of chemotherapeutics that are currently dominant in the clinic, including docetaxel, paclitaxel, and doxorubicin [24-26]. Although most of these NCs alter the pharmacokinetics, toxicological profile, or solubility of drugs, few have also shown significant survival benefits and improvement in therapeutic efficacy over the parent drug in clinical studies.

This could be due to multiple physiological barriers facing NCs upon systemic delivery. Our current understanding of EPR effectiveness is limited by data obtained using preclinical tumor models that mostly do not recapitulate human tumors. In fact, the most commonly used subcutaneous tumor xenografts are rapidly growing, resulting in very high-EPR tumors that could provide a false impression of the therapeutic benefit of NCs in therapies that rely on EPR-based targeting[27]. There is also limited patient derived data on the extent of EPR and its effect on drug accumulation in the tumor site that translates into clinical efficacy [27]. Further investigation of the EPR in various human tumors and the development of better preclinical models is therefore essential for the design of NCs with better tumor penetration and therapeutic outcome[28]. Furthermore, the development of companion diagnostic tools to predict patient's response to the treatment and patient's stratification is greatly needed.

Current and Future Challenges

The therapeutic efficacy of systemically administrated NCs is hampered by the heterogeneity of the EPR effect within and between different tumors. Variable endothelial gaps (ranging from one to hundreds of nanometers) result in non-uniform extravasation of NCs into the tumor [29]. Contradicting data suggesting

NCs extravasate more frequently in the tumor periphery or the hypoxic core suggesting on non-uniform diffusion of NCs in different solid tumors [30,31]. This data stress the importance of a better understanding of tumor structure and penetrance to NCs prior to treatment. Furthermore, permeability is not the only limiting factor; NC extravasation has also been shown to be governed by perfusion, which displays both spatial and temporal heterogeneity within a tumor, adding another level of complexity to controlling NC extravasation [32]. Additionally, physicochemical properties such as size and shape also affect NC extravasation and accumulation [33,34]. Reduction in the particle size significantly reduces the diffusional hindrance, thereby improving its penetration into the interstitial matrixs. One major technological challenge is the lack of quantitative tools and imaging techniques for NCs tumor accumulation and penetrance in patients. Multiple preclinical studies have demonstrated the advantages of utilizing imaging techniques for the evaluation and characterization of EPR in tumors as a prognostic tool for treatment success[35,36]. Bringing these technologies to the clinic could eventually enable clinicians to pre-select patients with high-EPR tumors who are likely to respond to NCs based treatments, thus remarkably improving therapeutic outcomes. Furthermore, collecting this data might enable the development of better preclinical models that will recapitulate human tumors and to design of NCs with better tumor penetration and therapeutic outcomes.

Advances in Science and Technology to Meet Challenges

Several strategies are currently being explored to improve tumor accumulation of administrated NCs. One such strategy is by augmenting the EPR using angiotensin II-induced hypertension, or heat-based vasodilation could be one solution. However, either technique could complicate the clinical translation of NCs. Applying active cellular targeting can improve tumor accumulation and increasing retention in the tumor, thus increasing the effective dose of the drug [20]. However, to date, no actively targeted NCs have been approved for clinical use. An additional approach is by utilizing cell-mediated delivery of NCs, thus bypassing the EPR. This approach exploits the ability of certain cell types to home or migrates to such tumors such as leukocytes [37]. Utilizing this approach, Huang et al. used the inherent ability of T-cells to traffic to tumors by conjugating nanocapsules encapsulating the topoisomerase I drug SN-38 to the cell surface [37] demonstrating a 90-fold increase of the drug in tumors sites compared to the free drug. This approach can be used for tumor targeting in low-EPR tumors or certain metastatic tumor locations that are unreachable by free NCs. However, this approach is limited to therapeutics with low toxicity to normal carrier cells. Certain compartments in the body, including lung, bladder, brain, peritoneum, and eye can be accessed locally for the administration of therapeutics. In these locations, local administration can help overcome some of the physiological barriers of systemic delivery. Local delivery can significantly improve the effective dose at the disease site and reduce systemic toxicity.

Harnessing of current imaging technologies (e.g., PET/CT, Ultrasound, etc.) and the development of more precise and quantitative techniques together with companion diagnostic NCs would enable clinicians to stratify patients suitable for NCs based therapies. Lee et al. utilized ^{64}Cu-labeled HER2-targeted liposomes and PET/CT to quantify drug accumulation in 19 patients with HER2 positive metastatic breast cancer [30]. Patients with high 64Cu-liposomal lesion deposition were associated with more favorable treatment outcomes. Such studies highlight the great potential of pre-selection of patients with high-EPR tumors using companion diagnostics or by theranostic approaches (Figure.1). This may remarkably improve therapeutic outcomes and reduce treatment-related toxicity and adverse reactions.

Concluding Remarks

Numerous preclinical studies utilizing NCs for cancer therapy have published in the last three decades. Since the approval of Doxil™ 25 years ago, more than 30 NCs have been approved for clinical use. This number is quite optimistic considering the relative infancy of clinical nanomedicine. However, the enormous potential of cancer therapy using NCs has been limited by the heterogeneity of the EPR effect

and the physiological barriers associated with it. The development of quantitative tools for EPR assessment and the application of such imaging technologies in humans should be enthusiastically pursued. Furthermore, such technologies could provide clinicians with companion diagnostic tools useful in pre-selection of patients who would respond to EPR-based therapies, and thus improving therapeutic outcomes. Local delivery of therapeutics using NCs can bypass physiological barriers associated with EPR, acting as an attractive strategy in the treatment of locally accessible tumors. Finally, the development of better and more predictive preclinical animal models, together with improving our quantitative diagnostic tools can increase the success rate of translation of novel NCs into clinical practice.

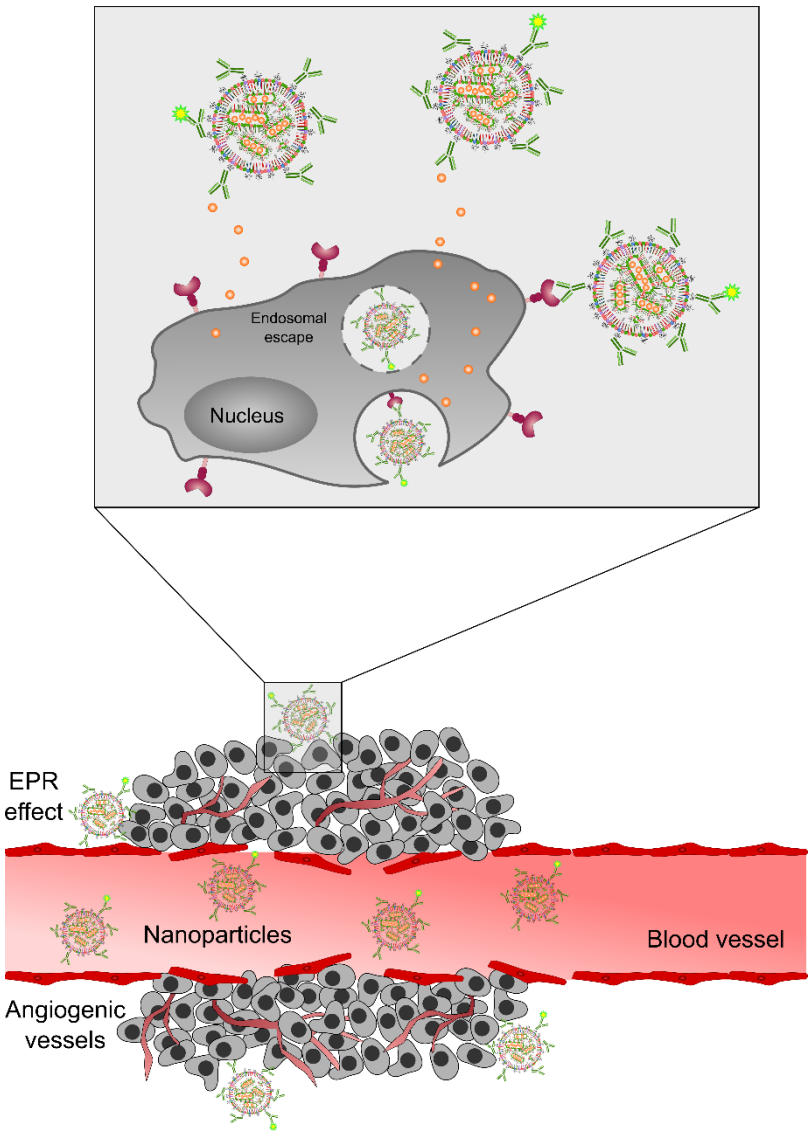

Figure 1. Schematic illustration of EPR based delivery of NCs. NCs can deliver drugs and imaging agents (represented by a green star bound to the monoclonal antibodies that decorate the NCs) to tumors.

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5. Nanotechnology against diabetes

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Status.

Diabetes mellitus [1], a metabolic disorder characterized by the abnormally elevated blood glucose level (BGL), causes 451 million cases worldwide in 2017, which was predicted to reach 693 million by 2045 [2]. The rising prevalence rate and difficulties in thorough curing impose great necessity to its deep-going investigation. Insulin, a hormone produced by beta cells of the pancreatic islets, plays a critical role in the homeostasis of glucose levels. Based on the physiological performances of this regulating molecule, diabetes mellitus is typically classified into insulin-dependent type 1 diabetes that results from insulin insufficiency and non-insulin-dependent type 2 diabetes that credits to insulin resistance [3]. Owing to the determinant effect of insulin treatment in both type 1 and late stages of type 2 diabetes, insulin delivery strategies that achieve dynamic regulation of glucose levels stand out to lead the way, which, however, also faces great challenges due to the complicated nature of physiological regulation.

The past few decades have witnessed the burgeon of nanotechnology. In the field of disease treatment, nanomaterial-based delivery systems have proven to be competent in improving the solubility, pharmacokinetics and therapeutic index of drugs. Up to now, great efforts have been dedicated to pushing forward the clinical translation of nanomedicines, especially for cancer therapy [4, 5]. Relying on their superior performance in protecting cargos from degrading and achieving on-demand release at right time with proper dose, the nanoparticulated formulations have also greatly benefited insulin delivery for diabetes treatment.

Current and future challenges.

Since the blood glucose level varies dynamically in accordance with metabolic activities, enhanced glycemic control within the healthy range (70–140 mg per dl or 4–8 mM) is the primary goal in diabetes treatment [1]. To achieve this, following challenges must be properly handled:

1) Physiological barriers [3]. Different administration routes may be confronted with several barriers *in vivo*. Physical barriers such as skin that includes epidermis and dermis layer can hinder bioavailability of insulin in the transdermal delivery. While pH change in GI tract would unavoidably disrupt the activity of insulins administrated through the oral route. Besides, enzyme degradation in the nasal delivery and immune recognition in pulmonary delivery also constrains adequate accessibility.
2) Poor patient compliance. When subjected to subcutaneous injections, patients will have to suffer from pain and some other syndromes, including tissue necrosis and microbial contamination, which would cause mental stress that comprises the therapeutic outcomes.
3) Lack of fast response. The blood glucose level fluctuates with metabolic states that are influenced by dietary intake. Therefore, flexible adaptation of insulin release that quickly responds to the changes in glucose level still remains to be the toughest challenge.
4) Safety concerns. The side effects in the treatment mainly derive from two aspects. On one hand, if overdosed, the hypoglycemia arises to be the potential risk that may threaten the physical conditions

of patients. On the other hand, the uncertain biocompatibility of materials used for delivery can also present biosafety issues that spark massive concerns.

Advances in science and technology to meet challenges.

In front of above-mentioned obstructions, many studies have been accomplished with nanotechnologies to surmount them, which greatly facilitated the therapy against diabetes [1].

Diversified nanomaterials such as lipids, polymers and inorganic particles have been employed in the insulin delivery in order to provide protective revisors and achieve sustained release. For instance, silica nanoparticles coated with lipid layers were shown to protect insulin from enzyme degradation, where the release profile was also improved [6]. A polymeric nanoparticle made of hydrophilic *N*-(2-hydroxypropyl) methacrylamide copolymer (pHPMA) derivatives and cell penetrating peptide (CPP) was demonstrated to overcome the physical barriers in oral insulin delivery [7]. Polymersome-based vesicles (d-GRPs) formed by self-assembly of hypoxia and H_2O_2 dual-sensitive diblock copolymer was also demonstrated to enhance the insulin release rate *via* transdermal administration [8]. These optimistic results indicated that enriched delivery strategies significantly advanced the diabetes treatment.

Instead of traditional subcutaneous injection, the non-invasive oral, inhaled and transdermal delivery are more acceptable owing to the reduced distress. For example, the recent smart microneedle patch-based transdermal delivery [9] represents a strategy towards painless insulin administration.

For the sake of precise control over insulin release, closed-loop smart insulin delivery systems [10] that can mimic normal pancreatic function and secrete insulin in a continuous and self-regulating manner have been comprehensively explored. Glucose-specific reactions such as oxidation by glucose oxidase (GOx), recognition by phenylboronic acid (PBA) or glucose binding proteins (GBP), have been adopted for glucose sensing and modulating subsequent insulin release [3]. To build fast insulin release platforms, biomimetic vesicles with hypoxia-sensitive capacity or multi-factor responsive properties were put forward as new type of insulin nanocarriers [9, 11]. Moreover, liposomal vesicles-in-vesicle superstructure-based synthetic beta cells equipped with a glucose sensing system and membrane fusion machinery have been developed for tuning dynamic insulin release in response to changes in glucose levels [12]. More importantly, the dose of delivered insulin also requires prudent manipulation to avoid side effects. Recently, a smart *i*-insulin was developed by conjugating insulin with a glucose transporter inhibitor to mitigate hypoglycemia [13]. Besides, biocompatible materials are exploited to reduce the damage to normal organs and tissues as well [14].

Concluding remarks.

Tremendous progress has been made against diabetes during last few years. Particularly, accompanied with micro- and nanotechnology, insulin delivery has gained favorable circumstances to step forward. With the purpose of long-term control toward insulin release, diversified materials with improved biocompatibility, non-invasive administration and close-loop based smart delivery have been fully investigated to answer arising challenges. Although remarkable progress has been achieved in dealing with harsh environment, poor patient compliance and inflexible responsiveness, there is still a long way to go in the treatment of this metabolic disease. Under the assistance with advanced nanotechnology, more groundbreaking innovations with high glucose-sensitivity, excellent biocompatibility and great momentum in clinical translation are expected to be realized to help people with diabetes in the near future.

Acknowledgements

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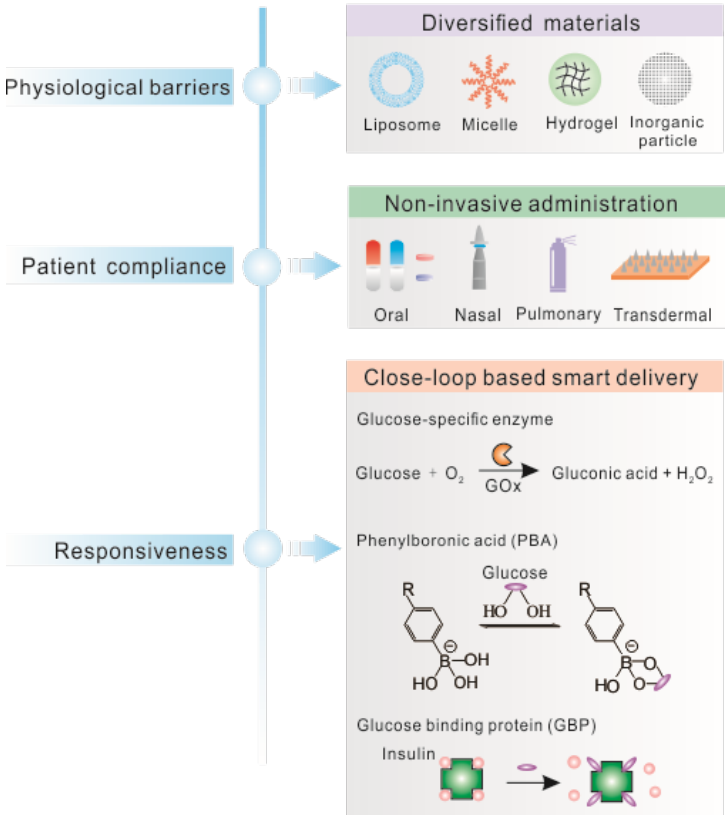

Figure 1. Major challenges and corresponding advances to meet these challenges in insulin delivery.

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6 - Nanomedicines for thrombolysis

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Status

Acute obstruction of blood flow as a result of a clot or an embolus is a leading cause of death and long-term adult disability in the western world and is associated with a variety of diseases such as: myocardial infarction, acute pulmonary embolism (PE) and ischemic stroke. In these acute medical conditions, a clot busting drug can be infused systemically or locally using a catheter, to restore normal blood flow. Currently, the only FDA approved thrombolytic drug for treatment of stroke, myocardial infarction and acute PE is tissue plasminogen activator (tPA). tPA acts by enzymatically converting circulating plasminogen into plasmin, which in turn degrades the fibrin mesh within the clot. Although its benefit for stroke patients has been proved more than 20 years ago, tPA usage is also associated with an increased risk of haemorrhagic transformation and bleeding complication [1]. Thus tPA treatment is given within a defined time window (<4.5hr from the onset of symptoms for stroke patients) and only after intracranial haemorrhage is ruled out via CT/MR imaging – thus delaying treatment which in turn increases brain damage. Moreover, tPA is also known to promote brain edema and enhance blood-brain barrier leakage. In addition to the risks associated with tPA, reperfusion is not always achieved, particularly for large vessel occlusions. As a result of these limitations, only <9% of stroke patients are treated with tPA, while for PE only patients presenting with acute, massive PE are advised to receive thrombolytic treatment. Altogether, there is a critical need for improving thrombolytic treatment by increasing lysis efficacy while reducing side effects.

Hence targeted thrombolytic nanomedicines, can potentially revolutionize thrombolytic treatment by maximizing its on-target activity while minimizing its off-target side effects, see figure 1. Thrombolytic nanocarriers can deploy large amounts of clot-busting drug directly at the clot site, utilize multivalent adhesive interactions with the clot surface, provide stability and long circulation half-life, trigger release of drug via exogenous and endogenous stimuli, and also possibly image and treat clots simultaneously. However, in-spite of recent advancement in thrombolytic nanomedicines, showing potential superiority in lysing clots and reducing bleeding, so far thrombolytic nanomedicines have not translated towards a commercial/clinical pathway.

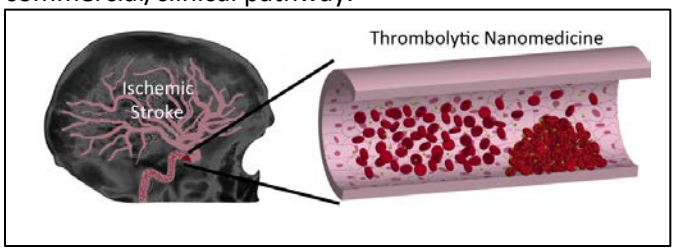

Figure 1. A clot or an embolus blocks the flow of blood in the brain leading to an ischemic stroke (left). Treatment via a thrombolytic nanomedicine can localize a clot busting drug at the occlusion site allowing efficient on target restoration of blood flow (right) while minimizing off-target side effects, such as: brain haemorrhage.

Current and Future Challenges

Recently, there has been growing interest in leveraging new approaches for improved thrombolytic treatment using nanomedicines. Some of the main issues in the field include:

1). Endogenously Activated Thrombolytic Nanomedicines: Use of nanomedicine that can activate locally via an external stimuli, such as ultrasound or magnetic forces, have been pursued for over more than two decades, however, their clinical translation is challenging. On the other hand, endogenously activated thrombolytic nanomedicines offer the ability to act autonomously upon injection and be responsive to

disease condition. These 'smart' carriers can be designed to respond to a biological stimulus or be based on a bio-physical stimulus such as: shear stress or platelet contraction forces [2,3]. Although these carriers are usually more complex to fabricate and characterize, smart responsive carriers can revolutionize thrombolytic treatment.

2). Improved Lysis of Platelet Rich Clots: Functionalized nano-carriers can utilize specific interaction with different constitute of clots, including activated platelets. Recently, promising results have been shown with platelet mimetic approaches and with ligands interacting with platelet-based receptors [4,5]. These approaches may also be valuable in demonstrating lysis of platelet rich clots, in which tPA has been shown to be ineffective.

3). Imaging and Theranostics: Thrombolytic nano-carriers can be designed to allow their imaging in vivo, which is key to understanding their pharmacokinetics. Additionally, via molecular imaging clot composition and size can be evaluated which may provide valuable information on patient specific conditions and be used to optimize treatment accordingly.

4). Prophylactic: A safe thrombolytics can potentially be utilized for prophylactic treatment, as demonstrated in previous work using tPA coupled to erythrocytes [6]. Long circulating drug carriers may offer similar prophylactic potential [7], thus expending thrombolytic beyond its current usage in acute settings. However, prophylactic thrombolysis requires that the nanomedicine demonstrates minimal risk of bleeding complications.

5). Enhancing Lysis Kinetics: Recently, magnetic or self-propelled particles have demonstrated improved clot lysis in occluded vessel by altering the transport kinetics at occlusion sites [8]. Though still in its initial research phase, such approaches modulating the transport physics in the lysis process are very interesting.

Advances in Science and Technology to Meet Challenges

Current state-of-the-art nanomedicines for thrombolytic treatment fall short of providing significant clinically relevant improvement for treatment of thromboembolic conditions, such as stroke. Advances in science and technology to address the challenges in thrombolytic nanomedicine include:

In vitro Physiological Assays: Further development of advanced microfluidics and physiological mimetic models recapitulating human thromboembolic disease condition as well as bleeding and haemorrhage are instrumental to develop, test and optimize thrombolytic nanomedicine. These models enable the study of nanomedicines in a controlled dynamic environment relevant to human disease, while incorporating the relevant human biological elements such as: plasma, blood cells and endothelial cells [9].

In vivo Models and Imaging: In addition to these dynamic *in vitro* models, there is also a need for improved *in vivo* models that can be used to test thromboembolism disease conditions relevant to humans. For example, in mimicking embolic stroke conditions, clots of different relevant compositions need to be examined including platelet rich clots or patient derived/reconstructed emboli. Better models of brain haemorrhage are also needed to enable the study of thrombolysis even under this severe clinical scenario. Realtime *in vivo* monitoring of the lysis process and drug carrier's distribution is a technological challenge that needs to further advance as well. More studies with such carriers are required to explore both the therapeutic potential of nanomedicines *in vivo* as well as possible off-target side effects of such nanomedicines. Moreover, there is a need to combine molecular imaging with nano-carriers to allow characterization of the *in vivo* disease conditions. This could also be a valuable clinical tool to provide patient specific information.

Nanocarriers Engineering: Technologically there are many challenges associated with fabricating and designing efficient thrombolytic nano-carriers. Parameters such as: size, shape and deformability are known to affect the carriers' performance [10] including their circulation time and there is a need for simple, scalable fabrication methods to allow control over these parameters. Though the field of active and responsive nano-carriers is very promising, these carriers are usually more challenging to fabricate as

well as to characterize.

Concluding Remarks

In-spite of the problems associated with tPA, it is currently the only acceptable thrombolytic therapy for a wide range of life-threatening acute conditions. Nanomedicines offer new unprecedented opportunities for improving thrombolytic therapy as well as expending its clinical usage. However, to enable a paradigm shift in thrombolytic therapy via nanomedicines important technological and scientific challenges need to be addressed. More research is fundamental to allow better understanding of biological and physical mechanisms governing thromboembolism and thrombolysis as well as complications associated with thrombolysis. Leveraging this knowledge in combination with technological advancement can allow the design of improved thrombolytic nanomedicines with strong potential to impact clinical treatment.

Acknowledgements

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7. Nanomedicine for Atherosclerosis

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Status

Tackling cardiovascular diseases is a public health priority. Earlier diagnoses and improved therapies are required and nanomedicine has the potential to improve current clinical practice. Indeed, atherosclerosis is the buildup of a waxy plaque on the inside of blood vessels, and plaque deposits can block the flow of blood. Plaques can also rupture or crack open, causing the sudden formation of a blood clot (thrombosis). Atherosclerosis can cause a heart attack if it completely blocks the blood flow in the heart (coronary) arteries. It can cause a stroke if it completely blocks the brain (carotid) arteries. These diseases of the arterial wall leading to acute arterial thrombosis and cardiovascular events are responsible for the majority of deaths. Although considerable progress has been made in the prevention and treatment of cardiovascular diseases, they remain the main cause of death in the world [38]. It is estimated that 30-35% of deaths worldwide are due to cardiovascular diseases, twice more than cancer.

There is a medical need for new approaches for early diagnosis, improved or novel targeted therapies, and therapy monitoring [39]. Novel non-invasive imaging agents could better identify patients at risk for (recurrent) thrombotic diseases. Better and/or more personalized treatments are also required with the aim is to move from conventional drugs with systemic exposure to targeted drug delivery using nanosystems that minimize the systemic side effects and enhance drug localization and efficacy in atherosclerotic and thrombotic lesions. Targeting the drug using a nanosystem seems possible, as demonstrated in preclinical research works [40].

In the field of atherosclerosis, the concept of hybrid nanoparticles (so called “theranostics”) combining imaging and treatment in the same entity is more difficult to follow for a clinical purpose, the products will be either a contrast agent or a drug used sequentially. To carry a drug and an imaging agent together seems not an obvious clinical strategy since the imaging will be performed first in the patient, and according to the results, a drug treatment will be or not administered. However, the monitoring of patients’ responses to therapy with (separate) companion diagnostics could be highly valuable. Triggering a drug release or efficacy by internal/external signals could also be obtained.

These considerations open the way to nanomedicine for treatment or imaging of cardiovascular diseases. Intriguingly, little attention has been developed in this area of research (Fig. 1), and hence in future clinical developments.

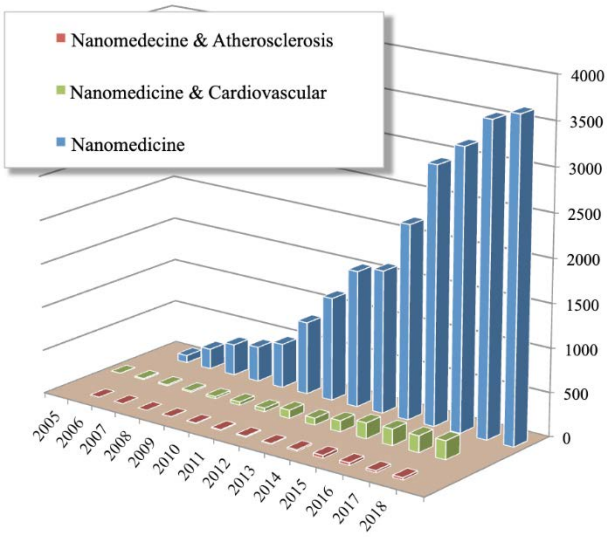

Figure 1 : Search on Medline performed on August 2019 with "Nanomedicine" as keyword gives more than 25,000 references. We found 1336 references for "Nanomedicine & Cardiovascular", and only 211 for "Nanomedicine & Atherosclerosis". Results obtained each year in each category are presented from 2005.

Current and Future Challenges

Atherosclerosis lesions are dangerous when a thin fibrous cap and a large necrotic core appear. At this stage, plaques may break exposing thrombogenic substances to the circulation driving the formation of intraluminal thrombi. Despite decades of research, the clinical and biological knowledge of these diseases did not provide enough diagnostic tools and therapeutic treatments.

Early diagnosis is mandatory to promptly initiate specific care and reduce clinical consequences. After moving from morphological approach to functional approach, the challenge for imaging technology is to shift from functional morphology to molecular imaging. This is true for all fields of pathology, including cardiovascular diseases in human. With the ageing of the population the prevalence of atherothrombosis will ineluctably increase in the next years. Early diagnosing patients at cardiovascular risk will significantly improve their quality of live because having been checked and treated appropriately, the risk in coming years is reduced profoundly.

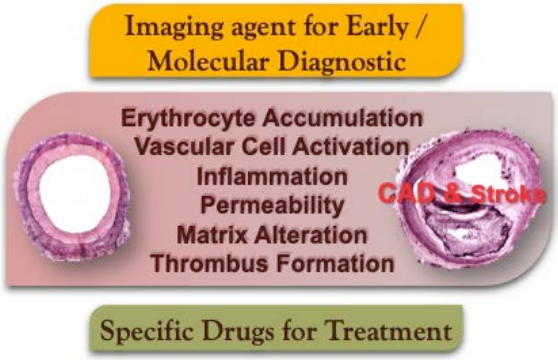

Figure 2: The evolution of blood vessels to the atheroma will lead to coronary artery diseases (CAD) or cerebrovascular diseases in the brain (stroke). 85% of CVD deaths are due to heart attack and stroke. New nanodiagnostics/therapies are required to improve the management of these cardiovascular diseases.

Accurate diagnosis is critical, partly because patients who have been discharged inappropriately from emergency departments, and are subsequently diagnosed with ACS, represent a high-risk group, with a

rate of fatal or potentially lethal complications of up to 26%. On the other hand, spontaneous and interventional revascularization and/or thrombolysis are not completely devoid of specific deleterious consequences due to acute pressurized blood reperfusion on ischemic tissue and the resulting tissue damage. Prevention of thrombotic events will reduce morbidity, reduce disability and its social cost.

One of feared conditions of an ageing population is also to have a stroke followed by serious mental and physical disablement. Successful nanomedicine would constitute a major societal advance in addressing the health and well-being of the population, especially (but not only) in the quality of life of the increasing proportion of elderly people prone to these debilitating events.

One main plausible application of nanoparticles in the management of atherosclerosis is the targeted delivery of atheroprotective or thrombolytic drugs. However, considerable challenges for nanomedicine in atherosclerosis concern the optimization of nanoparticle properties in order to enhance targeting of appropriate drugs while minimizing non-specific tissue residence. As it is the case for any novel pharmacological agent undergoing clinical trials, the investigation nanoparticles will also require thorough evaluation for toxicity, pharmacokinetics and biodistribution [41]. The design of safe (non-toxic) materials is a main issue in this field since the main biological effects should obviously not be on killing cells, as in the case of cancer cells. Indeed, the reported clinical trials remain very scarce in this field likely due to the rational design of non-cytotoxic nanosystems and hurdles related to scale up, GMP-grade production, quality control and full preclinical assessments which are required before clinical studies can be started.

Despite huge amount of pharmaceutical developments, the appropriate drugs are not all far from been fully optimized. For instance, the side effects of intravenous injection of recombinant tissue plasminogen activator (rt-PA) to induce vessel recanalization in acute thrombotic events are well known. Because of its short half-life, high doses of rt-PA need to be injected and intracranial hemorrhages are observed in the treated patients causing 50% of subsequent mortality. Due to the bleeding complications and reported neurotoxic effects, the benefit-to-risk ratio of rt-PA administration in human also rapidly decreases with time. In this context, a targeted rt-PA nanoformulation could represent an interesting therapeutic strategy [42].

The understanding of the progressive shift reported on Figure 2 from a healthy blood vessel to an atheroma has been largely supported by mouse models. In this context, it is noteworthy that there is also a need for better and more reliable preclinical models. Emerging concepts and new related treatments appropriate to human pathologies could rise from more observational studies on human sample biopsies and the corresponding images obtained directly on patients [43].

Advances in Science and Technology to Meet Challenges

Integrating an efficient transport mechanism using nanosystems, a stealth coating, a targeting and an active molecule is not fully clinically validated in the field of atherosclerosis. Indeed, no specific nanoparticle-based system is yet approved for diagnosis or therapy in cardiovascular diseases. The potential of nanotechnology-based therapies to overcome the disadvantages of systemic drug administration has been well recognized in the field of oncology, but not approved for diagnosis or therapy of cardiovascular diseases. To demonstrate its feasibility for patients, we have conducted several Phase I clinical trials (Table 1) in a Large EU-funded project, NanoAthero [44].

We evaluated the tolerance, dosimetry and SPECT imaging on healthy human subjects after injection of a macromolecular construct for molecular imaging of the thrombus. A GMP-grade Low Molecular Weight Fucoidan [8] was radiolabeled with technetium-99m (^{99m}Tech-Fucoidan). Ten healthy volunteers were included for administration of this microdosed radiotracer. ^{99m}Tech-Fucoidan had a favorable biodistribution with only 5% of the injected activity remaining after 24 hours and a safety profile [9]. A Phase IIa clinical trial for the validation of the ability of this SPECT radiotracer based on Fucoidan to demonstrate acute thrombogenic activity in patients with acute Deep Vein Thrombosis (12 patients) is ongoing and is expected to be completed by the end of the year.

Another achievement in NanoAthero project was to perform the feasibility study for delivery of GMP-prednisolone liposomes coated with polyethylene glycol (PEG) in human patients with atherosclerosis [10]. This nanoformulation improved the pharmacokinetic profile in humans (n = 13) of prednisolone with an increased plasma half-life of 63 hours (1.5 mg/kg). To prove the feasibility of prednisolone delivery to plaque macrophages in patients with atherosclerotic disease a randomized, placebo-controlled, double-blind trial in 14 patients with iliofemoral atherosclerotic plaques who were scheduled for endarterectomy was performed. Intravenously infused liposomal nanoparticle encapsulating prednisolone appeared in 75% of the macrophages isolated from iliofemoral plaques of patients. However, the liposomal nanoparticle treatment did not reduce inflammation or arterial wall permeability in patients as assessed by multimodal imaging.

Another Phase I clinical trial evaluated the suitability of using 'endogenous' HDL particles as nano-delivery systems. CER-001 is an engineered lipoprotein complex mimicking natural pre-beta HDL, consisting of recombinant human apoA-I and phospholipids. Eight patients received each a single infusion of CER-001 (3 mg/kg) which was co-administered with ^{89}Zr-labeled CER-001. Using serial PET/CT imaging, Zheng *et al.* showed that arterial uptake of CER-001 was higher in plaque compared with non-plaque segments [10].

We could also noticed, outside NanoAthero, the first-in-man trial (NANOM-FIM trial) to assess the safety and the efficacy of silica-gold nanoparticles for atheroprotective management of plaques [11]. The primary outcome was Total Atheroma Volume (TAV) at 12 months. 180 patients were assigned to receive either i) nano-intervention with delivery of silica–gold NP in a bioengineered on-artery patch (Nano group) (n = 60), or (ii) nano-intervention with delivery of silica–gold iron-bearing NP with targeted micro-bubbles and stem cells using a magnetic navigation system (Ferro group) (n = 60) versus (iii) stent implantation (Control group) (n = 60). This trial demonstrated that plasmonic resonance therapy using silica-gold nanoparticles has an acceptable level of safety for clinical practice and is associated with significant regression of coronary atherosclerosis.

Products	Aims	Patient number	Result #1	Result #2	Result #3
^{9m}Tech-Fucoidan	Safety / SPECT imaging	10	Favourable biodistribution	Safety profile	SPECT imaging
Prednisolone PEG-liposomes	Atherosclerotic plaque treatment	57	Pharmacokinetic profile improvment	plaque location	No reduction of inflammation nor arterial wall permeability
CER-001	Atherosclerotic plaque targeting	8	Increase of plasma apoA-I levels and of the plasma cholesterol efflux capacity	plaque location	No adverse events and no changes in blood pressure nor heart rate
Silica-gold nanoparticles	Safety / Atherosclerotic plaque treatment	180	Reduction of the Total Atheroma Volume	Lower risk of cardiovascular death	Safety profile

Table 1. Phase I clinical trials main results of four nanoparticle-based systems for diagnosis or therapy of cardiovascular diseases.

Concluding Remarks

The goals to use nanomedicine to fight atherosclerosis require a multidisciplinary approach, combining complementary skills and providing an interface between chemists, biologists, pharmaceutical scientists,

imaging experts, clinicians, analysts, and ethicists. In order to realize these objectives, large consortia associating medical science of atherothrombotic diseases in humans, with cutting edge technology of nanocarriers in the cardiovascular system, enriched by ethicians and academic/private interactions have to be effective by developing know-how, concepts, and new molecular contrast agents and appropriate drug formulations in this field. Such an association of medical, biological and technological high standards, to successfully put forward diagnostic and therapeutic nanocarriers in the field of human atherothrombotic diseases will provide a solid basis for a new nanosystem paradigm as a valid strategy for the development of personalized cardiovascular medicine.

The use of targeted nanocarriers represent a major step towards targeted therapies in cardiovascular applications, and later on to some other major diseases like auto- and allo-immune diseases, infectious diseases, and cancer. Moreover, the clinical demonstration of safety complying all regulations combined with efficacy of these nanocarriers in humans should prompt the pharmaceutical industry to embrace these novel approaches and provide the society with rational criteria to support further developments.

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8. Patient Stratification to Improve Nanomedicine Performance and Clinical Translation

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Status

Nanosized carrier materials are extensively employed to improve the stability, solubility, pharmacokinetics, biodistribution and target site accumulation of (chemo-) therapeutic drugs. As a result, nanomedicine formulations typically display less side effects and, in preclinical settings, enhance therapeutic efficacy as compared to non-formulated drugs. The first cancer nanomedicines, including most prominently PEGylated liposomal doxorubicin (Doxil® / Caelyx®), were approved for clinical use in the 1990s. In the 2000s and 2010s, several additional nanomedicinal anticancer drugs reached the market, such as Abaxane® (albumin-based paclitaxel nanoparticles), Onivyde® (liposomal irinotecan) and Vyxeos® (liposomal daunorubicin plus cytarabine). Notably, Vyxeos® was the first agent to significantly improve overall survival as compared to standard-of-care in patients with refractory acute myeloid leukemia [1].
Nanosized carrier materials are also enabling the clinical translation of novel drug classes, based e.g. on nucleic acids. Successfully employing nucleic acids as therapeutics crucially depends on nanocarriers that can prevent their degradation in the bloodstream and ensure their delivery into target cells. In 2018, the first small interfering RNA (siRNA) therapeutic, i.e. Onpattro® (patisiran; lipid nanoparticles containing anti-transthyretin (TTR) siRNA), was approved for the treatment of amyloidosis. Onpattro® inhibits the hepatocytic production of mutant TTR and the resulting amyloid fibril formation in the body, halting or reversing a fatal hereditary disease for which hardly any other therapeutic options are available [2]. Although not an anticancer drug, the delivery technology underlying Onpattro's® approval is currently facilitating the development of nucleic acid-based nanotherapies for oncological applications, including e.g. vaccines for messenger RNA (mRNA) delivery.

As opposed to the above success stories, several high-profile cancer nanomedicines that showed promise in preclinical trials failed to demonstrate significant therapeutic efficacy in patients, include e.g. BIND-014 (docetaxel-loaded prostate-specific membrane antigen-targeted polyethylene glycol-polylactic acid nanoparticles), CRLX101 (campthotecin-loaded polyethylene glycol-cyclodextrin nanoparticles) and NK105 (paclitaxel-loaded polyethylene glycol-polyaspartate-based micelles). These disappointing outcomes and the relatively small number of anticancer nano-drugs that have managed to obtain FDA and/or EMA approval in the past decade are in sharp contrast with the huge amount of time, money and effort invested in the development of cancer nanomedicines. While this imbalance has multiple underlying reasons, including those related to traditional hurdles in drug development and translation, we argue that the most pressing issue is the lack of considering biomarkers (and companion diagnostics and theranostics) for patient stratification in clinical trials [3].

Current and Future Challenges

Given cancer's heterogeneity, it has become common practice in oncology drug development to employ biomarkers for guiding patient selection and clinical trial design. Most anticancer drugs currently in development are molecularly targeted therapeutics, such as tyrosine kinase inhibitors and monoclonal antibodies. Because these agents target specific cellular proteins, determining the expression levels of these proteins in tumor tissue (via histopathological stainings) helps to identify patients who are most likely to respond. It is clear that patient stratification cannot guarantee that all selected patients will eventually have good therapeutic responses, but it will definitely reduce the likelihood of including non-responders in clinical trials. As an example, patient stratification (i.e. exclusion of non-responders based on immunohistochemistry) has substantially contributed to the successful clinical development of human epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor (EGFR) -targeted therapeutics, such as Herceptin® (trastuzumab) and Erbitux® (cetuximab). In recent years, also genetic testing is increasingly used to determine a patient's eligibility for molecularly targeted therapies, as well as for matching certain immunomodulatory drugs to patients based on the number of mutations in their tumors. As an example, the anti-programmed cell death protein 1 (PD-1) antibody Keytruda® (pembrolizumab) has been shown to work best in patients with tumors characterized by high microsatellite instability (MSI-H) and DNA mismatch repair deficiency (dMMR) [4]. This notion has contributed to Keytruda® becoming the first FDA-approved cancer therapy based on a patient's specific genomic signature as a biomarker, rather than on the patient's tumor histology.

Considering these rationales and developments, it is striking that no biomarkers or companion diagnostic tests are available to guide the clinical translation of cancer nanomedicines. It is likely that the lack of probes and protocols for patient stratification, together with the high inter- and intra-individual variability in the tumor accumulation of drug delivery systems, explains the relatively poor therapeutic responses achieved with nanomedicine formulations in advanced-stage clinical trials. The effectiveness of treating solid tumors with cancer nanomedicines largely relies on their ability to accumulate in tumorous tissues, via the so-called Enhanced Permeability and Retention (EPR) effect [5]. While the extent of the EPR effect in humans (especially as compared to mouse models) and the appropriateness of the terminology are an ongoing subject of debate, it is clear that long-circulating cancer nanomedicines do accumulate in tumors and metastases in patients, at least to some extent. However, as a result of tumor and metastasis heterogeneity, a high variability in nanomedicine target site accumulation exists. To address this issue, and to improve the translation and clinical performance of cancer nanomedicines, probes and protocols are needed to predict and monitor their tumor accumulation and antitumor efficacy.

Advances in Science and Technology to Meet Challenges

Several strategies are currently being explored to predict and/or assess the tumor accumulation of cancer nanomedicines. These include liquid, tissue and imaging biomarkers (Figure 1). Liquid biomarkers can be based on circulating tumor cells [6], on serum protein/cytokine levels and on circulating genetic material providing information on the tumor's accessibility for nanoparticles, such as angiogenesis markers to indicate neovascularization or enzymes that are responsible for stromal remodeling [7]. Histopathology features, such as vascular density, can be exploited to establish tissue biomarkers. As it is common practice to obtain and examine tumor biopsies in cancer patients, investigating tissue samples enables the analysis of vascular and tumor microenvironmental features as indicators for the ability of nanomedicines to accumulate, penetrate and/or be retained in cancerous lesions. In this context, besides looking at blood vessels, which are responsible for carrying drugs and drug delivery systems to tumors, it seems worthwhile to also analyze tumor-associated macrophages, as these are (co-) responsible for retaining nanomedicines at pathological sites [8]. Establishing tissue biomarkers for patient stratification in cancer nanomedicine may profit from several recent technological developments, such as automated

whole-slide image processing and deep-learning models, which increasingly assist pathologists in analyzing tissue biopsies [9].

Non-invasive imaging holds particular promise for monitoring and predicting the accumulation and efficacy of nanomedicines. As an example, Ramanathan and colleagues at Merrimack Pharmaceuticals recently demonstrated that the tumor accumulation of Feraheme®, i.e. 30 nm-sized FDA-approved iron oxide nanoparticles, positively correlated with reductions in tumor size following treatment of patients with Onivyde® (liposomal irinotecan) [10]. Similarly, Perez-Medina et al. showed that quantitative PET imaging using a ^{89}Zr-labeled liposomal companion nanodiagnostic relatively accurately predicted Doxil® accumulation and efficacy [11]. In a theranostic setup, Lee and coworkers at Merrimack Pharmaceuticals demonstrated that PET-CT imaging enables the visualization and quantification of the accumulation of ^{64}Cu-labeled HER2-targeted liposomal doxorubicin in patient tumors and metastases, and that higher nanomedicine concentrations in malignant lesions tended to be associated with better therapeutic outcomes [12].

While it is too early to conclude which biomarkers are the most practicable and cost-effective for predicting nanomedicine accumulation and efficacy, the above studies do clearly demonstrate that it is both feasible and meaningful to employ companion diagnostics and theranostics to promote patient stratification in cancer nanomedicine clinical translation.

Concluding Remarks

Several strategies have been proposed to improve the translation and performance of cancer nanomedicines, including the use of more predictive models in preclinical research, establishing methods to enhance data reproducibility and standardization, increasing our understanding of bio-nano interactions, implementing artificial intelligence in cancer nanomedicine design and development, and more extensively integrating nanomedicines in combination therapy regimens. Addressing all of these issues, however, will be in vain if the developed nanomedicinal drug products are subsequently evaluated in unstratified patient cohorts, as in such settings, they will likely fail to demonstrate improved therapeutic efficacy as compared to standard-of-care treatment. Consequently, identifying biomarkers and establishing companion diagnostics and nanotheranostics for patient stratification will be of crucial importance, in order to pre-select those patients who are most likely to benefit from cancer nanomedicine treatment and to only include those in clinical trials. As such, patient stratification will help to enhance the efficiency of cancer nanomedicine clinical translation and it will contribute to the development of nanomedicinal anticancer drugs that are able to improve therapeutic outcomes in patients.

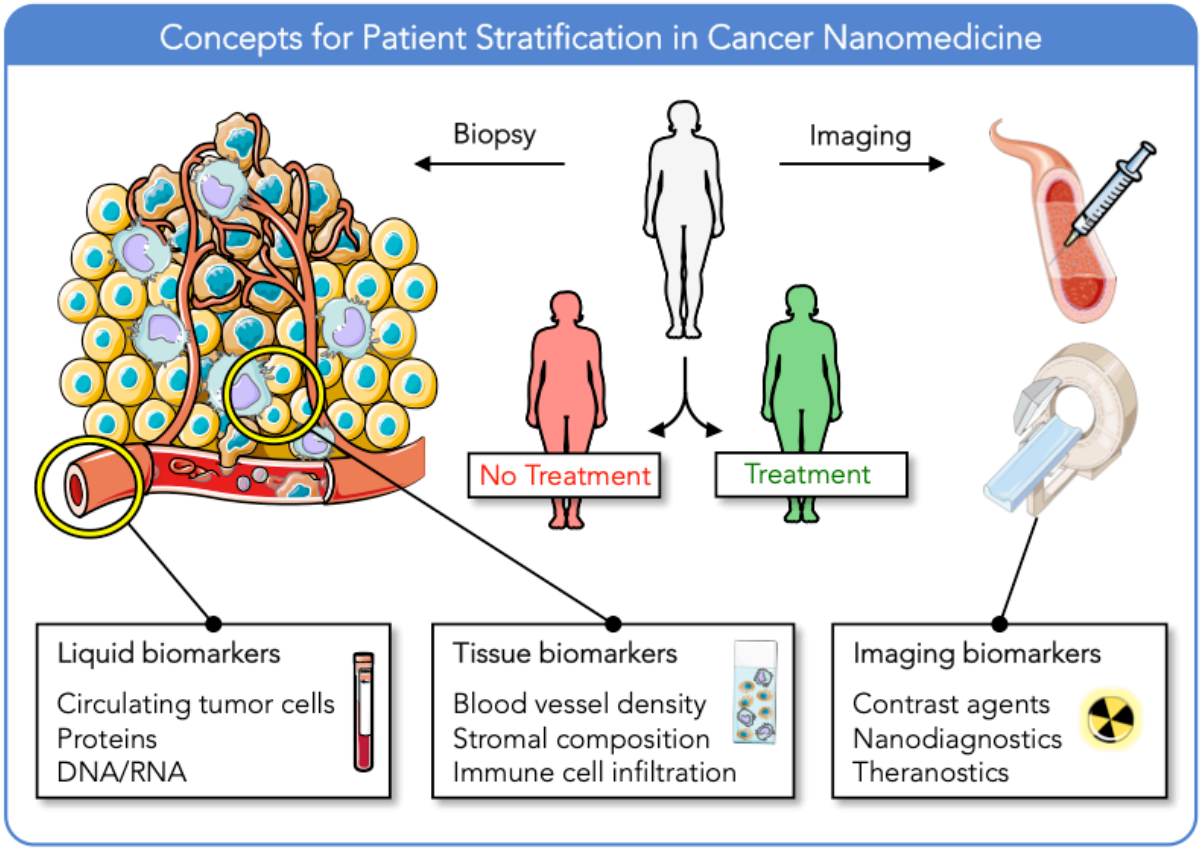

Figure 1: Patient stratification in cancer nanomedicine. Various tools and technologies can be conceived to enable patient stratification in cancer nanomedicine clinical trials. These include liquid biomarkers (e.g. circulating tumor cells and cytokines), tissue biomarkers (e.g. vessel and macrophage density) and imaging biomarkers. The latter can encompass standard imaging probes and protocols to noninvasively and quantitatively assess tumor blood vessel perfusion and permeability, as well as companion nanodiagnostics (e.g. iron oxide nanoparticles) and nanotheranostics (e.g. radionuclide and drug co-loaded liposomes). The overall goal of including such probes and protocols for patient stratification is to differentiate between individuals who are likely to respond to such targeted therapies and individuals who are unlikely to respond, thereby contributing to nanomedicine performance, translation and product development.

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