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Targeted nanomedicine: Lessons learned and future directions

Nuphar Veiga [a,1], Yael Diesendruck [b,1], Dan Peer [b,c,d,e,*]

[a] Laboratory of Angiogenesis and Vascular Metabolism, Center for Cancer Biology (CCB), VIB, Department of Oncology, Leuven Cancer Institute, KU Leuven, Leuven 3000, Belgium
[b] Laboratory of Precision Nanomedicine, The Shmunis School of Biomedicine and Cancer Research, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel-Aviv, Israel
[c] Department of Materials Sciences and Engineering, Iby and Aladar Fleischman Faculty of Engineering, Tel Aviv University, Tel Aviv, Israel
[d] Center for Nanoscience and Nanotechnology, Tel Aviv University, Tel Aviv, Israel
[e] Cancer Biology Research Center, Tel Aviv University, Tel Aviv, Israel

A B S T R A C T

Designing a therapeutic modality that will reach a certain organ, tissue, or cell type is crucial for both the therapeutic efficiency and to limit off-target adverse effects. Nanoparticles carrying various drugs, such as nucleic acids, small molecules and proteins, are promoting modalities to this end. Beyond the need to identify a target for a specific indication, an adequate design has to address the multiple biological barriers, such as systemic barriers, dilution and unspecific distribution, tissue penetration and intracellular trafficking. The field of targeted delivery has developed rapidly in recent years, with tremendous progress made in understating the biological barriers, and new technologies to functionalize nanoparticles with targeting moieties for an accurate, specific and highly selective delivery. Implementing new approaches like multi-functionalized nanocarriers and machine learning models will advance the field for designing safe, cell -specific nanoparticle delivery systems. Here, we will critically review the current progress in the field and suggest novel strategies to improve cell specific delivery of therapeutic payloads.

1. Introduction

The delicate balance between therapeutic efficacy and tolerability is the main bottleneck limiting the beneficial treatment of various diseases, including cancer, metabolic autoimmunity, neurodegenerative and infectious diseases. In the complex microenvironment of the human body, developing approaches to deliver a certain drug to a certain organ, tissue, or cell type is crucial for both the therapeutic efficiency and for drug safety, by limiting off-target adverse effects. Considering the tumor microenvironment as an example, delivering a cytotoxic drug exclusively to malignant cells will ensure the elimination of the tumor without risking toxicity or mutagenesis in healthy cells and will avoid critical adverse effects [1]. Likewise, treatment directed at immune cells to treat autoimmune conditions, should be directed to the specific autoantigens reactive cells, while avoiding the inhibition of a protective immune response. Many precision nanomedicine therapeutics were designed to answer this need. While free drugs simply diffuse in the body, exposed to quick clearance and degradation mechanisms, leading to limited effective dose in the disease site, nanoparticles (NPs) can enhance the therapeutic potential and safety of a drug in various aspects. NPs improve the bio-distribution and pharmacokinetics of drugs by stabilizing and protecting them from degradation and clearance by the kidney, liver and phagocytic cells [2]. NPs can overcome chemical and biological barriers such as solubility of hydrophobic drugs and the delivery of nucleic acids through the cellular membrane, respectively; and finally, increase the accumulation of a drug in the desired destination while reducing the effects on healthy sites[3,4].

Multiple obstacles are limiting the therapeutic potential of drug-loaded nanoparticles, and should be considered when designing a drug-delivery approach (Fig. 1): (i) size dependent clearance by the liver and kidneys; (ii) clearance by mononuclear phagocytic cells; (iii) biological membranes, including epithelial or endothelial barriers, (iv) specific binding to the target cell; and finally (v) cellular barriers, such as membranes, efflux pumps, endosomal escape, and lysosomal degradation. By tailoring the physiological characteristics of drug carriers or decorating the nanoparticles with molecules to actively breach through the barriers, nanomedicine can induce the therapeutic effect of a drug while limiting off target effects. For instance, nanoparticles can be functionalized with various molecules, including antibodies, peptides, oligonucleotides, polymers and more, to ensure the accumulation of a drug in a certain cell type or organ.

In this review, we will detail the multiple barriers that NPs encounter

* Corresponding author at: Laboratory of Precision Nanomedicine, The Shmunis School of Biomedicine and Cancer Research, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel-Aviv, Israel.
E-mail address: peer@tauex.tau.ac.il (D. Peer).
[1] Nuphar Veiga and Yael Diesendruck contributed equally to this manuscript

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on their way to the target cell and discuss the rationale for designing targeted drug delivery systems with different types of targeting moieties to maximize the potential clinical impact. We will further discuss how to increase the potential of a targeting strategy beyond one specific target, to ensure a versatile and flexible therapeutic approach for multi-factorial diseases.

2. Designing nanomedicines for targeted delivery

The physicochemical characteristics of the cargo should be taken into consideration when designing nanotechnology-based therapies, including the solubility, charge, and stability in various conditions. Based on the characteristics of a drug, the optimal targeting carrier should be chosen [5].

Due to the negative charge of oligonucleotides, such as siRNA, mRNA and sgRNA, they can be efficiently encapsulated in nanoparticles via ionic interactions with positively charged molecules like cationic lipids or polymers. Pluronic/poly(ethylenimine)(PEI2K) cationic nanocapsules were utilized for the loading of siRNA-PEG molecules on the surface, via electrostatic interactions[6]. As another example, cationic polyethyleneimine (PEI)-coated gold nanoparticles (AuNPs) where synthesized to form stable complexes with siRNA molecules [7].

While cationic compounds can facilitate the encapsulation of nucleic acids, positively charged nanoparticles can be immunogenic[8] and promote toxic adverse effects. Ionizable lipids were designed to overcome this issue, with Heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)- butanoate (DLin-MC3-DMA) as the most widely used lipid for gene therapy[9](Fig. 2). These lipids possess pKa values of 6.4-6.7, having a positive charge at low pH to allow for efficient oligonucleotides loading, and a close to neutral surface charge at physiological pH [4,10–14].

Likewise, hydrophobic drugs, poorly soluble in aqueous media, can be easily loaded into the lipid layer of a lipid nanoparticle, or be incorporated in hydrophobic polymers via hydrophobic interactions [5,15,16].

Moreover, some materials combine different strategies, allowing encapsulation of several drugs with different characteristics. For example, polymeric core-shell NPs have been designed to ensure a hydrophobic cholesterol core for the encapsulation of hydrophobic drugs, and a cationic shell allowing the binding of DNA molecules [17].

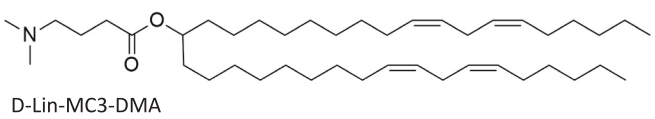

Fig. 2. The structure of D-Lin-MC3-DMA ionizable lipid.

3. Bio-distribution and in-vivo fate of NPs

Achieving desired site-specific accumulation of the administered drug is hindered by many obstacles and biological barriers, most notably clearing by filtering organs, the mononuclear phagocyte system (MPS), tissue penetration and adsorption of protein corona. Over 99% of systemically administered NPs will be cleared by the various clearance mechanisms of the body[18]. 30-90% of NPs accumulate in the liver, which is specialized in metabolism and clearance of foreign molecules and particles [19]. Other NPs are cleared, for instance, by phagocytic immune cells, primarily by Kupffer cells in the liver and macrophages in the spleen, scanning the body for foreign particles [20]. This un-specific interaction and clearance results in reduced therapeutic effect and increased side effects. Aside from clearance, interaction with components of the immune system may lead to toxicity due to secretion of inflammatory interleukins and interferons or activation of the complement system [21,22].

The size of NPs play a major role in dictating their in-vivo fate. It is generally accepted that NPs averaging at 100 nm demonstrate reduced clearance and longer half-life in the circulation, thereby increasing the chance of reaching the target organ[23]. NP larger than 150 nm are rapidly distribute to the lungs, liver and spleen, while much smaller NPs (<5 nm) are cleared by the kidneys. Micrometer size NPs tend to accumulate in the lungs, offering an advantage for targeting pulmonary related conditions or sites of metastatic disease [24]. Specifically for tumor targeting, sub-100 nm particles demonstrated better penetration;

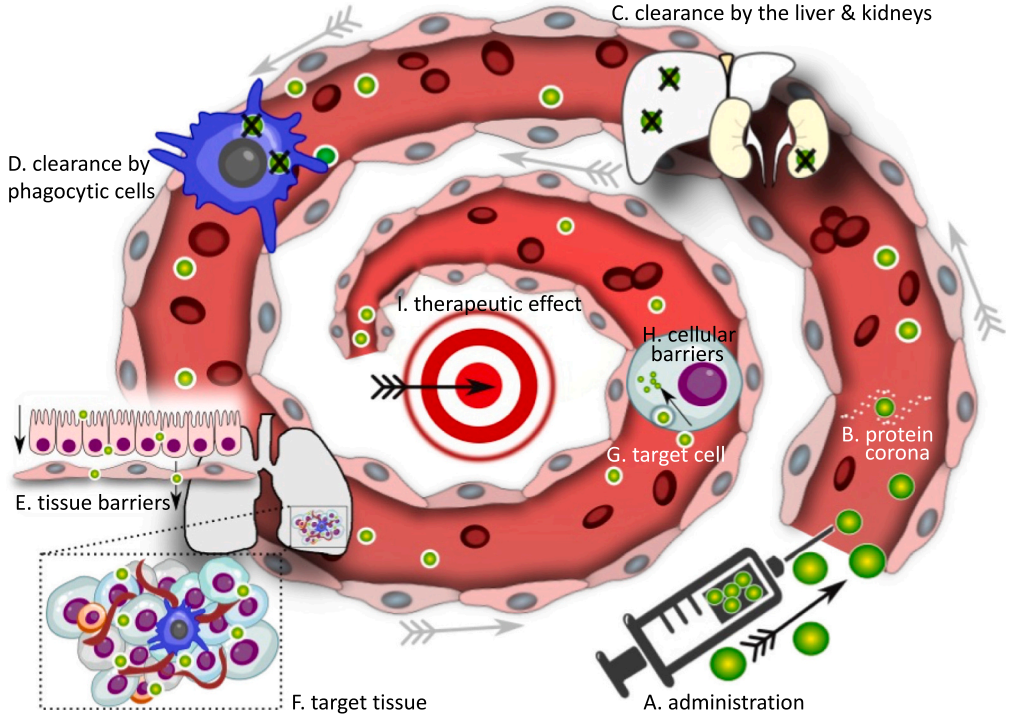

Fig. 1. Overview of the barriers facing NPs. Upon administration of NPs (A), they first encounter and bind plasma proteins (B). Then, the NPs are distributed throughout the body, depending on their characteristics, and are facing clearance by circulating phagocytic cells, the liver and the kidneys (C-D). Before reaching the target tissue (F), the NPs have to pass also through tissue barriers (E), such as endothelial cells. The NPs are then capable of binding the target cell (G) and, depending on their mechanism of action, need to overcome cellular barriers (H), including the plasma membrane, intracellular compartments and efflux pumps. Finally realizing their therapeutic potential (I).

the more permeable cancer types allowing wider range of NP sizes, while in low permeable tumors only particles smaller than 50 nm were effective [25].

The surface charge of particles also plays a major role in biodistribution, as it is a key factor in cellular uptake mechanisms and tissue accumulation. Positively charged nanoparticles show higher adsorption of serum proteins, resulting in shorter circulation time, while neutral or slightly negatively charge nanoparticles showed lower accumulation in the liver and spleen [23]. However, positive surface charged nanoparticles mediate favorable endosomal release mechanisms such as the proton sponge effect[26], which will be discussed later in this manuscript. The development of ionizible lipids addressed issues in this aspect as well; these lipids facilitate a surface charge close to neutral at physiological pH, while regaining a positive charge in acidic pH, such as the environment of the endosome [23].

The shape of NPs also affect their circulation time. While spherical particles are most commonly used, other shapes have been explored and showed favorable kinetics. For example, it was shown that filamentous polymer micelles persist longer in the circulation compared to spherical micelles [27], an observation explained by the fibril-like particle align with the blood flow. These filamentous micelles, containing paclitaxel, also showed higher accumulation in tumors [28]. Hemorheological behavior and cellular uptake are also affected: some non-spherical shaped NPs, such as disc-shaped, demonstrate higher binding to the endothelium and slower phagocytic internalization compared to spherical particles [24].

Another factor that highly effect the drug's biodistribution is exposure to plasma proteins. When a therapeutic agent is exposed to a biological environment, it encounters and adsorbs proteins and biomolecules, a phenomenon termed protein corona [29–31]. Here as well, the composition of the proteins interacting with the surface of the NP depends on factors such as nanoparticle size, surface charge, hydrophobicity and surface chemistry. Plasma proteins, including serum albumin, apolipoproteins, complement components and immunoglobulins will effect biodistribution, cellular uptake mechanism, and sometimes intracellular localization. Often protein corona results in opsonization and uptake by cells of the MPS, which are mainly resident macrophages in the liver, spleen and lymph nodes [31]. In our experience, when utilizing an active targeted NPs, protein corona might serve as a 'blocker' between the targeting moiety and cell-surface molecule, thus limiting, or altering, the intended targeting affect. It is of high importance for further investigate the exact nature of protein corona in different NPs, i.e. accurately identify and quantify each component and its interactions with the NP or the targeting moiety. In depth understanding of these issues is critical to achieve adequate targeting.

Several interesting studies demonstrated harnessing protein corona for specific purposes. For example, high-content vitronectin particles were shown to enhance targeting to melanoma cells over-expressing the vitronectin receptors, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Poly(butyl cyanoacrylate) nanoparticles coated with polysorbate 80, a nonionic surfactant absorbing apolipoproteins from the blood, were able to penetrate the BBB v via a receptor-mediated endocytosis by brain capillary endothelial cells, interacting with the apolipoproteins coated on the surface of the NPs [32].

The most widely used approach to reduce rapid clearance is including polyethylene glycol) (PEG) in NPs formulation. While indeed PEG increases the half-life of the NPs in the circulation due to reduced MPS uptake [33], cellular uptake by the target cells is reduced as well [3]. Additionally, the formation of anti-PEG antibodies may increase the clearance of PEGylated NPs [34], an effect which might be enhanced if a patient was previously exposed PEG (e.g. treated with other PEGylated drugs, and many cosmetic and hygiene products(. Another suggested approach to reduce rapid clearance of NPs is mononuclear phagocytic system blockade by 'blocker', non-functional particles. It was shown to result in a temporarily decrease in macrophage endocytosis, and 18-fold increase in blood circulation time of the active administered particles. [20].

Consideration of size, charge, and other physical properties of NPs is crucial for designing an effective drug product for a specific indication. The composition and formulation of the NPs will determine the stability of the encapsulated cargo, the drug's pharmacokinetics, safety profile and play a key role in efficacy.

4. Biological membranes and microenvironment as barriers between NPs and the target tissue

It is clear that designing nanocarriers with longer circulation time and decreased phagocytic uptake will result in improved accumulation in the target organ. As the nanoparticle reaches the designated site, consideration must be made on how it will exit the blood vessel and reach the organ (or tumor) environment.

Specifically for tumors, many strategies relay on the Enhanced Permeability and Retention (EPR) effect, a proposed hypothesis for the observed extraversion of NPs to tumors. It has long been known that tumors promote angiogenesis in a fast and unorganized manner, resulting in incompetent, 'leaky' blood vessels, harboring pores of up to 1 μm. While this hypothesis is certainly true in some cases, the extent of the leakiness is heterogeneous within different areas of the tumor and between different types of cancers [3,4]. Moreover, it is not relevant at all for low-vacularazied tumors with other mechanisms of nourishment and proliferation. Only a small subset of tumor endothelial cells allow transportation of nanoparticles, and this population is scarcely distributed on a small number of vessels [35]. In fact, for some types of tumors, compromised vasculature and poor lymphatic drainage results in elevated interstitial fluid pressures, which hinders extravasation of NPs to distal regions in the tumor [24]. The heterogeneity of tumors is relevant also regarding the properties of the extracellular matrix (ECM), in which the density and composition of proteins serve as a physical barrier preventing NPs penetration to tumor cells [36].

Therefore, in the design of nanoparticle the point of crossing the endothelium needs to be addressed. It was shown [24] that a disc-shaped particle has favorable margination characters, as it has more interactions with the vessel wall than a traditional spherical shaped NP. On the other hand, when designing nanomedicine to treat cancer, the phenomenon of Nanomaterials-induced endothelial leakiness (NanoEL) must be taken into account. This is the mere administration of a nanomaterial that causes gaps in the blood vessel endothelial walls. While allowing extravasation of the therapeutic vehicle, these gaps can also facilitate the intravasation of resistance cancer cells, thus potentially promoting metastasis [37].

A special case of the endothelial barrier is the blood-brain-barrier (BBB), comprised of tightly-packed specialized endothelial cells, astrocytes and interneurons with unique extracellular proteins that together contribute to the structure of the BBB as the gate-keeper for the brain microenvironment. One approach to enable nanoparticles crossing of the BBB is utilizing ultrasound/ microbubbles and osmotic pressure to inflict temporary permeability; this approach poses risk of CNS toxicity as it allows materials other than the administered drug to enter the brain. A second strategy is designing NPs with physio-chemical characteristics that relate to specific BBB mechanisms, for example favoring adsorptive transcytosis, using positively charged NPs that readily interact with the negatively charged endothelial cell plasma membrane, and using NPs smaller than 200 nm for clathrin-mediated endocytosis. And finally, promoting specific receptor-mediated transcytosis by functionalizing NPs with targeting ligands [38]. An alternative approach explores an intranasal administration route instead of intravascular, utilizing olfactory and trigeminal neural pathways to transport NPs to the CNS[39].

Designing NPs that display a desirable biodistribution, pharmacokinetics and efficient accumulation in the target tissue is highly dependent on physical characteristics and formulation. Considering these elements when tailoring a drug carrier for a specific organ or

tumor site is referred to as passive targeting, which is imperative to the successful delivery of the encapsulated drug. However, to manipulate or effect selectively a specific type of cell population, modifications to actively target NPs are required.

5. Specific binding to the target cell

Once the NPs reached the target tissue, passing through the numerous barriers and establishing a desired biodistribution, the NPs should reach their target cell. Specific binding of NPs to the cell of interest can be achieved by functionalizing the particles with active targeting moieties, such as proteins, peptides, oligonucleotides, polymers, saccharides, and small molecules. The targeting molecule should be chosen based on various biological parameters: selectivity and off target effects, binding affinity, stability in different conditions, size, and charge. Technical considerations, such as availability, production and purification processes complexity and cost, should also be taken into consideration.

Often, coating NPs with a specific ligand or antibody results in a tight binding to the cell surface receptor and limiting the internalization of the desired payload. In addition, some of these binders can cause outside-in signaling events when receptors are crosslinked. Therefore, we highly recommend to first study the ligand – receptor interactions under sheer-flow condition. This will increase the likelihood of finding the best ligand-receptor couple and will eventually cause a specific internalization of the payload into the cells via receptor-ligand endocytosis.

5.1. Monoclonal antibodies

Monoclonal antibodies (mAbs) are widely used as moieties for targeted delivery of NPs [4,10,11,14,40–44]. Their selectivity, high affinity, versatility, and wide availability gained their high popularity in the field and positioned them as the ultimate targeting moieties. Antibodies are bound to NPs mostly via chemical conjugation of functional groups on the surface of the particles and a suitable group on the antibodies [45]. This conjugation of mAbs to the surface of NPs allows for a selective binding to a target cell and the delivery of drugs, including, for instance, RNA molecules for gene therapy. Over the years, many studies demonstrating the use of antibodies as an approach to deliver NPs-based gene therapies to leukocytes or tumor cells, or cytotoxic drugs to tumor cells were reported [14,42,43,46]. Also, numerous clinical trials were initiated based on this approach. For instance, in a clinical trial utilizing P53 gene therapy via liposomes with an anti-transferrin receptor (TfR) scFv as the targeting molecule, an accumulation of the transgene in metastatic tumors but not in normal skin tissue was observed [47]. In another trial, miRNA therapy for malignant pleural mesothelioma was delivered by targeting EGFR[48], a third study delivered paclitaxel and docetaxel prodrugs formulated as ephrin A2-targeted liposomes to tumor cells[49], and another study reported blocking DNA replication using targeted nanoparticles loaded with siRNA against M2 subunit of ribonucleotide reductase [50,51].

The limitations of the antibodies-based approach include the high cost and demanding purification processes, large size, immunogenicity, and sensitivity to changes in pH, temperature, solvent, and salt concentration[4]. As the field of recombinant proteins and mAbs developed, multiple modifications have been made, addressing some of the limitations of the therapeutic use of antibodies in nanomedicine. This includes the use small antibodies fragments (such as Fab and scFv) without the immunogenic Fc area, to reduce immunogenicity and avoid Fc receptors binding and clearance by phagocytic cells, Fc engineering, and novel mammalian expression and purification processes [52,53]. In our opinion, the well-known structure elements of mAbs, established development and engineering process, the large body of knowledge of antibody-ligand interactions and the multiple clinically approved mAbs, position them as the most promising targeting approach, with the highest chances of overcoming the barriers and reaching a clinically approved therapy.

Yet, while delivering drug-loaded antibodies-conjugated nanoparticles to a target cell might provide the solution for targeted delivery in the short term, developing a versatile, flexible platform to deliver multiple drugs to multiple cell types of choice, is the Holy Grail in the nanoparticles field and is hoped to answer a broader long-term need. Developing such a flexible approach will enable to overcome resistance with great ease as well as save resources when generating a complete treatment to multifactorial conditions. With the wide availability of multiple libraries of a theoretical infinite number of antibodies against a wide variety of targets, antibodies-based targeting platforms have a potential to enable a quick, flexible and uniform conjugation of antibodies to nanoparticles. Our laboratory recently developed the ASSET platform (Anchored Secondary scFv Enabling Targeting), which consists of a lipidated secondary antibody that can self-assemble into the membrane of lipid nanoparticles, and facilitates a rapid and flexible coating of various primary antibodies to the surface of LNPs [41] (Fig. 3). With the efficient ASSET approach we can limit production and purification processes and have a better control on the conjugation of mAbs. For instance, the ASSET linker governs the orientation of the antibody on the surface of the NPs; preserveing the binding sites of mAb are exposed, while the Fc is shielded from being recognized by Fc receptors, thus limiting the immunogenicity of the targeted NPs. Thus, we believe the ASSET platform can be a game-changer in the clinical utilization of targeted NPs.

5.2. Peptides, biomimicry and other proteins

Peptides, short sequences of amino acids, are attractive targeting moieties due to their small size, low immunogenicity, and simple production compared to antibodies. Yet, peptides generally suffer from low stability due to enzymatic degradation and the masking effect of protein corona, which is more prominent due to the peptides small size [54]. Peptides are either designed from binding regions of certain proteins (e.g. biomimicry [55]) or screened from peptide libraries for binding to a the target, for instance using a phage display method [56,57]. Similarly to antibodies, nanoparticles are commonly being decorated with peptides by chemical conjugation of functional groups.

Over the years, multiple biomimetic targeted nanoparticles were designed for various aims, for instance to promote tumor targeting [55], penetrate the blood brain barrier (BBB) [58,59] or promote specific binding to certain types of cells [60,61]. For example, somatostatin and somatostatin analogues have been used for targeted delivery of paclitaxel to tumor cells and tumor blood vessels in glioma and breast cancer animal models [62,63]. Also, many peptides have been found to have the capacity to breach the BBB and promote drug delivery to the brain [64]. One such strategy utilizes peptides of rabies virus glycoprotein (RVG) for an efficient brain-targeting delivery [65–72]. Similarly, cell-penetrating peptides (CPPs) [73] have been used to overcome cellular barriers [74]. TAT peptide, a typical example of CPPs, is derived from the human immunodeficiency virus. TAT was reported to facilitate translocation through the cell membranes and to accumulate in the nucleus [75], thus facilitating cellular drug delivery via nanoparticles [76–80]. Peptides based on the ‘don't eat me’ marker CD47 [81] were used to delay macrophages-mediated clearance of nanoparticles, demonstrating a targeted approach for avoiding MPS. Targeted arginylglycylaspartic acids (RGD) nanoparticles have been also widely used as a tumor-targeting drug delivery approach [82,83]. RGD peptides specifically bind to cancer-related integrins, for instance $\alpha_v\beta_3$, and are thus being investigated as ligands for integrin-targeted drugs [84].

Formulating drugs with albumin was shown to be effective in overcoming the endothelial barrier and promoting accumulation in the tumor site. Albumin-bound paclitaxel (nab-Paclitaxel, Abraxane) promotes transcytosis by interacting with glycoprotein 60 and accumulation in the tumor is achieved via binding to SPARC (secreted protein,

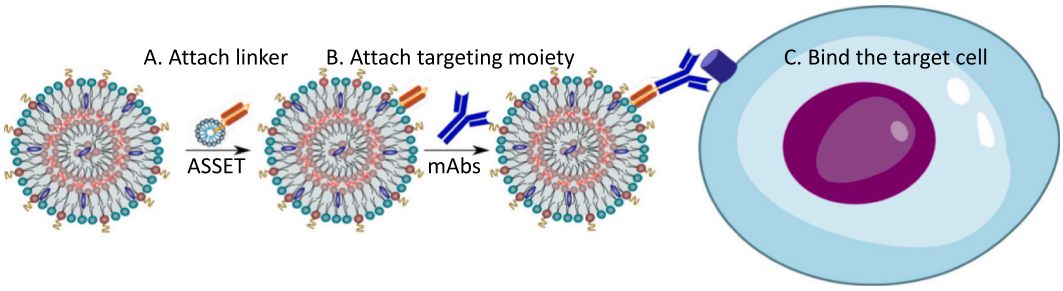

Fig. 3. ASSET targeting platform. The ASSET linker in self-assembled into the lipid layer of the NPs by a simple mixing of the two solutions (A). Followed by an efficient binding of primary mAbs through their Fc region to preserve their active conformation and control their orientation on the surface of the NPs (B). The targeted NPs finally bind the target cell in antibody-receptor specific link to allow a selective delivery of a drug to the target cell (C).

acidic and rich in cysteine) [85].

In clinical studies, peptide-targeted nanoparticles encapsulating siRNAs against TGF-b1 and Cox-2 were tested in subjects with hypertrophic scar[86]. Likewise, targeted polymeric nanoparticles, utilizing somatostatin analogue to deliver anti-tumor therapy to colon cancer, have been tested [87].

5.3. Aptamers

Aptamers are short single stranded oligonucleotides, with a defined 3-dimensional structure, capable of selective and strong interactions with numerous entities, including proteins, nucleotides, cells and small molecules. Aptamers display several advantages compared to other approaches, including their small size, rapid and simple synthesis and modifications, flexibility, low immunogenicity, and higher stability compared to antibodies [88]. Aptamers libraries are being screened in vitro, in a process called systematic evolution of ligands by exponential enrichment (SELEX) [89,90], using both positive and negative selection approaches, against a specific target of interest or control sample, respectively. Recent progress in the selection process can identify aptamers uniquely binding multiple receptors, as well as computational approaches to predict the structure and binding of aptamers [91–94].

Aptamers can be conjugated to the surface of nanoparticles via attachment of functional groups, which interacts with the appropriate functional group on the surface of the nanoparticles (e.g. COOH and NH2 creating an amide bond, SH groups creating S—S bond, avidin-biotin interactions etc. [95]).

Aptamers have been used as targeting moieties for multiple pathologies. For instance, poly(lactic-*co*-glycolic acid) (PLGA)-PEG nanoparticles, loaded with cisplatin, were functionalized with prostate-specific membrane antigen (PSMA)-targeting aptamer to deliver the cytotoxic drug to prostate cancer cells [96]. Likewise, aptamer-conjugated magnetic nanoparticles were used as a nano-surgeon approach for a selective magnetic field-dependent removal of cancer cells [97] and for a photothermal destruction of cancer cells using aptamer-conjugated nanorods [98]. Unfortunately, this technology of aptamers-targeted nanoparticles has yet to reach the clinical trials.

5.4. Polymers

Due to their high flexibility and variability, polymers are frequently used for drug delivery applications. Their multiple functional groups can interact with a wide variety of payloads that can be incorporated into or decorate the surface of nanoparticles. By using rational design, considering the solubility, molecular weight, stability, hydrophobicity, polydispersity, charge, and the relevant functional groups, polymeric nanoparticles can be tailored for a specific cargo and target [99]. Although highly promising, the knowledge gap preventing the optimal utilization of such a method is still substantial. Likewise, while screening synthetic polymer libraries is an available technique, and there are multiple known natural polymers, the complex synthesis and purification processes might hinder the flexibility and high throughput use of polymers as targeting moieties.

Hyaluronic acid, a natural polymer of the extracellular matrix, is widely used for both increasing biocompatibility of nanoparticles, and for binding the hyaluronic acid receptor CD44, overexpressed by tumor cells [100,101]. Another example is 7C1, a synthetic polymer, was discovered by a library screen to induce endothelial selectivity. 7C1 enabled gene therapy application in endothelial cells in vivo, by delivering siRNA, sgRNA or mRNA molecules selectively to endothelial cells, with minimal off target effects in immune cells [99,102,103]. Although several clinical studies with polymeric nanoparticles were initiated, we did not find any study demonstrating a polymer-based active targeting approach, but rather passive targeting or local administration.

Many other molecules can be used for active targeted delivery of NPs, including small molecules, sugars, and metabolites. For instance, GalNAc, amino-sugar derivative, ligand to the asialoglycoprotein receptor (ASGPR) highly expressed by hepatocytes, is widely used for a targeted delivery of therapeutics to hepatocytes [104,105].

6. Drug release in the target tissue or cell

Once overcame the systemic barriers and reached the target tissue and cell, targeted NPs should either promote an extracellular release of their cargo or internalize into the cell and release its payload. The selected approach should be defined based on the drug's characteristics, stability, and function, where a clear distinction between two types of drugs should be made: (i) extracellularly active drugs, for which no intracellular barriers are relevant; and (ii) intracellularly active drugs, in which several cellular barriers may hinder the efficiency of the treatment [106]. For intracellular active drugs, the plasma membrane is the first cellular barrier to overcome, by either the NP or the drug itself. Next there are intracellular membranes and organelles to breach in order to reach the active site of the drug (e.g. cytoplasm, nucleus, etc.) and avoid degradation. And finally, efflux pumps can remove a drug from a cell, thus reducing the therapeutic effect.

The receptor-ligand based interactions of NPs with the target cell can determine the fate of the encapsulated drug and the extracellular or cellular path to follow. Different receptors can promote the retention of the NPs on the surface of the cell, to allow for a slow release of a drug, or induce the cellular uptake of the NPs. Unfortunately, not every receptor allows optimal internalization of NPs by pathways promoting an intracellular release rather than degradation. Thus, the biology of targeted receptor plays a crucial role in determining its suitability as a target, both at the rate of internalization, the intracellular path to follow internalization, the recycling of the receptor and thus the ability to bind more NPs, and the induction of beneficial or harming signaling pathways [107]. The affinity of the receptor-ligand interaction should be high enough to allow the targeted delivery of the drug, yet still allow the release of the receptor and recycling. Thus, the specific receptor to target should be selected carefully and functionally tested to ensure either extracellular retention in case a drug-diffusion out of the NPs is

preferred, or cellular internalization of intracellular active drugs. Considering these aspects, and based on our experience, mAbs-targeted NPs have the highest potential to achieve a desired, selective, and efficient binding of a receptor with the required characteristics. The wide availability of mAbs binding different receptors, and the ability to control their affinity, makes them the optimal strategy for designing targeted nanotherapeutics.

6.1. Extracellular drug release independent of NPs internalization

Extra-cellularly active drugs, as well as stable small molecules capable of crossing or incorporating into the cellular membrane, are classical examples of drugs for which there is no need for an intracellular release mechanism. Still, NPs allow for higher therapeutic effect and low toxicity by navigating the drugs to the tissue of interest[1]. For this purpose, drug releasing NPs, potentially triggered by environmental cues, such as temperature, pH or enzymatic activity, should be utilized [108–110].

Collagenase delivery by NPs to the tumor site is an example of extracellular active compounds delivered by NPs. The overexpression of collagen, the main structural protein of the extracellular matrix, in the tumor area is limiting the penetration of drugs into the tumors, and thus resulting in impaired therapeutic effect. To overcome this challenge of drug delivery into dense tumors, collagenase-loaded NPs were designed to efficiently release collagenase in order to degrade collagen selectively in the tumor area [111]. Collagenase release is not only a stand-alone approach but can be combined with the delivery of other drugs or NPs to improve their intra-tumor delivery. This strategy has been proven efficient for extracellular matrix penetration and doxorubicin release in the tumor area [112,113], and can be further applied to overcome the insufficient intra-tumoral delivery of many other drugs. Likewise, a dense extracellular matrix within the tumor was shown to corelate with poor biodistribution of gold nanoparticles, and the combination of collagenase gold nanoparticles with metformin gold nanoparticles, improved the therapeutic effect of metformin in breast cancer spheroids [114]. Moreover, hyaluronidase [115] delivery via NPs to the tumor area was proposed as another approach for extracellular matrix manipulation to enhance anti-cancer treatments [116].

Intracellular active molecules delivered to the extracellular space should have a sufficient rate of crossing the plasma membrane, measured by the permeability coefficient value [106]. The compounds could be delivered actively, via energy-dependent transporters [117], or passively. Passive delivery is mainly controlled by the size and polarity of a compound; The bigger and more polar the molecule is, the smaller its permeability potential[106]. Yet, some exceptions to these rules were found [118]. One example for extracellular drug release by NPs is Doxil. Doxorubicin was proposed to be released from Doxil NPs in the interstitial fluid and then uptake by tumor cells as a free drug [1109]. Similarly, synthetic lipid-NPs were design to enable the deposition of active compounds directly into cellular membrane [119]. Upon binding to a specific cell, the lipophilic drug in the NP's lipid monolayer can be delivered directly to the plasma membrane of the target cell. For this aim, the NPs should optimally retain on the surface of the cell to allow for an efficient release of the drug. To achieve that and ensure sufficient retention, various receptor-ligand interactions should be normally tested.

6.2. NP internalization for an intracellular release of a desired drug

Nanoparticles can internalize into the cell by several mechanisms: (i) clathrin-mediated endocytosis to the endosome, via receptor-mediated endocytosis; (ii) caveolin-mediated endocytosis to the endosome, via receptor-mediated endocytosis; (iii) clathrin-caveolin independent endocytosis to the endosome, via receptor-mediated endocytosis; (iv) phagocytosis to a phagosome via phagocytic receptors, such as Toll-like Receptors, Mannose/Lectin Receptors, scavenger receptors and Fc Receptors [120],; or (v) micropinocytosis to the macropinosome and the endosome. The internalization mechanism of a NP depends on the size, surface charge, shape and targeting moieties present on the NPs [121–123]. Most NPs internalize via endocytosis, either caveolin-dependent, clathrin-dependent, or caveolin-clathrin independent internalization, potentially via both lipid-rafts dependent and independent processes [124]. Yet, determining the NP structure that promote each mechanism is not trivial. Multiple contradictory results are available through different studies [121,125–128], highly depending on the target cell and the composition and the structure of the NPs. Likewise, positively charged NPs have a higher capacity of internalization [121,123]. Also, surface modification, including the attachment of targeting moieties, such as antibodies, can modulate the internalization pathway of NPs. For instance, the attachment of antibodies to NPs promotes receptor-mediated internalization of NPs in a mechanism that depends upon the specific targeted receptor. Yet, exposed Fc regions can induce phagocytosis and clearance via binding of Fc receptors on phagocytic cells [41]. Thus, to ensure optimal internalization of NPs and find the best receptor to target, multiple ligand-receptor interactions should be carefully studied.

6.3. Intracellular barriers & drug secretion

Following internalization, NPs are allocated to intracellular compartments [129]. NPs internalized by endocytosis will be allocated to the endosome and finally the lysosome, where degradation of drugs, especially nucleic acids, macromolecules, peptides, and proteins, will occur [130]. Similarly, exocytosis mechanisms are a further barrier limiting the efficiency of NPs [131]. Thus, designing delivery methods to avoid and escape these barriers has the potential to enhance the therapeutic potential of a drug. And finally, efflux pumps, and metabolism are methods by which a cell could further eliminate drugs, resulting in low efficiency. These mechanisms are drug-specific and thus should be addressed at that level [3].

Several approaches have been developed to enhance endosomal escape and evade lysosomal degradation of the NPs' cargo. Endosomolytic peptides and proteins were shown to form pores in the endosomal membrane, thus promoting translocation and endosomal escape of the NPs' cargo, such as siRNA molecules [132]. Likewise, the conjugation of pH sensitive fusogenic molecules, such as the GALA peptide, was shown to accelerate endosomal escape [133]. Similarly, positively charged molecules, including poly-histidine sequences [134], have been used to destabilize the endosomal membrane and promote the “proton sponge effect”. The proton sponge effect refers to proton absorption by molecules allocated to the endosome, such as polyamines, thus inducing ATPase proton pumps to enhance the endosomal acidification and transport more protons into the endosomes, ending up with osmotic swelling, rupturing and endosomal escape [26]. Finally, pH sensitive ionizable lipids, such as DLin-MC3-DMA, have been utilizing their conditional positive charge to interact with endogenous endosomal anionic lipid and facilitate endosomal release of drugs [135,136]. Yet, with only limited improvement [129], this limiting factor remains one of the greatest challenges in nanotechnology. Our understanding of the endosomal/lysosomal barriers is still lacking, thus preventing further improvements in the field. Additionally, several strategies were demonstrated to bypass the endosomal allocation and directly release the NPs' content to the cytosol [137,138]. Still, these strategies are yet to provide an efficient broad solution for intracellular drug delivery.

7. Conclusions and future prospective

Nanotechnology holds great promise for an efficient and flexible therapy of various conditions, while ensuring accuracy, selectivity, and minimal side effect. Yet, with the multiple barriers to overcome, such as systemic barriers, clearance, reaching the target.

organ and the target cell, and several cellular barriers, designing

nanomedicine strategies is challenging and resource and time consuming. Over the past decades great achievements were made in the nanotechnology field, starting from a better understanding of the barriers to overcome, to the design of strategies successfully overcoming the challenges (Fig. 4). Still, to ensure the biocompatibility, efficiency and selectivity of new targeted therapeutics, a combination of multiple factors is needed. Despite the great achievements reported, determining the right combination of components in multi-functionalized NPs is mostly not straightforward, hard to predict and requires careful optimization.

While focusing on one specific therapeutic strategy might be the realistic approach in the short term, designing a multi-targeted flexible platform, which could be easily tailored for a variety of aims, has the highest potential in the long term. Designing a targeted delivery platform is a complex approach that holds several advantages: (i) versatility, including the availability of targeting moieties libraries, for a wide variety of receptors. This process is well established with several targeting moieties, such as antibodies, peptides and aptamers[89,90]; (ii) multi-functionalized nanoparticles to overcome multiple barriers (iii) quickness and ease of modifying the nanoparticles to direct a drug to varied targets; (iv) accessibility; (v) stability; and (vi) affordability. Considering all the requirements, we believe platforms for mAbs-based targeting of nanoparticles have the highest potential of reaching the clinics to enable selective and effective targeted therapeutics in multiple diseases.

While several NP-based drugs are available in the clinics [139], mostly untargeted NPs are used. Some are given by local administration, for which targeting is not necessary: NPs for vaccination (e.g. Spikevax, Nuvaxovid, Pfizer-BioNTech COVID-19 vaccine, ARCoV), skin care (e.g. Octinoxate), anti-bacterial treatments (e.g. Astodrimer; Arikayce) and analgesia (e.g. Bupivacaine, AeroLEF). Other are administered as systemic treatments based on un-specific passive targeting: used for cancer treatment (e.g. Doxorubicin, Onivyde, LErafAON) or liver pathologies (e.g. Onpattro). Regardless of the great hype around targeted nanomedicine, multiple publications, and various different approaches to overcome the discussed barriers, there are still very few clinical trials using targeted nanoparticles.

Chemistry, manufacturing, and controls (CMC) problems are the main limiting factor in advancing targeted nanomedicine to the clinic. CMC include the manufacturing process and product specification. Among the specific aspects limiting the clinical utilization of targeted NPs are the multifactorial complexity of targeted nanoparticles, only partially understood; low predictability, low reproducibility in the conjugation of targeting moieties to the surface of NPs, both at the efficiency, the accessibility and function levels, and high production and purification costs. Overall, a better control over the efficiency, orientation and active conformation of the targeting moiety in needed to support the clinical use of targeted NPs. The ASSET platform was specifically designed to address these needs, providing efficient, controllable and reproducible conjugation of mAbs to the surface of lipid NPs, without affecting their active conformation. From the efficient self-assembly of the ASSET linker into the NPs, which does not require chemical conjugation with purification processes, to the affinity-based interaction with mAbs, allowing a control over the orientation of mAbs and preserving their active conformation, we believe the ASSET platform has a high potential to overcome the CMC barrier of targeted NPs.

In addition, the animal models used to evaluate potential

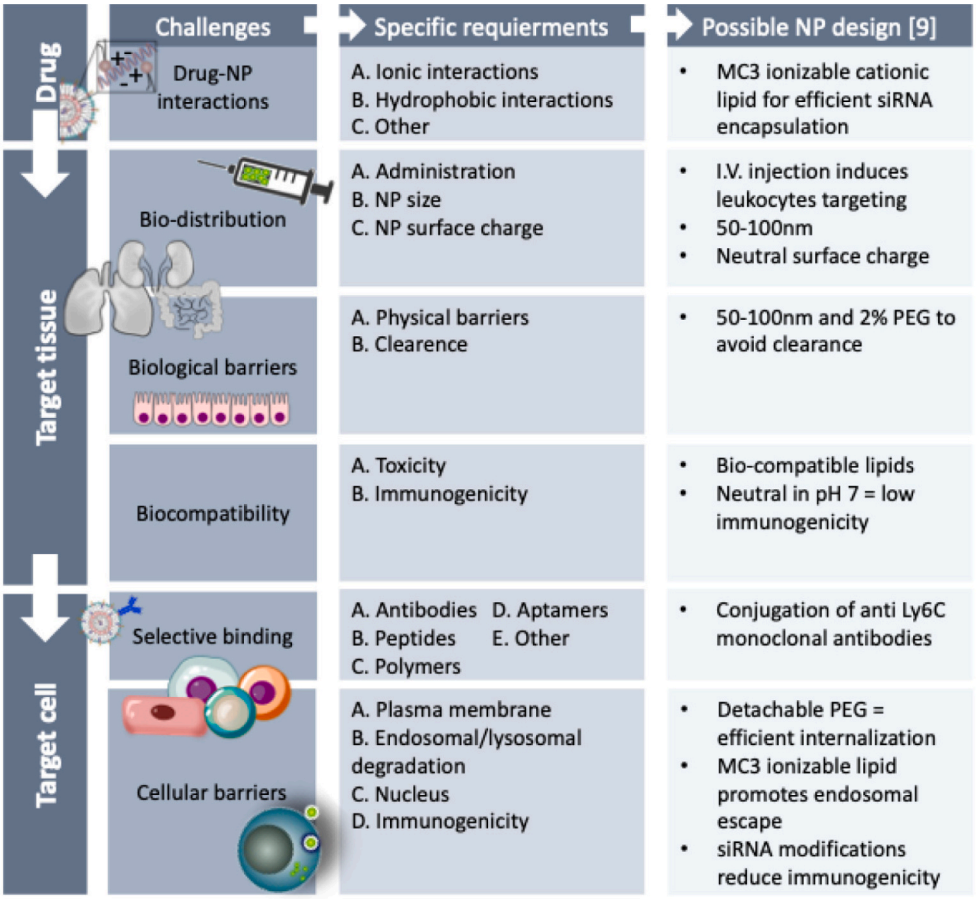

Fig. 4. Flow chart indicating the requiered elements for NP design. The NPs' formulation and modifications should be designed based on their requiered function, from the characteristics of the drug, to the target tissue and the target cell, and considering the relevant challenges to overcome in each aspect. Including an example of the design of siRNA-loaded Dlin-MC3-DMA based LNPs.

technologies and strategies for targeted delivery in the preclinical stage are often not representative of the investigated indication or condition, which further limits the translation to the clinic[3]. This gap goes beyond the differences in clinical parameters of a certain disease, but also how that condition influences the behavior and functionality of the administered drug.

The most challenging aspect of nanomedicine is to foresee and optimize the efficiency and the off-target effects of the NPs. Streamlining the process of NPs design, and specifically targeted-NPs, can accelerate the development of new nanotechnology-based therapeutic applications. Thus, there is a great need to be able to predict the behavior of a designed formulation, especially the ability to overcome the biological and cellular barriers ahead and forecast the efficiency and the toxicity of the nanoparticles. Machine learning (ML) and artificial intelligence (AI) have the potential of changing the way we design nanomedicine approaches (Fig. 5). A smart design of a targeting platform, predicting the function and selectivity of a formulation, and promoting the use of multiple moieties in one nanoparticle, has the potential to generate optimal multi-functionalized nanoparticles. Yet, a major limitation will be to create a meaningful and consistent training set of experimental data for the machine learning algorithms. Currently, the techniques used in the nanoparticles.

field are highly diverse: wide variety of materials and techniques for constructing NPs, different injection techniques, analysis method and timing, lack of a broad unbiased analysis in multiple papers, and multiple disease models used. Thus, the first crucial step will be to collect a set of standardize experiments, with data collected in a suitable way to train ML models.

As creating such a standardized set of NPs studies will require massive efforts and resources, the optimal approach will involve a funded consortium of multiple labs collaborating for a greater cause by: (i) creating a standardized set of experiments; (ii) performing experiments in multiple research centers; (iii) analyzing the experiments by set criteria; and (iv) creating and training a machine learning algorithm with a free user-friendly portal. Moreover, ML could enhance the prediction of novel targeting moieties, including new artificial peptides, proteins, polymers, or aptamers, as well as new therapies. For instance, new ML-based therapeutic approaches include the design of new mRNA sequences for 'fake' proteins or peptides, aiming to answer a particular needed function. These predictions are possible, for example, via deep learning methods, including structure predictions, function-to-sequence and sequence-to-function predictions[140–143]. Overall, the link between the fast-evolving ML and the versatility of drug delivery systems is holding a great promise for future highly efficient and selective novel therapeutics. This unique combination is indicating on the beginning of an exciting era in which we could dream and mold the biology more efficiently than ever.

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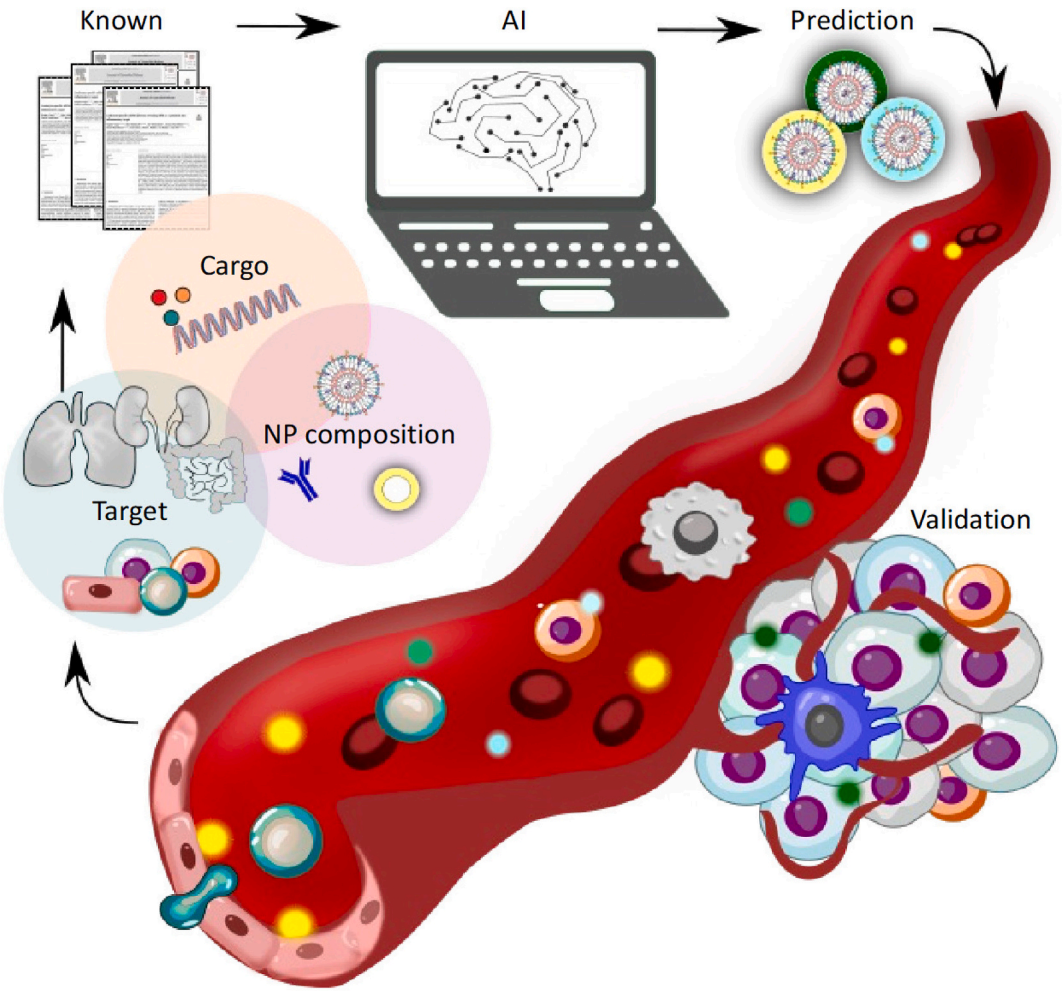

Fig. 5. The utilization of AI in NP design. The AI's prediction of the NPs' formulation is calculated based on known, published, data on the cargo, NP composition and the target tissue and cell. The validations of the predictions will supply further data to continue and improve the prediction ability of the AI.

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CRediT authorship contribution statement

Nuphar Veiga: Conceptualization, Writing – original draft, Writing – review & editing. **Yael Diesendruck:** Conceptualization, Writing – original draft, Writing – review & editing. **Dan Peer:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

D.P. declares the following competing financial interest(s): D.P. receives licensing fees (to patents on which he was an inventor) from, invested in, consults (or on scientific advisory boards or boards of directors) for, lectured (and received a fee) or conducts sponsored research at TAU for the following entities: ART Biosciences, BioNtech SE, Eleven Therapeutics, Kernal Biologics, Merck, Newphase Ltd., NeoVac Ltd., RiboX Therapeutics, Roche, SirTLabs Corporation, Teva Pharmaceuticals Inc.

All other authors declare no competing financial interests.

Data availability

No data was used for the research described in the article.

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