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# Personalized Cancer Nanomedicine: Overcoming Biological Barriers for Intracellular Delivery of Biopharmaceuticals

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**The success of personalized medicine in oncology relies on using highly effective and precise therapeutic modalities such as small interfering RNA (siRNA) and monoclonal antibodies (mAbs). Unfortunately, the clinical exploitation of these biological drugs has encountered obstacles in overcoming intricate biological barriers. Drug delivery technologies represent a plausible strategy to overcome such barriers, ultimately facilitating the access to intracellular domains. Here, an overview of the current landscape on how nanotechnology has dealt with protein corona phenomena as a first and determinant biological barrier is presented. This continues with the analysis of strategies facilitating access to the tumor, along with conceivable methods for enhanced tumor penetration. As a final step, the cellular barriers that nanocarriers must confront in order for their biological cargo to reach their target are deeply analyzed. This review concludes with a critical analysis and future perspectives of the translational advances in personalized oncological nanomedicine.**

## 1. Introduction

Personalized medicine encompasses treatment and diagnosis based on patients' distinctive biological characteristics, i.e., genomics and proteomics.[1] This approach concurrently decreases patients' heterogeneity and fine-tunes the dose–response balance, consequently leading to an enhanced therapeutic response and a reduced occurrence of resistance. Within this framework, therapeutic strategies often rely on the use of biological drugs, which are particularly complex in their interplay with the biological systems.[2,3]

In this scenario, the field of nanomedicine has contributed to the potential benefits of personalized medicine through the design of nanoparticles (NPs) that are capable of helping biological drugs defeat specific biological barriers. In this sense, the Precision Medicine Initiative along with related programs such as precision FDA would help to generate knowledge enabling optimized treatment following patient stratification. Notably, the specificity and potency of biologicals have propelled them to the forefront of personalized medicine, with mAbs[4–6] and siRNA[7,8] emerging as leading therapeutics in this field. In particular, mAbs have shown an unquestionable clinical efficacy, however their use has been restricted to the targeting of extracellular targets. The use of nanotechnology opens up the possibility for their access to intracellular targets, this having an important impact in personalized medicine. On the other hand, in the realm of gene therapy, siRNA emerges as a highly promising therapeutic platform, primarily due to its capacity to selectively silence oncogenes through sequence-specific mechanisms. Nonetheless, the beneficial features of RNA molecules are hampered by the cell barrier itself together with the multiple barriers encountered before reaching intracellular domains.[6,9] Consequently, nanotechnology has emerged as a promising tool to overcome biological barriers, thereby expanding the horizons of personalized medicine.

Here, it is crucial to understand the potential of personalized nanomedicine in oncology through the critical analysis of the determinant barriers preventing the NP from reaching the targets' intracellular compartment. This includes the comprehension of the protein corona and its correlation with in vivo outcomes, the quest of achieving tumor targeting and permeability and ultimately, reaching the intracellular target.

While other works have aimed to discuss the biological barriers curtailing the therapeutic potential of nanomedicine candidates, very few articles analyze the whole trajectory from the perspective of delivering biological drugs to their intracellular targets. Thus, we have focused our analysis on delivering mAbs and siRNA, not yet subjected to extensive scrutiny. Ultimately, both mAbs and siRNA confront a shared critical biological barrier: reaching intracellular domains. Recognizing that crossing this barrier is not cargo-specific and heavily depends on the characteristics of the

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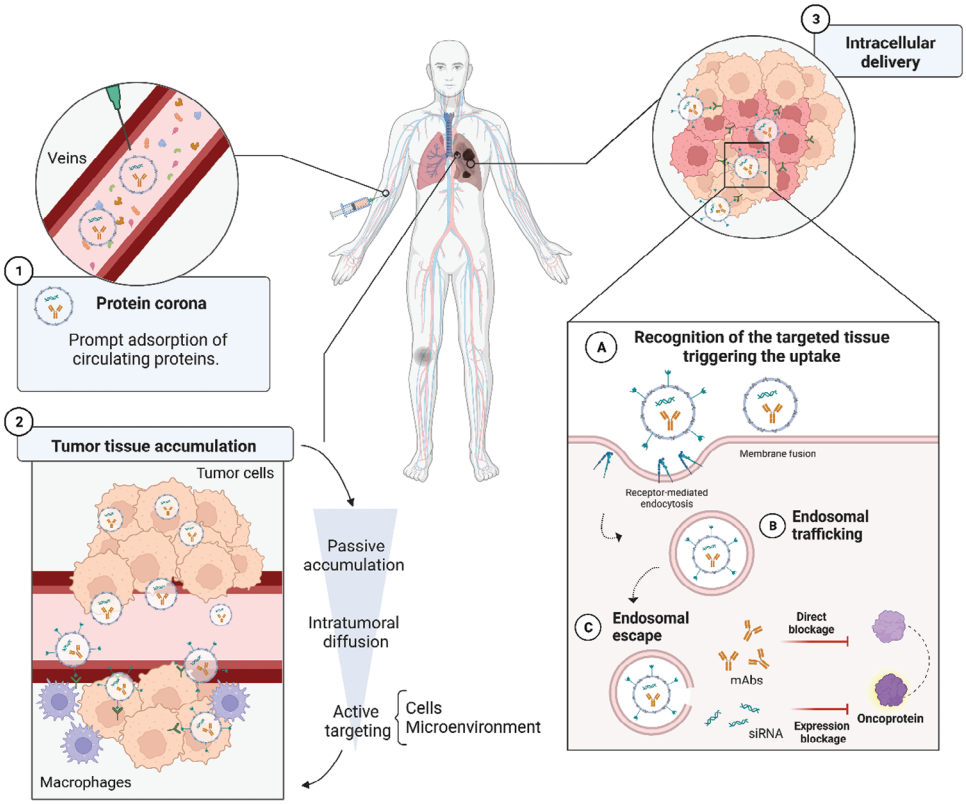


**Figure 1.** Overview of the biological barriers that nanosystems carrying biological therapeutic entities (mAbs and siRNA) face after intravenous administration. This includes 1) protein corona, 2) tumor tissue accumulation and intratumoral diffusion, and 3) intracellular delivery.

nanocarrier, we felt challenged to delineate and dissect the junctures at which and to what extent these technologies converge.

Hence, our purpose is to discuss the most relevant nanocarriers classified based on the biological barrier faced in the context of personalized nanomedicine for cancer therapeutics (**Figure 1**). We draw attention to the prevailing strategies that have demonstrated notable efficacy in targeting intracellular oncoproteins, coupled with an assessment of their current clinical progress. Ultimately, we outline future perspectives within the field, seeking to identify opportunities to advance the development of more efficacious therapeutic strategies.

## 2. Interaction with Proteins as a Limiting Step for the Targeted Biodistribution of Nanocarriers

Following in vivo administration, NPs promptly engage with biological components, particularly proteins, resulting in the formation of a protein corona which decisively affects the NPs' biological trajectory.[10,11] Accurate comprehension of their protein corona composition is now considered indispensable for unraveling the mechanism of action and the resulting efficacy of nanomedicine candidates.[12]

The first evidence of the protein interaction with drug nanocarriers emerged in the late 1970s. In 1977, Tyrrell et al. reported the interactions of albumin and the $\alpha$- and $\beta$-globulin fractions of serum with liposomes.[13] The same year, Davis and his team illustrated how the conjugation of 2 or 5 kDa polyethylene glycol (PEG) to bovine catalase decreased the protein's immunogenicity and prolonged its circulation time in the bloodstream.[14,15] Nonetheless, it was not until 2007 that Dawson and collaborators coined this phenomenon as "protein corona,"[16] opening up the space for its in-depth study.

In this section, we aim to provide an overview of current strategies employed to harness the protein corona for the targeted biodistribution of nanocarriers (**Figure 2**).

### 2.1. PEGylation as a Strategy to Reduce Protein Attachment

In recent years, there has been significant advancement in comprehending the corona-mediated in vivo fate and its correlation with NP composition. However, the strategies to manipulate the protein corona remain limited, with PEGylation being the main strategy to extend blood circulation time as evidenced the number of marketed PEGylated NPs.[17,18] This scenario is also in agreement with the good regulatory profile of PEG.

Numerous studies have attempted to delineate the optimal composition and configuration of PEGylated surfaces for minimizing protein corona formation. Among the factors influencing protein adsorption, PEG length and its density, as well as conformation on the surface of NPs have been recognized for decades. More recently, the PEG desorption kinetics and the presence of an active targeting motif have been analyzed as key determinants of protein corona formation. These factors cannot be viewed independently from the described carrier system, making definitive conclusions challenging. However, general trends can be

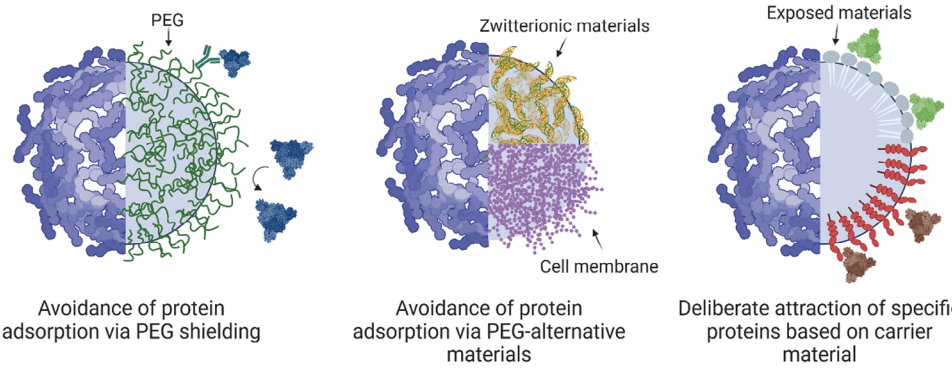


**Figure 2.** Illustration of strategies aimed at conferring stealth properties to NPs by mitigating or controlling protein adsorption. PEGylation represents the classical strategy to prevent protein attachment (left). Zwitterionic polymers and constituents of cell membranes have emerged as possible alternatives to PEG (middle). Controlling the attraction of specific proteins within the context of endogenous targeting (right) is a strategy still in its early investigative stages.

discerned. In spite of the incapacity to agree on the ideal PEG density as particle size and outer shell composition, among others, also play into the equation, high PEG densities have been linked to reduced protein adsorption.[18,19] Nevertheless, this observation requires careful consideration since the units employed to describe these technologies hinder direct comparisons. For instance, a $PEG_{2k}$ density of 10 lipid mol% was found to reduce the ApoE adsorption in lipid-nanoparticles (LNPs).[19] Similar conclusions have been drawn for PEG–poly(lactide-*co*-glycolide) (PLGA) self-assemblies, with densities of 0.45 $PEG_{5k}$ chains $nm^{-2}$ leading to reduced adsorption of ApoE.[18] Similar trends regarding increased PEG density resulting in decreased protein adsorption were observed for inorganic NPs.[20] Although PEG density holds greater importance than its length, a threshold of between 2 and 5 kDa has been established for optimal stealth properties. However, this conclusion has been based on specific types of PEGylated materials and nanocarriers and, therefore, cannot be extrapolated in a generic form.

In addition to PEG length, the detachment kinetics provide a means to regulate the composition of the adsorbed corona. In numerous cases, the carbon anchor length dictates the PEG detachment, as exemplified in LNPs. When comparing a series of alkyl chain lengths (18, 16, 14) in PEGylated lipids, an inversely proportional desorption rate (0.2, 1.3, 45% $h^{-1}$) to the chain length was observed.[21] This trend is potentially translatable to other systems, although specific rates are applicable only to the evaluated carrier and for specific PEG lengths. Furthermore, the significance of conformational alterations in NPs upon protein adsorption should not be underestimated,[22–24] as these changes could generate a modified NP during circulation, thereby influencing anticipated effects by altering intracellular trafficking.

Ultimate limitations inherent to active targeting pertain to the impact of an anchored targeting motif on PEGylated NPs, including its potential to alter the protein corona or the possibility for the ligand to be buried beneath the layer of adsorbed proteins. This discouraging outcome has been observed with transferrin-targeted PEG–silica NPs by Dawson and colleagues.[25] Chan and colleagues have proposed an alternative approach to ensure the active ligand exposure by introducing shorter PEG additionally to the longer, ligand-carrying PEG chains.[26] Although there is an increasing interest in how active targeting approaches are hindered by protein corona formation, most studies focus on in vitro formation and target recognition. Considering the critical importance of the biological identity of NPs in vivo, improved understanding thereof is necessary.[27]

There is a substantial lack of attempts to correlate the in vivo protein corona profile to the actual biodistribution of administered NPs, a matter of utmost importance that needs to be addressed to improve translational advances of delivery systems. Improved methodology regarding the recollection of corona-coated NPs might solve one of the intricacies contributing to this knowledge gap.

In addition to the problems highlighted above, from the experimental point of view, the results reported about protein corona should be cautiously interpreted, considering that the PEG–protein corona formation is highly dependent on study conditions.[28,29] For example, the use of dynamic flow incubation versus static conditions for specific compositions of PEGylated liposomes, led to significantly different results.[28] Experimental implications on protein corona formation, including critical factors as protein to NP surface ratio and incubation time[30] have been considered by Farokhzad and colleagues. Although their specific results on corona formation of diverse inorganic NPs for plasma proteome profiling,[31] also in disease context,[32] cannot be directly translated to organic systems, they still give valuable insights into correlations between NP surface characteristics and protein corona profile.

Finally, discussions around potential immunological reactions are also to be considered.[33] This immunogenicity has been associated to the complement activation observed for some compositions of PEG-liposomes.[34] Despite certain reports on this aspect,[34–37] there are discrepancies about the doses necessary to elicit this undesired effect.[35,38] Along these lines, recent contributions have reported complement activation triggered by FDA-approved PEGylated liposomes Doxil and Onivyde.[39–41] Despite of this, the unquestionable success of these therapies coupled with the widespread administration of PEGylated LNP-mRNA vaccines against SARS-CoV-2 have relativized the concerns about PEG immunogenicity.

Nevertheless, in the quest for sophisticated alternatives to PEG, various approaches are under exploration.

### 2.2. Alternatives to Conventional PEGylation

In the following subsections, we outline various biological and nonbiological approaches suggested over the past decade to mitigate interactions with biological proteins.

#### *2.2.1. Hydrophilic and Zwitterionic Polymers*

Zwitterionic materials such poly(carboxybetaine acrylamides) and poly(sulfobetaine methacrylate) have been proposed as alternative polymers to provide the nanosystems with stealth properties.[42] These materials share three fundamental chemical properties: hydrophilicity, hydrogen bonding capability, and a net-neutral surface charge, which are crucial to prevent protein attachment.[43] The use of certain zwitterionic polymers has been inspired by the zwitterionic phosphatidylcholine (PC) headgroups found in cell membranes.[44] These materials possess alternating positively and negatively charged groups along their carbon chain, resulting in an overall neutral charge.[45] Compared to PEG, zwitterionic materials have a greater capacity to attract and maintain a larger number of water molecules, forming a more robust hydration shell.[46,47] Their super hydrophilic nature contributes to minimizing nonspecific binding of proteins to the NPs surface.[48–51] Despite their extensive use as stealth coatings for drug delivery, only limited research has thoroughly examined protein adsorption in the context of zwitterionic polymers. Another claimed property of these materials is their low immunogenicity.[52] However, the number of studies oriented to investigate their safety is still testimonial.

Polyglycerol (PG), particularly hyperbranched PG (hPG) shielding, has also emerged as an alternative to PEG to prevent protein corona formation.[53–57] However, the studies reported so far do not claim a superiority versus PEG in terms of preventing protein attachment. On the other hand, while some claims have been made that this material does not provoke any immune response after multiple in vivo administrations,[53] additional studies are requisite to substantiate the potential of hPG.

Recently, some authors have reported that certain polypeptides can be used as alternatives to PEG. Notably, polysarcosine (pSar), composed of repetitive units of the natural amino acid sarcosine (*N*-methylated glycine), has shown promising outcomes when associated to LNPs and polymeric NPs.[58,59] In specific studies comparing the protein adsorption patterns of pSar-coated and PEG-coated NPs, remarkably similar results were obtained.[60,61] Despite of this while PEG-liposomes were rapidly cleared from blood circulation after a second administration, there was no discernable difference in the circulation time of pSar-NPs after two consecutive administrations.[62] Nevertheless, as previously suggested for zwitterionic and hPG polymers, a more rigorous characterization of these modified NPs with proteins as well as in-depth research into the safety of these materials is warranted.

#### *2.2.2. Surface Modification of Nanocarriers Using Cell Membrane Components*

It has been argued that cells possess the ability to prevent the uncontrollable adsorption of exogenous proteins, phospholipids, and other biological molecules[46] due to the zwitterionic headgroup of cell membrane phospholipids.[42] Inspired by these inherent properties, an alternative approach that has emerged is to camouflage NPs behind cellular membranes, instead of modifying the NPs' surface with hydrophilic and zwitterionic materials. In this sense, various cell membranes including platelet, red and white blood cell, stem cell, and cancer cell membranes have been employed to fabricate nanosystems.[63–65] Recent reports showed that the coating of PLGA NPs with human platelet derived plasma membranes successfully produced biomimetic NPs with enhanced immunogenetic properties. Compared to nonmodified NPs, biomimetic particles displayed no complement activation upon incubation.[66] In a similar approach, the adsorption of cellular membranes derived from leukocytes or red blood cells onto silica NPs has resulted in a reduction of protein adsorption.[67,68] In addition, the advantage of utilizing biomimetic red blood cells (RBC)-derived NPs was illustrated through in vivo protein corona analyses, showing negligible protein adsorption after multiple administrations of RBC-derived NPs, whereas PEG-modified NPs exhibited a pronounced increase in the attachment of biological proteins, particularly IgM and IgG.[69]

Collectively, these findings underscore the potential of biological camouflage to augment the stealth properties of NPs. Nonetheless, numerous challenges still lie ahead, encompassing rigorous characterization, ensuring reproducibility, managing the limited supply of biological components, and addressing clinical safety concerns. Additionally, a more substantial body of evidence is essential to thoroughly comprehend the precise components responsible for the diminished protein corona formation.

In summary, a consensus prevails within the scientific community concerning the imperative need to fabricate materials aimed at mitigating interactions between nanocarriers and proteins. Nevertheless, the approaches devised thus far have not conclusively exhibited superiority over PEG. Hence, there is a need to thoroughly investigate the quantities and conformation of these materials on the surface of nanocarriers, as they have been suggested to play a crucial role in the binding of biological proteins to the NPs.

### 2.3. Controlling the NP–Protein Corona Interaction for Endogenous Targeting

Instead of viewing protein corona formation as exclusively detrimental to successful NP distribution, growing evidence indicates the potential for controlled attraction of distinct proteins to aid accumulation in the targeted tissue, a concept termed endogenous targeting.[70] Indeed, this approach has been successfully applied in the case of Onpattro, the first FDA-approved siRNA-LNP for treating hereditary disease transthyretin-mediated amyloidosis. The liver affinity of Onpattro has been strongly linked to its ApoE-rich protein corona, particularly through the interaction with hepatocytes via ApoE-binding receptors like low-density lipoprotein receptors.[71] Very recently this hallmark has been updated, as ApoE-containing high density lipoprotein enriched in NP–corona complexes was shown to be a better predictor of in vivo liver efficacy compared to sole ApoE.[72]

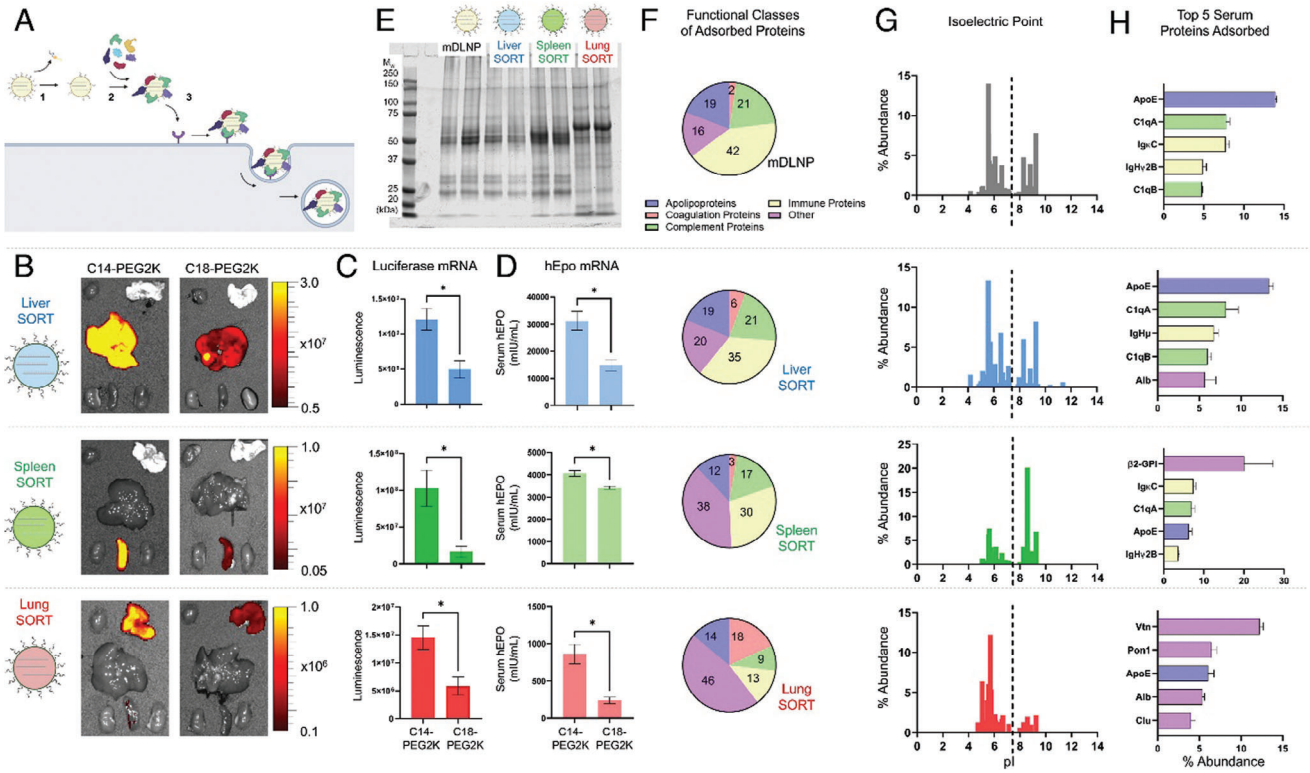


**Figure 3.** Overview on mechanism of SORT LNP tissue targeting. A) Schematic overview on three-step mechanism, including PEG–lipid desorption (1) resulting by distinct protein adsorption (2) followed by receptor-mediated uptake in target tissues (3). B) SORT particle induced bioluminescence in target organs depending on PEG-lipid identity, suggesting the importance of PEG-lipid desorption. C) Quantification of luminescence in target organ. D) Serum hEPO quantification following treatment with SORT LNPs. E) SORT LNPs bind distinct plasma proteins. F) SORT molecules cause shift in abundance of functional classes of adsorbed corona proteins. G) The isoelectric point distribution of adsorbed proteins is affected by the SORT lipid identity. H) The top five most enriched plasma proteins are determined by SORT lipid chemistry. Reproduced with permission.[77] Copyright 2021, National Academy of Sciences.

The structural analysis of LNPs unveiled that a notable content of DSPC and cholesterol on their surface[22–24] was correlated with the attraction of ApoE. This could be explained by ApoE's involvement in cholesterol transport.[73] A similar observation emerged when introducing cholesterol into DNA tetrahedrons, leading to hepatocyte accumulation.[74] Interestingly, other studies demonstrated that substituting neutral cholesterol with positively charged DC-cholesterol on the surface of LNPs leads to a transition from ApoE- to vitronectin-based corona profile, diminishing the NPs' ability to target and transfect hepatocellular carcinoma.[75] Furthermore, a shift in the tropism toward the lungs was achieved by introducing quaternary ammonium containing lipids like 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), dimethyl-dioctadecylammonium (DDAB) or 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine (EPC). The concept of incorporating a tropism-shifting lipid component altering biodistribution was termed selective organ targeting (SORT) by Siegwart and colleagues (**Figure 3**).[76] Vitronectin was identified as the predominant protein corona constituent of these LNPs containing the aforementioned cationic lipids.[77] This protein bears the Arg–Gly–Asp (RGD) sequence by which it recognizes integrin $\alpha_v\beta_3$, commonly overexpressed in pulmonary endothelium, solid tumors, and tumor neo vasculature.[78,79] This specific targeting of cancer cells overexpressing the $\alpha_v\beta_3$ receptor has been demonstrated for DOTAP/DNA NPs[79] and association of vitronectin with DOTAP liposomes has also been established.[80] Overall, these works reveal that ascribing a functional role to specific corona constituents proves to be a more intricate endeavor than previously assumed.

The role of LNPs' protein corona profile in influencing in vivo targetability has been subjected to deeper investigation in a library screen approach. Findings from screening indicated that LNPs containing ionizable lipids with distinct bonds in their tails, O-series (ester bonds) versus N-series (amide bonds) lipids shifted the delivery from liver to lung. Analysis of the protein corona composition revealed that N-series LNPs were enriched in vitronectin, fibrinogen, fibronectin, and proteins that are known to bind the vitronectin receptor $\alpha_v\beta_3$,[81] possibly accounting for their lung tropism. Conversely, the O-series LNPs' corona exhibited a high ApoE enrichment, a fact that might explain their liver tropism.[82] This study underscores the impact of seemingly minor changes in the lipids' chemical features on corona constituents.

The mentioned SORT approach has demonstrated its applicability beyond lung targeting, as on a similar note the incorporation of a negatively charged phospholipid like phosphatidic acid into LNPs was reported to promote the attachment of $\beta_2$-glycoprotein I. As a result, specific delivery to the spleen was

achieved.[77] The interaction between $\beta_2$-glycoprotein I and acidic phospholipids has been described previously, reinforcing the rational incorporation of these lipids into NPs to specifically attract serum proteins facilitating spleen targeting.[83]

Some authors have argued that it is possible to extend the circulation time of PEGylated liposomes through their conjugation with an albumin-binding domain (ABD). This led to eightfold enrichment in albumin content within the corona, contributing to increased tumor accumulation.[84,85] Similarly, arginine-lipid based siRNA polyplexes, which exhibited notable albumin association on their surface, were also associated with prolonged circulation times.[86]

The examination of the concepts around endogenous targeting necessitates cautious assessment in terms of their translatability, given the multifaceted factors contributing to specific protein corona formation. Among these, hydrophobicity, surface chemistry, and charge do not depend only on NP components but on their structural arrangement.[87,88] For instance, there exists substantial evidence to infer a discernable relationship between the incorporation of materials carrying quaternary ammonium groups (e.g., DOTAP, DDAB, EPC) and the presence of vitronectin, even across different classes of carrier systems such as LNPs, lipoplexes, and nanocapsules (NCs). However, the question whether solely this protein governs the tropism shift has been addressed by Siegwart and colleagues by evaluating the impact of different biophysical classes of lung SORT lipids, based on their headgroups trimethylammonium-propane (TAP) or EPC with diverse tail chemistries, on protein corona formation and tissue tropism. They unveiled significant positive correlations between not only vitronectin but also clusterin, albumin, and prothrombin, and their tropism to the lung. These proteins are attracted by both TAP and EPC lung SORT lipids.[89] However, the specific reason underlying the exhibited behavior of certain cationic compounds warrants further elucidation.

Our group has dedicated substantial efforts to shedding light on the interaction of polymeric NCs, originally developed in our laboratory, and serum proteins. In accordance with other authors, we have corroborated that the incorporation of PEGylated surfactants in polymeric NCs generally diminished the number of adsorbed proteins. However, we also found that the combination of PEGylated materials with other hydrophilic polymers dramatically changed the amount and type of proteins adsorbed. Namely, our investigations found the combination of a short linear PEG surfactant D-$\alpha$-tocopherol PEG 1000 succinate with HA resulted in a prominent protein corona with a high content on Apo-A4. Contrarily, its combination with poly-$\alpha$-glutamic acid (PGA) resulted in a very low amount of Apo-A4 adsorbed. On the other hand, branched PEG, introduced via polysorbate 80, was linked to a very low amount of complement protein C3. Finally, as expected, the incorporation of DOTAP in the formulation was translated into a significant adsorption of vitronectin. Moreover, when comparing HA and polysialic acid coatings, we observed fairly similar corona fingerprints, with ApoE, ApoC2, and ApoC3 constituting more than 75% of total protein.[90]

Generally, the mere presence of a certain lipid does not reliably predict enrichment of the theoretically associated serum protein, given that its surface display is a prerequisite. This adds complexity to the translation of findings across different NPs, since not only the components but also their ratios affect their structural organization,[91] and consequently, the corona formation of these particles. Thus, a more thorough analysis of the relationship between NP structure and corona composition is essential. Expanding on this, the presence of certain proteins within the corona does not imply a functional role. Indeed, protein binding to NPs can cause structural alterations of said proteins, rendering them biologically inactive.[92] While it is exceedingly challenging to predict how the protein corona might alter the NPs fate, incremental progress in understanding presents opportunities for novel therapeutic and diagnostic avenues.

Thus far, endogenous targeting has proven effective in directing NPs to the liver, spleen, and lungs. To extend the application of this strategy toward additional tissues, further investigations connecting protein corona composition and biodistribution are required.

## 3. Reaching the Tumor Tissue

Despite the considerable efforts directed toward the design of nanomedicines for cancer therapy, the majority of formulations fall short in achieving the intended therapeutic efficacy. A predominant challenge lies in the insufficient accumulation of NPs within the tumor tissue, together with the limited diffusion of the NPs across the tumor matrix. This challenge is further exacerbated by the difficulties in identifying ligands that are expressed exclusively in the target tissue. It is recognized that successful tumor accumulation entails preventing undesired protein corona formation and adequate diffusion across the tumor. Therefore, in this section we will consider passive targeting, tumor diffusion, and intratumoral active targeting.

### 3.1. Passive Targeting: Enhanced Permeability and Retention (EPR) Effect

The first passive targeting strategy found its way into the clinic with the commercialization of Doxil,[93] a formulation of PEGylated liposomes containing doxorubicin. This formulation was described to exhibit the EPR effect, coined in 1986 by Matsumura and Maeda.[94] Since then, four passively targeted PEGylated oncological formulations have entered the market,[95,96] Onivyde, Genexol-PM, Lipo-Dox, and Doxil. It is now well-established that not only the surface composition (stealth materials) but also the particle size, surface charge, and NP elasticity determine the EPR effect.[12,97,98] In general, very small NPs (less than 10 nm) undergo rapid clearance,[99] while those with a size larger than 150 nm may suffer from significant accumulation in the liver and spleen.[100] The mentioned properties and characteristics of nanosystems are also affected by the respective technique employed for NP manufacturing, a topic which has been covered by numerous articles to which the reader is referred for in-depth information.[101–104] Apart from this generic statement, PEGylation has emerged as a pivotal strategy to modulate the biodistribution of both small molecules and biological drugs. This phenomenon can be attributed, in part, to the influence of PEGylation on the formation of the protein corona, stemming from its extended circulation time, as previously elaborated; a key factor in driving passive accumulation. Aside from reducing corona

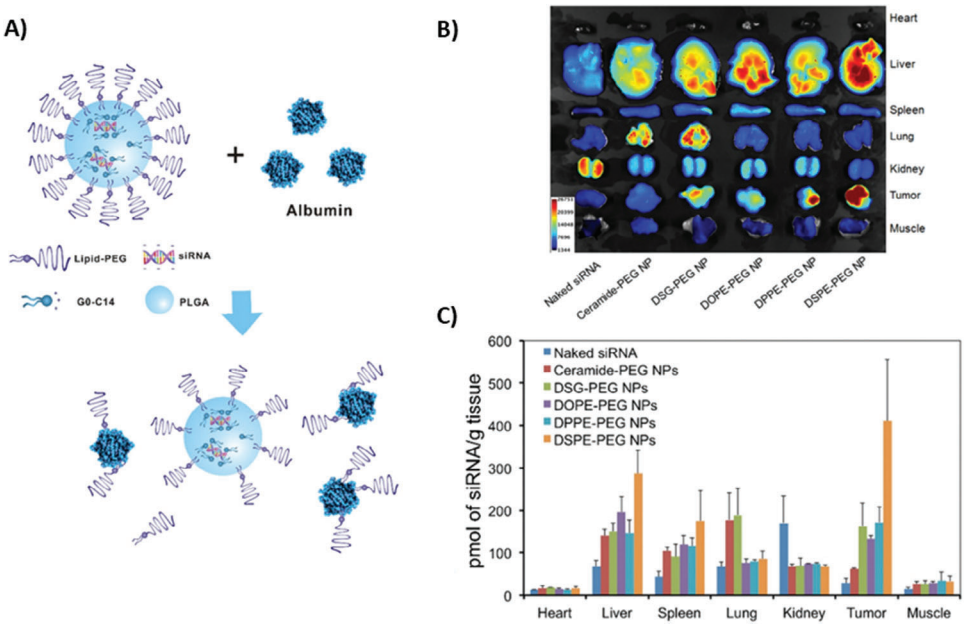


**Figure 4.** The balance between PEG-lipids and its dissociation rate drives the in vivo outcome of lipid–polymer hybrid NPs for siRNA delivery. A) NP configuration and lipid-PEG behavior upon incubation with serum albumin. B) By screening the PEG-lipid that shells the NPs, the biodistribution of the NPs and consequently, C) the amount of siRNA is modulated. Reproduced with permission.[119] Copyright 2017, Ivyspring International Publisher.

formation to improve passive accumulation, the deliberate attraction of circulation-enhancing proteins such as albumin[84,85] or proteins detargeting the liver[89] might lead to enhanced passive accumulation, as discussed extensively in Section 2. Besides, the insights provided in Section 2.3 are applicable here, given that passive targeting involves preventing protein corona formation. Despite this theoretical basis, the reality is that few studies have established a clear correlation between protein corona formation and biodistribution to the tumor.

In this context, it has been well-established that DSPE-PEG has been the most utilized form of PEGylation for NPs.[105–116] However, other materials like DMG-$PEG_{2k}$ or PEG-polymers have gained popularity, especially in the context of siRNA delivery.[117,118] One of the primary factors driving the widespread adoption of DSPE-PEG is its hydrophobic segment, which plays a role in retarding detachment from various NPs, including LNPs.[21] This property contributes to the extended circulation time and enhanced accumulation of these NPs at tumor sites. In fact, in vivo studies intended to investigate the importance of PEG-lipids with alkyl chains of varying lengths (C14, C16, and C18) such as DMG-PEG, DPPE-PEG, and DSPE-PEG, concluded that the lipophilic segment of PEG-lipids not only governs their dissociation rate from siRNA polymer–lipid hybrid NP surface but also exerts tumor accumulation and silencing efficiency (**Figure 4**). Notably, surface modification with DSPE-PEG exhibited the most extended blood circulation time and the highest levels of tumor accumulation and gene silencing effectiveness.[119] Certainly, PEGylated LNPs, which are extensively used as carriers for RNA, have also been explored for the delivery of mAbs,[111–116] although this exploration has mainly centered around liposomes incorporating DSPE-$PEG_{2k}$. However, the correlation between biodistribution and efficacy has been overlooked for these compositions.[114–116] Instead, a number of pharmacokinetics studies have shown the possibility to correlate the tumor accumulation of PEGylated nanostructures carrying different mAbs with a great antitumor outcome. This is the case for $PEG_{5k}$-(−)-epigallocatechin-3-O-gallate micelles containing trastuzumab,[120] $PEG_{12k}$-lysine and -aspartamine micelles containing antinuclear pore complex or anti-myelocytomatosis oncogene cellular homolog (MYC) mAbs,[121] and poly(oligo-(ethylene glycol)methacrylate) $(POEGMA)_{20}$-dopamine reactive-oxygen-species (ROS)-responsive nanocomplexes carrying glucose oxidase and antiPDL-1 mAb.[122] In our view, the PEGylation of these nanocarriers contributed to the satisfying therapeutic responses reported.

Regarding the degree of PEGylation, densities ranging from 1.5% to 5% (expressed as a molar ratio relative to total lipid components) have proven the most favorable outcomes for achieving successful passive targeting.[114,123] Unfortunately, the actual composition of the NPs, namely, the amount of PEG that is associated to the NP, has generally not been characterized. Concerning PEG MW, the prevalence of $PEG_{2k}$ has led to a certain level of comfort, resulting in limited exploration of other MWs. It is presumed that higher MW could enhance its properties, but a deeper examination of the interplay between MW, PEG density, and the mechanical attributes of NPs is warranted, particularly in the context of delivering biologicals. This need arises from the significant influence of PEG MW and density on the interaction between NPs and cellular surfaces.[124–126] Thus, finding an optimal balance is of utmost importance. Lastly, in terms of structural conformation of PEG on NP surfaces, the field emphasizes the necessity of brush-like conformations.[127]

Collectively, these aforementioned optimal PEG properties also apply in the context of biologicals, where the same attributes have shown to result in the best tumor accumulation.[96] In this context, a tumor accumulation of ≈8% of the injected dose per

gram of tissue represents the most noteworthy accumulation reported in the delivery of biologicals[115,120] in murine models. However, direct comparisons between all these technologies are complicated due to differences in the units used for measurement.

In our perspective, the mechanical and biophysical attributes of PEGylated NPs, along with their interplay with factors like MW, particle size, and density, have been set aside. It is reasonable to acknowledge that the techniques accessible for such characterization are restricted, and this challenge becomes even more pronounced with PEGylated polymers due to their heterogeneity. However, undertaking comparative studies could serve as an alternative approach to at least establish trends.

Furthermore, it is noteworthy to mention that it is now well understood that the nature of the carried biological payload should not significantly affect the passive accumulation of the NP. However, the positioning of the biological payload in the outer layers of the particle might potentially alter the adsorbed protein corona, thereby influencing passive accumulation. A definitive strategy for achieving optimal passive accumulation remains elusive, given the persistent issue of capture by the liver and spleen. Determining whether this limitation can be overcome within the field or requires the formulation of strategies to enhance uptake within the target tissue remains uncertain.

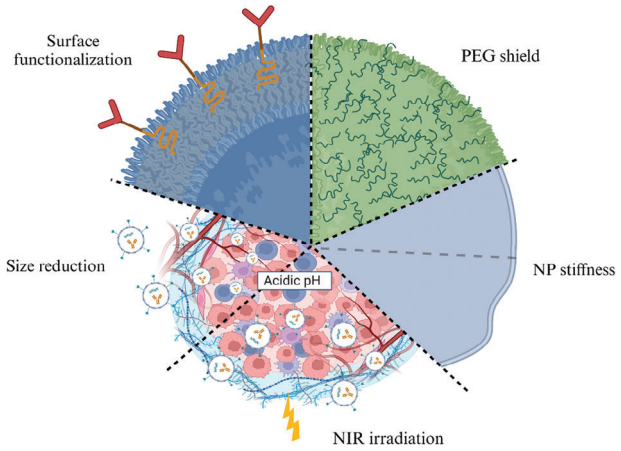


**Figure 5.** Diverse approaches for augmented tumor penetration of NPs. Addressing the challenge of efficient NP diffusion within the tumor stroma has been pursued through three strategies. First, the physicochemical properties of the NPs, comprising particle size and rigidity, have demonstrated improved access to intratumoral regions. Conversely, the incorporation of targeting ligands onto the NP surface has augmented diffusion and distribution across the tumor stroma. Lastly, NP design can be tailored to take advantage of the natural TME acidic pH, thereby prompting NP disassembly. In this regard, external stimuli, including photothermal therapy, could bolster tumor diffusion.

### 3.2. Intratumoral Diffusion

Despite the numerous advantages that nanomedicine offers over conventional drug delivery, various challenges continue to impact their clinical application. Among them, inadequate tumor penetration represents one of the most substantial obstacles. The efficient delivery and penetration of NPs within tumor are hindered by a disorganized tumor vasculature, elevated interstitial pressure, and a dense extracellular matrix characterized by collagen-enriched stroma.[128,129] Therefore, an imperative exists to design novel strategies to enhance the diffusion of nanosystems within the tumor tissue. Recent endeavors have investigated approaches to address this issue, encompassing the exploitation of unique TME properties, employing rational nanomedicine design, and leveraging externally applied stimuli such as radiation or ultrasound. In the forthcoming subsections, we will delve into the methods employed to achieve enhanced intratumoral diffusion, classifying them according to tumor characteristic or NP features responsible for the observed outcomes (**Figure 5**).

#### *3.2.1. Physicochemical and Mechanical Properties of NPs that Influence Diffusion of Nanocarriers across the Tumor*

*Surface Composition and Particle Size*: The symbiosis between particle size and the presence of a PEG shell has also been reflected regarding NPs access to intratumoral regions, seen in paclitaxel-loaded nanodiscs with 2, 5, or 10 mol% $PEG_{5k}$-lipid coating. This platform has demonstrated its ability to penetrate into orthotopic breast tumors, followed by an apparent extravasation. The authors concluded that augmented tumor penetration was contingent upon both the degree of PEGylation and concomitant particle size, as particles with 10 mol% PEG exhibited favorable results.[130] There are other works that have shown how PEGylation favors diffusion in biological fluids and tissues. For example, our group was first in showing that PEGylation limits the interaction with proteins, i.e., mucin, facilitating mucopermeation.[131,132] This was further corroborated by Hanes and co-workers confirming that PEG modification minimized NPs' mucoadhesion.[133] On a similar note, they also confirmed the enabling role of PEGylation in the diffusion of NPs across brain tissue.[134] Finally, our group has also verified that PEGylation and fluidity of nanocarriers are essential for the diffusion of drugs across the subcutaneous tissue into the lymphatic system, thereby promoting lymphotargeting.[135–137]

Subsequent developments in the field revealed that anchoring a ligand to the NP surface would not only allow for active targeting but also enhanced diffusion across the tumor. To achieve this goal, the field has moved from traditional ligands such as folic acid to more sophisticated alternatives, as is the case for tumor-penetrating peptides (TPPs) (e.g., truncated LyP-1, tLyP-1). The success of tLyP-1 not only relies on its tissue targeting capacity but also relies on the ability to drive the NPs to intratumoral regions. For instance, tumor sections at different depths obtained from breast tumor bearing models demonstrated significant distribution of tLyP-1-modified micelles anchored to HA nanogels across the tumor tissue. The superior properties of dual versus single targeting have already been reported for improved tumor accumulation. However, here, micelles embedded in HA nanogels without tLyP-1 seemed to penetrate to a similar extent as the dual targeting strategy.[138]

The use prostate-specific membrane antigen (PSMA) became recognized by the field after being targeted by docetaxel NPs. This technology (BIND-014) had previously reached Phase 2 clinical trials (NCT01792479),[139] and more recently, it has inspired other platforms such as 4-armed starPEG nanocarriers. By anchoring only a single S,S-2-[3-[5-amino-1-carboxypentyl]-ureido]-

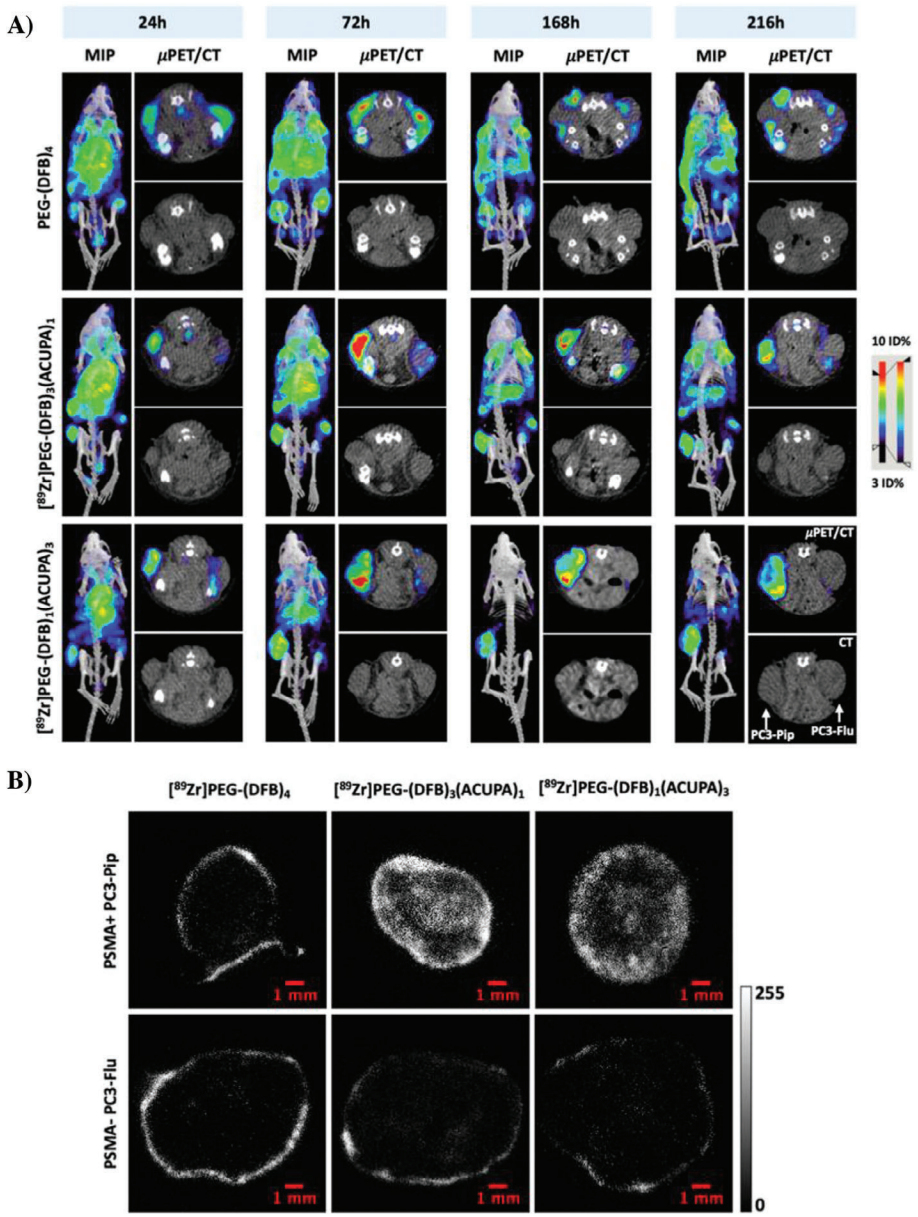


**Figure 6.** The effect of ACUPA functionalization 216 h postadministration. A) Maximum intensity projection μPET/CT, axial μPET/CT, and axial CT images of $^{89}$Zr-labeled starPEG nanocarriers in xenografts of PSMA+ PC3-Pip (left flank) and PSMA− PC3-Flu (right flank) mice bearing model. B) Autoradiography images of 20 μm tumor slices of PSMA+ PC3-Pip (top images) and PSMA− PC3-Flu tumors (bottom images). Reproduced with permission.[140] Copyright 2022, American Chemical Society.

pentanedioic acid (ACUPA) molecule per NP, deep tumor penetration comparable to that of starPEG nanocarriers targeted with three ACUPA molecules was achieved. Conversely, peripheral accumulation was observed for nontargeted NPs or PSMA negative prostate tumors (**Figure 6**).[140]

*NPs' Stiffness*: Considering that the mechanical properties of nanocarriers have been described to affect circulation $t_{1/2}$[141] and tumor accumulation,[142] Hammond and colleagues delved into the influence of stiffness of layer-by-layer (LbL) poly-L-arginine and HA-coated liposomes on tumor penetration in a subcutaneous ovarian cancer model. Atomic force microscopy was employed to determine LbL NPs as either stiff, when their liposome core was composed solely of C18 unsaturated phospholipids or fluid, when the core contained additional fluidity enhancing cholesterol. Their findings indicated that stiff liposomes owned enhanced penetration and diffusion within the tumor.[143] However, this statement should be approached with caution since the change in the core composition may influence the arrangement of the different components of the nanostructures, among other factors that affect tumor penetration.

The relevance of the physicochemical or mechanical properties of the NP such as surface charge or stiffness is controversial as attributing NP fate to a single property is questionable. Consequently, drawing conclusions is intricate as the underlying

mechanism of the reported intratumoral diffusion has not been disclosed. In our opinion, the crux lies within the multitude of factors contributing to intratumoral diffusion, and the limitations in currently available techniques in shedding light on them. Beyond this, further efforts in reporting delivery efficiencies of the biological rather than a percentage of accumulated NPs may be crucial to establish a correlation with the therapeutic outcome.

*Natural and Artificial Stimuli*: Distinctive attributes of the TME have been harnessed for the design of TME-responsive nanocarriers. As described below, the acidic pH of the TME has been leveraged to enhance tumor penetration. A recent contribution has reported deep penetration of NPs that disassembly into smaller particles prompted by the acidic TME. Upon arrival in the tumor tissue, pH-responsive micellar nanosystems experienced disassembly into smaller nanocarriers in response to the acidic TME.[144] For example, acid-sensitive polymeric micelles carrying cisplatin decreased from 130 to 40 nm in size upon encountering weakly acidic environment in vitro. Intratumoral injection in a liver tumor-bearing mice model of these responsive particles led to deeper penetration and distribution into the tumor than the free drug.[145] Similarly, doxorubicin-loaded polymeric nanoassemblies were found to disassemble in acidic environment into small, 30 nm sized particles, enhancing their tumor penetration.[146]

Instead of for example relying on the acidity of the TME as internal stimulus driving tumor penetration, relying on external stimulation is another commonly considered approach in overcoming tumor penetration as limiting factor toward successful anticancer therapy. A "cluster-bomb" nanohybrid was developed, featuring IR780- and 1-methyl-tryptophan loaded PAMAM which was crosslinked by ROS-responsive thioketals and further coated with chondroitin sulfate. Upon encountering ROS at the tumor sites in addition to local NIR irradiation, the 130 nm assemblies fragmented into sub-10 nm sized "bomblets" as suggested by ROS incubation in vitro, penetrating deeply into tumor tissue, as proven in a subcutaneous melanoma model.[147] Another relevant example was the design of PEGylated light-inducible nanocargos (LINCs), combining both photodynamic therapy and immunotherapy. The PEG layer facilitated EPR-driven accumulation of these nanosystems within the tumor. After tumor accumulation, LINCs migrated from the tumor periphery and diffused deep into the tumor mass due to the cleavage of the PEG shield under NIR laser irradiation.[148]

In brief, the synthesis of NPs with tunable particle size, either due to responsiveness to the biological features of the TME or external stimuli, has shown promising penetration capabilities. Through it, the generation of relatively small NPs of ≈40 nm or less have granted enhanced diffusion across the tumor. Nevertheless, despite the interest of this approach, so far authors have failed to provide clear evidence of the in vivo mechanism of disassembling, leaving all this information under the premise of hypothetical behavior.

### 3.3. Active Targeting

NPs primarily rely on the EPR effect to access the tumor site. However, the incorporation of targeting ligands onto NPs has been observed to enhance the interactions between the NPs and cells. This enhancement facilitates more efficient accumulation and delivery of drug-loaded NP within the cells. Active targeting involves the conjugation of different types of ligands, including Abs, peptides, or polymers on the NPs surface for specific recognition by the targeted disease cells. The interaction between these receptors expressed on the cellular surface and their complementary ligands attached to the NP can mediate the internalization of the nanocarriers into the tumor cells. Therefore, this strategy is mainly focused on enhancing the cellular uptake following specific accumulation at the tumor site.[145,149–151] Achieving targeted delivery toward desired tissues is considered the holy grail in nanomedicine, a sentiment underscored by the substantial number of over 8000 publications that have emerged since the inception of the active targeting strategy. Over 30 years ago, numerous studies initiated the practice of adorning the surface of NPs with ligands, aiming to surpass the limitations of the EPR effect and thereby enhance the therapeutic efficacy of these NPs.[152–154] Nevertheless, even after three decades the number of targeted NPs that successfully transitioned from animal studies to clinical trials in humans remains relatively low.[155]

The success of a targeted NP is not easily achieved, as it involves overcoming numerous challenges in their rational design. Among them, a key problem is the lack of exclusive overexpression of the target at the tumor tissue. Moreover, the ligand density not always has a linear correlation with the extent of recognition due to the appearance of steric hindrance.[156] In the same breath, the interplay of ligand-to-receptor density plays a crucial role in the recognition on cellular targets.[157] Therefore, fulfilling these critical requirements for successful active targeting is not straightforward. This section will comprehensively explore the leading approaches employed in the field to facilitate the accumulation of NPs within the tumor. These approaches encompass targeting both the tumor vasculature and tumor cells, while also enhancing the diffusion of NPs through the intricate tumor stroma.

#### 3.3.1. Targeting of Tumor Cells

Diverse ligand types have been employed for the design of active targeting nanomedicines, with peptides being one of the most exploited ones (**Table 1**). TPPs not only promote the accumulation but also enhance the drug distribution within the target tissue.[158,159] One of the TPPs at the forefront is iRGD, combining tumor endothelial cell targeting and tumor penetrating attributes.[160] Several studies using iRGD showed a clear improvement in the accumulation of NPs in tumors compared with nontargeted NPs.[161–163] Another TPP that has demonstrated a significant ability to improve the tumor accumulation of NPs is tLyP-1. Our laboratory was the first to show that tLyP-1 functionalized HA NCs enhanced the tumor accumulation and retention in an orthotopic lung cancer model, while they also facilitated the access to the lymphatics (**Figure 7**). In fact, the targeted-NPs exhibited notably high tumor accumulation, reaching ≈20% of the administered docetaxel dose per gram of tumor tissue. In contrast, the levels of docetaxel dose per gram of tissue acquired from other analyzed organs, including the liver, remained below 5%. On the contrary, Taxotere and its nontargeted

**Table 1.** Summary of functionalized nanosystems targeting tumor cells.

| Active ligand | Nanocarrier | Physicochemical properties (size, surface charge, AE) | Adm. route | Tumor model | Outcome | Refs. |
|---|---|---|---|---|---|---|
| iRGD | HSA–OEG NPs (STAT3) | 220 nm 96% | i.v. ($2.0 \times 10^{11}$ NPs/100 µL) | Glioma | Stronger tumor accumulation compared nontargeted NPs | [#0161] |
| iRGD | PolyMTO-DSPE-PEG micelles | 92 nm 40% | i.v. (5 mg $mL^{-1}$) | Prostate cancer | Higher tumor accumulation and therapeutic efficacy | [#0162] |
| iRGD | Polymeric NPs (survivin–siRNA) | 70 nm | i.v. (650 µg $kg^{-1}$ siRNA) | Prostate cancer | Tumor accumulation with modified-NPs was threefold superior | [#0163] |
| tLyP-1 (DS 8%) | HA NCs (DOX) | 149 nm (–) 27 mV | i.v. (5 mg $kg^{-1}$) | NSCLC (orthotopic) | Strong tumor accumulation and reduction of tumor load | [#0164] |
| tLyP-1 | LNPs (siRNA KRAS) | 59 nm (–) 02 mV | i.v. (1 mg $mL^{-1}$ siRNA) | Pancreatic cancer | Higher distribution to the tumor compared to nontargeted NPs | [#0165] |
| HA | Lipid–polymer hybrid NPs | 122 nm (–) 21 mV 85% ERL, 82% BVZ | i.v. (10 mg $kg^{-1}$ of ERL and/or BEV) | NSCLC | Strongest tumor accumulation and reduction of tumor volume | [#0170] |
| HA | LNPs (siPKL1 siRNA) | 60 nm (+) 5 mV | intraperitoneally (1 mg $kg^{-1}$ siRNA) | Ovarian cancer | Enhanced tumor targeting compared to uncoated NPs | [#0171] |
| HA and CD20 mAb | HA–PLGA–PLA NPs (BCL2 siRNA) | 135 nm (–) 39 mV | i.v. (1 mg $kg^{-1}$ siRNA) | Non-Hodgkin lymphoma | Improved siRNA delivery to tumor cells and enhanced survival | [#0172] |
| TfR | PLGA NPs (BCR/ABL mAb) | 296 nm (–) 13 mV 73% | i.v. (2 mg per mice) | Chronic myeloid leukemia | More extensive survival | [#0186] |
| MPC | NCs (Nimo or Tras) | 25 nm | i.v. (10 mg $kg^{-1}$) | Glioblastoma (orthotopic) | Effective mAb delivery to tumor site and prolonged survival | [#0178] |
| CXCL13 | PLA–PEG–PLA NCs (RTX) | 28 nm (–) 1 mV | Retro-orbital vein injection (4 mg $kg^{-1}$) | Non-Hodgkin lymphoma | Greater antitumor effect compared to nontargeted NCs | [#0179] |

AE association efficiency, NCs nanocapsules; NPs nanoparticles; tLyP-1 truncated LyP-1; i.v. intravenous; HAS human serum albumin; OEG oligomerized (-)-epigallocatechin; STAT3 activator of transcription 3; BVZ bevacizumab; PolyMTO polymitoxantrone; HA hyaluronic acid; DS degree of substitution; DOX doxorubicin; NSCLC nonsmall cell lung cancer; ERL erlotinib; PLK1 polo-like kinase-1; PLGA poly(lactic-*co*-glycolic acid); PLA poly(lactic acid); Tfr transferrin; MPC 2-methacryloyloxyethyl phosphorylcholine; Nimo nimotuzumab; Tras trastuzumab; CXCL13 chemokine ligand 13; RTX rituximab.

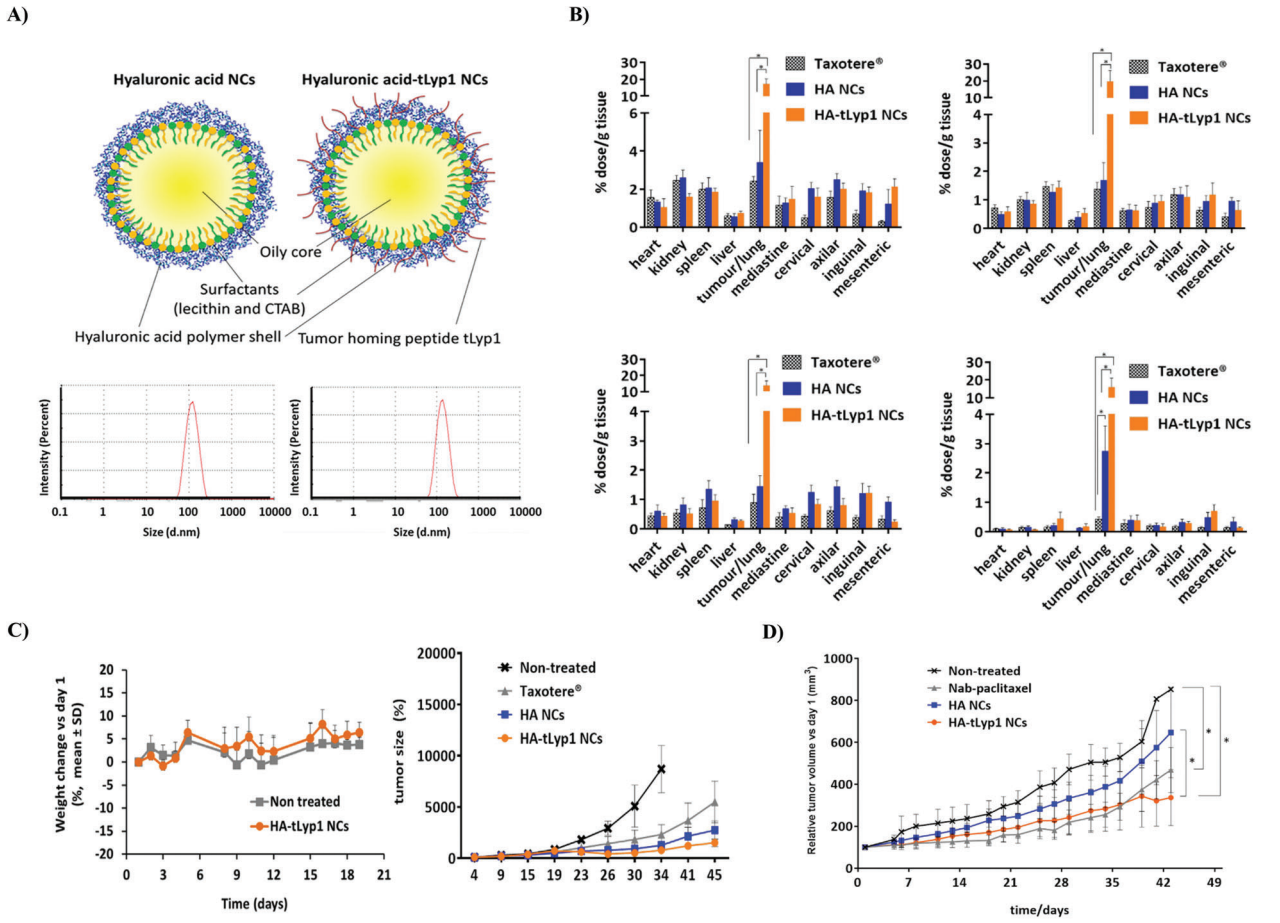


**Figure 7.** HA-based NCs modified with the tumor homing peptide ligand tLyP-1 exhibit strong tumor accumulation. A) Schematic representation of the HA and HA-tLyP1 NCs structure and their physicochemical characterization. B) Biodistribution studies at 1, 4, 8, and 24 hours in an orthotopic lung cancer model with lymphatic metastases after i.v. administration of different treatments. C) In vivo efficacy and toxicity studies in an orthotopic lung tumor-cancer model. D) Relative tumor volume of the pancreatic cancer patient derived xenograft model after treatment administration. Significant differences between the treatments (*) $p < 0.001$. Reproduced with permission.[164] Copyright 2022, Elsevier Ltd.

counterpart displayed limited targeting capacity.[164] These results encouraged us to move forward and develop LNPs encapsulating siRNA-KRAS (Kirsten rat sarcoma) functionalized with tLyP-1. Biodistribution assessments showed a clear accumulation of modified and unmodified LNPs in the liver. However, it became evident that the modification of these nanocarriers with tLyP-1 resulted in stronger accumulation in the tumor tissue compared to nontargeted LNPs.[165] In essence, although several NPs have demonstrated a preferential distribution to the tumor, the prevalent tendency remains the accumulation in peripheral organs.

Some authors have also claimed the active targeting capacity of HA due to its affinity toward the CD44 receptor.[166,167] Our laboratory has also reported positive results for HA NCs containing docetaxel.[168,169] A relevant example in the context of biological drugs has combined erlotinib and bevacizumab-loaded lipid–polymer hybrid NP with HA anchored to PEG. The administration of this combination to a nonsmall cell lung cancer tumor model led to an enhanced tumor accumulation of erlotinib.[170] Analogous outcomes were found with HA-modified LNPs encapsulating polo-like kinase-1 (PLK1) and eukaryotic translation-initiation factor 3c (eIF3c) siRNA in an orthotopic ovarian cancer model.[171] However, the intraperitoneal administration performed in this study may have facilitated a higher proportion of LNPs reaching the tumor due to the proximity of injection site and tumor. It should be noted that intraperitoneal administration bypasses the liver, thus enhancing the tumor accumulation possibilities. Despite the significant amount of work making use of HA to facilitate the access to tumoral tissues, we now know that relying solely on HA as a targeting ligand is not sufficient, given that CD44 receptor expression is not exclusive for tumor cells. Thus, the concept of dual targeting involving the incorporation of additional ligands alongside HA has been proposed to enhance the NPs targeting efficiency while reducing off-target effects. In this context, an array of NPs were designed with mAb as targeting ligands anchored to HA as outer layer to target tumor cancer cells via CD44 and an additional receptor.[172,173] Interestingly, this dual targeting approach significantly improved siRNA delivery to blood cancer cells compared to the single-targeted CD44 NPs, evident through a strong downregulation of BCL-2 expression in the tumor tissues analyzed ex vivo. In fact, this demonstrates that a combination of two or more targeting

ligands could be beneficial to improve the accumulation of the NP in the tumor.[172]

In addition to HA polymer, Abs that specifically bind CD44 receptors have been used for targeting purposes. In this regard, the remarkable tumor accumulation of the NPs in a breast and potential metastatic tumor bearing-mice model was triggered by the presence of anti-CD44 Abs on the liposomes surface. This effect was translated into an impediment of the metastasis in various breast cancer models.[113]

Other ligands have been explored intended to increase chronic myeloid leukemia (CML) cell targeting. This is the case for transferrin targeted-PLGA NPs carrying antiBCR/ABL mAb, which were found to reduce liver and kidney accumulation while increasing the bone-targeting ability in a CML mouse model.[174]

Moreover, the usage of cell-based delivery systems has become a disruptive approach in tumor-targeted therapy, which remains to be validated.[175] In an illustrative example, liposomes conjugated to T regulatory cells have elicited an apparent accumulation in the tumor area for up to 2 days.[176] Unfortunately, the value of this strategy needs to be further investigated since no quantitative information regarding the drug accumulation in tumor tissue has been reported.

The incorporation of ligand-type materials that are naturally transported through the BBB has recently been explored to reach brain tumor cells. This is the case in the choline and acetylcholine-analogue MPC (2-methacryloyloxyethyl phosphorylcholine). The versatility of this technology was validated for the entrapment of an array of clinically relevant mAbs (e.g., trastuzumab, rituximab) following slight optimizations.[177–179] For instance, the sole administration of mAb-loaded MPC-NCs in an orthotopic glioma model resulted in a seven- and seven- to tenfold enrichment in brain and cerebrospinal fluid, respectively, and mitigated liver and kidney accumulation as compared to free nimotuzumab.[178] Surprisingly, in another relevant example, surface functionalization with the CXCL13 motif (ligand with affinity for a chemokine receptor overexpressed on B-cell lymphoma) to PLA–PEG–PLA–MPC-modified NCs led to the highest brain accumulation and minor liver accumulation, in contrast to nontargeted NCs and free mAb.[179] Even though the drawn conclusions are based on fluorescence imaging studies, an alternative method to overcome the BBB besides the classical incorporation of targeting ligands to the surface has been proposed.

All the aforementioned studies utilized fluorescence imaging to analyze the biodistribution and accumulation of targeted and nontargeted NPs. While this technique has proven to be a valuable tool for in vivo studies, it comes with certain limitations that must be taken into consideration. These limitations include autofluorescence and the inability to perform an absolute quantification of NP tissue accumulation. Moreover, ensuring the stability of the link between the imaging fluorophore and the NP is of utmost importance to prevent unintended release and, consequently, inaccurate results.[180] While this limitation is a shared aspect with radiolabeled NPs, the metal-chelate affinity is presumably superior. The high sensitivity and absence of limitations related to tissue penetration in nuclear imaging underline its potential to study the biodistribution and pharmacokinetics of organic NPs in vivo.[181,182] These techniques open avenues of application to facilitate the comprehension of tumor efficacy results and establish in vitro–in vivo correlations.

In brief, concerning the targeting of tumor cells, the most advanced approach has relied on the use of TPP. While iRGD and tLyp1 have been the main ligands employed, this strategy has been extended to F3, iNGR or TT1.[165,172,183] The traditional approach of using TPP has been focused on attaching these ligands to the NPs' surface. Nevertheless, the coadministration of TPP has been also investigated with satisfactory outcomes.[184,185] Additionally, the combination of HA and an additional targeting ligand has been evidenced to be advantageous for improving the tumor accumulation of the NP. Lastly, the incorporation of ligand-type or biomimetic materials during the rational design of the NPs has shown great potential in the delivery of cancer therapeutics to the tumor site.

### 3.3.2. Targeting the TME

Upon reaching the tumor site, NPs face the challenge of navigating through the TME, a complex scaffold composed of extracellular matrix, angiogenic vascular cells, lymphatic vasculatures, immune cells such as tumor-associated macrophages (TAMs) and cancer-associated fibroblasts.[151,160,187] This tumor ecosystem plays a pivotal role in supporting the growth and survival of cancer cells.

*Targeting TAMs*: A highly explored strategy involves delivering NPs that specifically target M2-like TAMs, given their key role in tumor immunosuppression and, therefore, in the tumorigenesis process[188] (**Table 2**). Mannose, in this context, has been widely explored as a targeting strategy with promising results.[189–192] In our laboratory, the surface modification of HA NCs with mannose exhibited a notably higher tumor accumulation in an orthotopic fibrosarcoma murine model compared to nonmannosylated NCs. Ex vivo analysis of tumor sections demonstrated that this approach was also correlated with an augmented uptake by M2-like TAMs.[192] Conversely, the incorporation of the M2pep ligand onto the NP surface has also significantly increased their ability to target M2-like TAMs. These NPs not only reached the tumor tissue faster and in a more efficient manner, but they were also capable of efficiently remodeling TAMs' phenotype to a proinflammatory profile (M1 phenotype). This outcome highlights the potential of these modified NPs for immunomodulatory therapy and enhancing the antitumor immune response.[193,194]

Active targeting can be further explored by the inclusion of ligands recognizing various targets, therefore facilitating the TME and cell interaction or tumoral diffusion. However, from our perspective, the accumulation of NPs is indeed a team effort between passive, active, and endogenous targeting mechanisms, with the formed protein corona being a crucial driver of these strategies' success.

Despite the advancements in active targeting for the delivery of biologicals, the field is moving toward engineering materials with tissue specificity. The design and optimization of ionizable lipids have initiated the new era of LNPs, setting a precedent for the emergence of alternative materials such as polymers. The forthcoming challenge lies in extrahepatic delivery as it is essential for cancer therapeutics. In this sense, delivery systems

**Table 2.** Summary of functionalized nanosystem targeting TME.

| Active ligand | Nanocarrier (biological) | Physicochemical properties (size, surface charge, AE) | Adm. route | Tumor model | Outcome | Refs. |
|---|---|---|---|---|---|---|
| Mannose | Polymeric NPs | 183 nm (−) 27 mV | i.v. | Melanoma | Higher tumor accumulation and TAMs colocalization | [#0190] |
| Mannose | PEG–PLGA NPs (DOX) | 152 nm (−) 27 mV 63% | i.v. (5 mg $kg^{-1}$) | Triple-negative mammary (orthotopic) | Reduction of TAMs and tumor growth compared to unmodified NPs | [#0191] |
| Mannose (DS 16%) | HA NCs | 158 nm (−) 39 mV | i.v. | Fibrosarcoma (orthotopic) | Significant higher accumulation and 1.30-fold increase enhanced TAMs uptake versus unmodified | [#0192] |
| M2-pep | Micelles (BVZ and CSF-1R siRNA) | 799 nm (+) 5 mV 82% | i.v. (8 and 0.31 mg $kg^{-1}$, BVZ and siRNA) | Pancreatic tumor | Repolarization of TAMs toward M1 and tumor suppression | [#0195] |
| M2-pep and α-pep | PLGA NPs (Baicalin and Hgp) | 118 nm (+) 18 mV Baicalin, 44% Hgp, 30% | i.v. (500 µg/100 µL of formulation) | Melanoma (subcutaneous) | Remodulation of tumor microenvironment and enhance anti-tumor efficacy | [#0194] |

AE association efficiency; NCs nanocapsules; NPs nanoparticles; tLyP-1 truncated LyP-1; i.v. intravenous; HA hyaluronic acid; BVZ bevacizumab; CSF-1R colony stimulating factor-1 receptor; TAMs tumor associated macrophages; DS degree of substitution; DOX doxorubicin; Hgp tumor-associated antigen (Hgp10025-33); PLGA poly(lactic-*co*-glycolic acid).

containing zwitterionic and cationic sulfonamide amino lipids and SORT molecules, that are quaternary ammonium-based materials, have led to exciting findings in terms of lung targeting. Alternative strategies such as natural killer cells, RBC or T cells have been engineered for the delivery of an array of biologicals, currently in clinical trials.[196] Although these alternative approaches seem promising, further efforts need to be undertaken to evaluate their real potential. Indeed, when overviewing above-delivery strategies it is obvious that the insertion of active motifs onto the NP's surface might not be the most promising approach in terms of clinical translatability. The development of materials that can control or evade protein corona may also offer some exciting opportunities.

## 4. Overcoming Cellular Barriers for Efficient Intracellular Delivery of Biologicals

After accomplishing tissue targeting and assuming the ability to diffuse across the stroma, nanocarriers still need to cross the cell membrane, diffuse within the intracellular space, and release their cargo while preventing its inactivation. While these steps are rather simple for small lipophilic drugs, they pose a significant challenge for biological drugs such as proteins and RNA molecules.

Upon reaching the target cell, the majority of nanocarriers will enter the cell via an endocytic mechanism, thereby facing an additional barrier to overcome, the endosome.[197,198] Attempts to quantitatively asses the amount of biologicals reaching the intracellular compartment have reported that only a modest percentage of ≈1–3% of molecules successfully reaching intracellular domains following intravenous administration.[121,199] However, this minor amount has been found to be adequate for significant target inhibition in some instances.[121,200] Recognizing endosomal escape as bottleneck toward functional intracellular delivery, researchers have explored various approaches (**Figure 8**).[201,202] For a detailed review on endosomal escape of NPs and the role that carrier materials play, readers are referred to recent reviews.[203,204]

Transporting proteins and RNA molecules using nanocarriers represents a paramount challenge in the realm of drug delivery. Notably, proteins, particularly mAbs, have received increasing attention, constituting 21% of the FDA-approvals within the past five years.[205] Despite their clinical benefit, their inability to cross cellular membranes has prevented their full exploitation.[206,207] It is noteworthy that the marketed mAbs are primarily designed for extracellular antigens/receptors or membrane-bound receptors, which pose a relevant limitation considering that the majority of oncoproteins are localized inside the cancer cells.

Conversely, in the context of RNA delivery, further challenges arise. Their inherently polar nature along with their susceptibility to degradation prevent them from reaching intracellular compartments.[208] Given the intricate challenges posed by cellular barriers, the development of strategies facilitating intracellular delivery has become as crucial as the process of target identification itself. In response, nanotechnology has emerged as a promising avenue for the effective intracellular delivery of biologicals.

In this sense, our laboratory has understood the complexity of encapsulating mAbs and RNA and the need to develop customized drug delivery strategies. Thus, the work done in our laboratory involving the encapsulation of complex proteins into PLGA nanospheres,[209,210] PLA–PEG NPs,[211] and chitosan NPs[212] was the basis for the design of novel oncological therapies. This is the case of L-asparaginase-loaded PLGA NPs[213] and PLGA-based technologies for the delivery of interferon-alpha.[214] Building on the success of these strategies, we developed NPs capable of reaching intracellular targets, specifically delivery plasmid DNA in the context of ocular delivery, where successful intracellular delivery could be confirmed.[215,216]

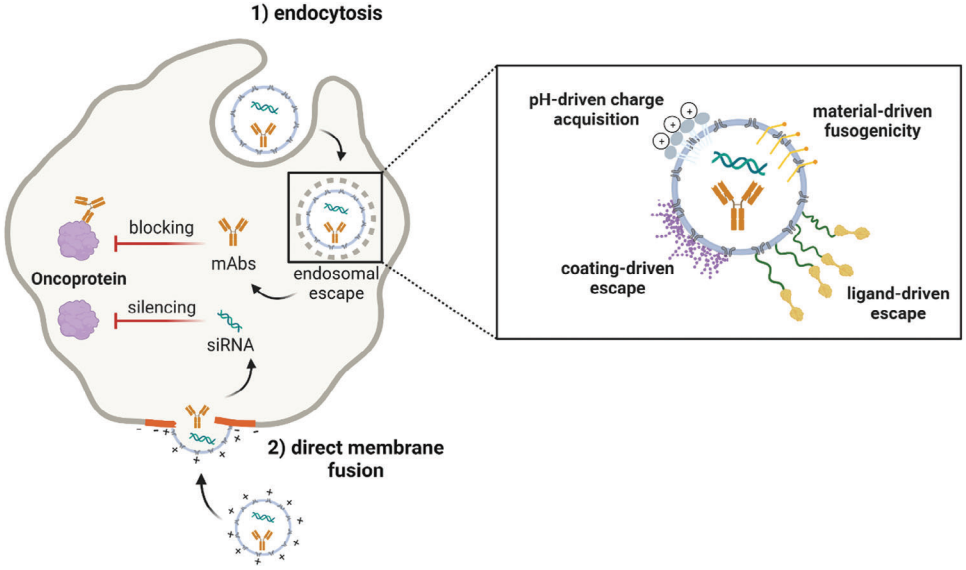


**Figure 8.** Intracellular delivery of biologicals. Successful therapeutic intervention relies on intracellular cargo release, enabled by endosomal escape. Various strategies are being explored aiming at enhancing endosomal escape rates, over charge acquisition in acidifying endosomes, incorporation of fusogenic materials, specific coatings enhancing escape, and ligand-based strategies. Once biologicals reach the cytosol, they act on their oncoprotein target.

Only a limited number of intracellular oncoproteins, considered as undruggable, has been addressed using nanocarriers for the delivery of biological drugs. Among these, RAS, MYC, PLK1, and Signal Transducer and Activation of Transcription 3 factor (STAT3) have emerged as particularly clinically relevant. Recent efforts have resulted in certain candidates advancing to clinical trials. For instance, the MYC inhibitor Oncomyc has reached Phase I//II trials (NCT04808362) for the treatment of advanced solid tumors.[217] On the other hand, $KRAS^{G12V}$ T-cell-based therapies have also entered phase I/II trials (NCT04146298 and NCT03190941), reflecting a growing potential in the inhibition of oncoproteins as a therapeutic option against mutant-driver cancers. This trend has coincided with the FDA-approval of the first $KRAS^{G12C}$ small molecule inhibitors, Lumakras, for NSCLC indication.[218] Krazati also found its way into the market[149] for the treatment of NSCLC. Despite their apparent success in the clinical practice, the efficacy of these small molecules is being questioned.[219]

The scarce number of approved therapies to address intracellular oncoproteins together with the apparent limited success of current therapies in clinical stage has forced the field to develop alternative strategies. Among them, the downregulation of the oncoprotein expression (via siRNA-based therapies) or its direct blockage (via mAbs) have been raised as the most promising alternatives. In subsequent sections, the most relevant delivery systems for siRNA and mAb delivery will be discussed in detail (**Table 4**).

### 4.1. Silencing Intracellular Targets

RNA-based therapies have gained widespread attention, notably through the recent development of COVID-19 vaccines, which may have accelerated the advancement of siRNA-based treatments for cancer therapy. As of 2023, 18 clinical trials focused on the treatment of cancer by siRNA have been reported (**Table 3**).[220,221]

Although several promising clinical trials are ongoing, their success rates have only shown moderate outcomes. For instance, PLK1-targeted stable nucleic acid lipid particles encapsulating siRNA PLK1 (NCT01437007, NCT02191878, and NCT01262235) were investigated in phase I/II. While preliminary tumor inhibition was observed, the results did not warrant further evaluation as single-treatment.[224,225] Trials involving the drug DCR-MYC (NCT02314052 and NCT02110563) were terminated as the clinical trials fell short of expectations. This highlights the significance of preclinical studies investigating alternative translatable delivery technologies to address current needs.

A variety of carrier classes have been explored in these clinical trials including lipid- and polymer-based systems, exosomes, and inorganic gold particles (Table 3). Among the lipid-based particles are liposomes, which are spherical vesicles with an aqueous core girdled by at least one phospholipid bilayer.[237] LNPs are on the other hand have been described to have a more electron-dense core.[24] The mentioned stable nucleic acid lipid particles are considered early-generation LNPs.[238] Exosomes, a subgroup of extracellular vesicles, are naturally derived, phospholipid-based NPs and therefore comparable to liposomes, although they contain various lipids and proteins integrated in or bound to their membrane.[239] For more detail on these and other classes of nanosystems and their morphology the reader is referred to recent reviews.[240,4]

Significant preclinical efforts have been dedicated to the investigation of nanocarrier-based siRNA delivery for the treatment of cancer, addressing various targets. They rely on leveraging RNAi to target a single or multiple targets by coencapsulating distinct siRNA molecules within one nanocarrier, often alongside conventional chemotherapeutic drugs.

**Table 3.** siRNA-based drugs in clinical trials for cancer therapy.

| Target | Drug | Cancer type | Phase/status | NCT number | Carrier | Refs. |
|---|---|---|---|---|---|---|
| KRAS | siG12D LODER | Pancreatic | Phase 1/completed | NCT01188785 | Polymeric matrix | [#0222] |
| KRAS | Mesenchymal stromal cells-derived exosomes with KRAS G12D siRNA | Pancreatic | Phase 1/recruiting | NCT03608631 | Exosomes | [#0223] |
| PLK1 | TKM-080301 | Liver | Phase 1/completed | NCT01437007 | SNALP | [#0224] |
| PLK1 | TKM-080301 | Cancer | Phase 1–2/completed | NCT01262235 | SNALP | [#0225] |
| PKN3 | Atu027 | Advances solid tumors | Phase 1/completed | NCT00938574 | Liposome | [#0226] |
| PKN3 | Atu027 | Pancreatic | Phase 1–2/completed | NCT01808638 | Liposome | [#0227] |
| MYC | DCR-MYC | Hepatocellular carcinoma | Phase 1–2/terminated (Sponsor decision) | NCT02314052 | LNP | [#0228] |
| MYC | DCR-MYC | Solid tumors, multiple myeloma, lymphoma | Phase 1/terminated (sponsor decision) | NCT02110563 | LNP | [#0228] |
| GSTP | NBF-006 | Nonsmall cell lung cancer, pancreatic cancer, colorectal cancer | Phase 1/recruiting | NCT03819387 | LNP | [#0229] |
| RRM2 | CALAA-01 | Cancer, solid tumor | Phase 1/terminated | NCT00689065 | Cyclodextrin NP, Tf targeted | [#0230] |
| EphA2 | EPHARNA | Advanced malignant solid neoplasm | Phase 1/recruiting | NCT01591356 | Liposome | [#0231] |
| TMPRSS6 | SLN124 | Polycythemia vera | Phase 1–2/recruiting | NCT05499013 | GalNAc conjugate | [#0232] |
| Bcl212 | NU-0129 | Gliosarcoma, glioblastoma | Phase 1/completed | NCT03020017 | Gold NP | [#0233] |
| KSP, VEGF-A | ALN-VSP02 | Solid tumors | Phase 1/completed | NCT01158079 | LNP | [#0234] |
| TGF-$\beta$1, COX-2 | STP705 | Squamous cell carcinoma skin cancer | Phase 2/active, not recruiting | NCT04844983 | Polymeric NP | [#0235] |
| TLR9, STAT3 | CAS3/SS3 | Lymphoma | Phase 1/recruiting | NCT04995536 | | [#0236] |

KRAS Kirsten rat sarcoma viral oncogene homolog; MYC myelocytomatosis oncogene cellular homolog; PLK1 polo-like kinase-1; PKN3 protein kinase N3; GSTP glutathione S-transferase P; RRM2 M2 subunit of ribonucleotide reductase; EphA2 Ephrin receptor A2; TMPRSS6 transmembrane serine protease 6; KSP kinesin spindle protein; VEGF-A vascular endothelial growth factor A; TGF-$\beta$1 transforming growth factor-beta1; COX-2 cyclooxygenase-2; TLR9 toll-like receptor 9; STAT3 Signal Transducer and Activation of Transcription 3 factor; SNALP stable nucleic acid lipid particles; LNP lipid nanoparticle; NP nanoparticle; Tf transferrin.

Particularly, the focus has extended to oncogenic KRAS, the most mutated RAS isoform, in pancreatic ductal adenocarcinoma (97.7%), colorectal adenocarcinoma (44.7%), and lung adenocarcinoma (30.9%).[241,242] RNAi has been shown to suppress the growth of KRAS-dependent cancer.[243,244] A combinatorial approach involving siRNA KRAS and the pan-PI3K inhibitor GDC-0941 (GDC) to simultaneously downregulate RAS and PI3K pathways was explored. The authors based their delivery system on thiol-modified polymerized siRNA, complexed by thiol-modified glycol chitosan into NPs. The combination of both GDC and siKRAS-carriers outperformed single-agent therapies and yielded complete attenuation of tumor volume over the study period in an allograft ovarian tumor model.[245] In another approach, miR-34a and siKRAS loaded NPs based on the polymer 7C1 developed by Jacks and colleagues, showing remarkable reduction in tumor volume in a lung cancer model. Enhanced survival, especially when combined with the conventional drug Cisplatin demonstrates the efficacy of small RNA combination.[244] The same combination, siKRAS and miR-34a, was delivered by liposomal layer-by-layer particles, developed by Hammond and colleagues, showing similar enhancement of survival in an NSCLC model in mice.[246]

Drawing upon our laboratories' expertise in the development nanocarriers for the delivery of polynucleotides, such as PEG–PGA, chitosan/HA NPs enveloped nanocomplexes[247] or polyarginine NCs,[248] we expanded our efforts to address oncogenic KRAS. More precisely, we engineered actively targeted LNPs composed of C12-200, DOPE, cholesterol, and Tween 80 as PEGylated lipid, encapsulating chemically modified and stabilized siRNA KRAS for the treatment of pancreatic cancer. This approach, combined with gemcitabine, yielded significant reduction in tumor volume in comparison to free gemcitabine alone in a subcutaneous mouse model.[165]

**Table 4.** Summary of the most relevant mAb- and siRNA-delivery nanocarriers intended to address intracellular targets.

| Target | Nanocarrier (biological) | Physicochemical properties (size, surface charge, AE) | Combination therapy | Adm. route [dose] | Tumor model | Refs. |
|---|---|---|---|---|---|---|
| Gasdermin B protein | PEG–HA–NCs (antigasdermin B mAb) | 130 nm<br>(−) 20 mV<br>>84% | – | i.p.<br>(4 mg $kg^{-1}$ of mAb) | Breast cancer (orthotopic) | [#0255] |
| VEGF | CD44v6 Fab–PEG–PLGA NPs (BVZ) | 150–250 nm<br>(−) 5–10 mV<br>86% | – | – | – | [#0261] |
| | Tween 80/Tween 20/Brij 97 NPs (BVZ) | 240–700 nm<br>(−) 5–26 mV | – | – | – | [#0262] |
| | Liposomes (BVZ) | – | Benzoporphyrin derivative-loaded PLGA–PEG NPs | – | Pancreatic cancer (orthotopic) | [259] |
| | Liposomes (BVZ) | 120 nm<br>(+) 15 mV<br>≈70% | Benzoporphyrin derivative | i.v.<br>(15 mg $kg^{-1}$ of mAb) | Pancreatic cancer | [#0112] |
| | Peptide-based NPs (siVEGF) | 300 nm<br>(+) 8 mV<br>≈100% | miR145-5p | i.v.<br>(10 µmol $kg^{-1}$ siRNA) | Ovarian cancer | [#0263] |
| EGFR | PBAE NPs (siEGFR) | 115 nm<br>(+) 18 mV<br>≈100% | siRobo1, siYAP1, siNKCC1, siSurvivin | i.t.<br>(0.6 µg siRNA /animal) | Glioblastoma (orthotopic) | [#0253] |
| | ERM-coated lipoplexes (siEGFR) | 188 nm<br>(+) 30 mV<br>≈100% | – | Peritumoral<br>(0.5 mg $kg^{-1}$ siRNA) | Breast cancer (orthotopic) | [#0264] |
| Ki-67 protein | Liposomes (antipKi-67 mAb) | 160 nm | – | – | – | [265] |
| hTERT | EM-coated self-assembling NPs (antihTERT mAb) | 197 nm<br>(−) 32 mV | – | i.v. | Lung cancer | [#0266] |
| S100A4 protein | Fusogenic liposomes (antiS100A4 mAb) | <200 nm<br>Not found<br>>90% | Doxorubicin | i.v.<br>(1 mg $kg^{-1}$ of mAb) | Breast cancer (immunocompetent) | [#0116] |
| BCR/ABL protein | Transferrin-targeted-PLGA NPs (antiBCR/ABL mAb) | 297 nm<br>(−)13 mV<br>73% | – | i.v.<br>(2 mg per week of NPs) | Chronic myeloid leukemia | [#0174] |
| MYC protein | PEG-pAsp(DET)-PLL polymeric micelles (antiNPC/anticMYC mAb) | 41 nm<br>(+) 2 mV | – | i.v.<br>(2.5 mg $kg^{-1}$ of mAb) | Colon cancer (immunocompetent) | [#0121] |
| STAT3 | iRGD-targeted PEI/HAS NPs (siSTAT3i) | 220 nm<br>96% | Focused ionized radiation | i.v.<br>(0.33 mg siRNA per animal) | Glioblastoma (immunocompetent) | [#0252] |
| KRAS | Pluronic F127 micelles (antiKRAS mAb) | 24 nm<br>(−) 5 mV<br>≈90% | – | i.v.<br>(300 µg $k^{-1}$ g of mAb) | Colon cancer | [#0257] |
| | tLyP-1-targeted LNP (siKRAS) | 60 nm<br>(+) 3 mV<br>93% | Gemcitabine | i.v.<br>(1.3 mg $kg^{-1}$) | Pancreatic cancer | [#0165] |
| | Poly-siRNA/chitosan NPs (siKRAS) | 232 nm<br>≈100% | GDC-0941 (Pictilisib) | i.v.<br>(1.5 mg $kg^{-1}$) | Ovarian cancer | [#0245] |
| PLK1 | Peptide-modified cyclodextrin/antisense DNA NPs (siPLK1) | 75 nm<br>≈100% | – | i.v.<br>(1.2 mg $kg^{-1}$) | Cervical carcinoma | [#0250] |
| | PBA-modified G5 PAMAM dendrimer NPs (siPLK1) | 187 nm<br>(+) 32 mV<br>≈100% | – | i.v.<br>(1 mg $kg^{-1}$) | Breast cancer | [#0251] |

AE association efficiency; NCs nanocapsules; NPs; BVZ bevacizumab; CPD cell penetrating disulfide; KRAS Kirsten rat sarcoma viral oncogene homolog; PLK1 polo-like kinase-1; EGFR endothelial growth factor receptor; NPC nuclear pore complex; HA hyaluronic acid; hTERT human telomerase reverse transcriptase; EM erythrocyte membrane; VEGF vascular endothelial growth factor; Pluronic F127 polyethylene oxide (PEO)–poly(propylene oxide) (PPO) copolymer; PBA phenylboronic acid; PEI polyethylenimine; PBAE poly(beta-amino ester); ERM endoplasmic reticulum membrane; tLyP-1 truncated LyP-1, i.v. intravenous, i.p.: intraperitoneal, i.t. intratumoral.

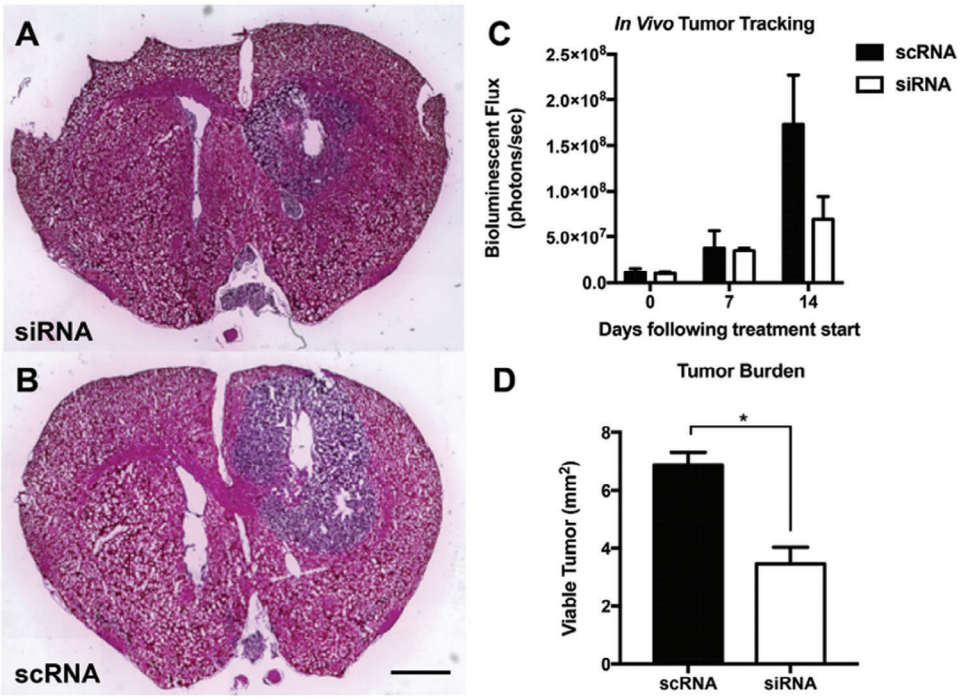


**Figure 9.** Codelivery of five distinct siRNAs via PBAE NPs in an orthotopic GBM model reduced tumor burden. A) Tumor slide post siRNA treatment showing reduced tumor burden. B) Slide of control siRNA treated tumor. C) Reduced growth of luciferase-positive GBM following siRNA-treatment. D) Significant tumor reduction due to siRNA combination treatment. Scale bar = 2 mm. Reproduced with permission.[253] Copyright 2019, Elsevier Ltd.

Another frequently addressed intracellular target is PLK1.[249] For example, siRNA PLK1-loaded nanocarriers made of folate-targeted $\beta$-cyclodextrin and PEGylated PAMAM carriers were shown to accumulate in tumor tissues and effectively silence PLK1, consequently inhibiting the increase in tumor volume in different animal models.[250,251]

For the delivery of Signal Transducer and Activation of Transcription 3 factor (STAT3i) siRNA in glioblastoma therapy, NPs based on polymerized human serum albumin and oligo(ethylene glycol) (OEG), polyethyleneimine (PEI) for RNA condensation and tumor penetrating iRGD peptide for enhanced BBB crossing and tumor penetration were designed. Albumin was selected as primary matrix component due to its interaction with gp60 or SPARC receptors, commonly overexpressed in tumor endothelium and glioma cells. Systemically administered and combined with radiation therapy, the siRNA delivering carriers induced tumor regression in a GBM mouse model.[252]

In another approach, Green and colleagues addressed GBM by delivering siRNA against five genes involved in GBM progression (Robo1, YAP1, NKCC1, EGFR, and surviving) using poly(beta-amino ester)-based NPs. In vivo efficacy evaluation in a human orthotopic GBM1A tumor model in mice resulted in significantly reduced tumor burden due to the siRNA combination treatment,[253] confirming the synergistic effects of silencing multiple targets model (**Figure 9**).

Although monotherapy with siRNA offers promising possibilities, the field gravitates toward combination therapy, either involving RNAi inducing molecules against multiple targets[253] or coadministration of siRNA with anticancer drugs.[165] Combination therapy holds potential for treating cancers in with multidrug resistance occurs, rendering tumors more homogenous and susceptible to conventional anticancer drugs due to down-regulation of resistance-causing genes by siRNA.[254]

As evidenced by these preclinical results, siRNA molecules offer great applicability for the treatment of cancer, considering their potency and specificity for several oncotargets previously considered undruggable. Regarding the design of nanocarriers enabling RNAi, a broad spectrum of materials, from lipid- and polymer-based systems over hybrids thereof, is being explored. Given the gaps in the existing literature including insufficient mechanistic insights into the NPs' intracellular fate and the lack of comparability between studies, clear assignment of definitive superiority to one of these options is prevented. While the variety of novel materials might pose regulatory challenges, it must be acknowledged that their diversity offers significant chances, considering their wide applicability.

### 4.2. Blocking Intracellular Protein Targets

Interestingly, despite the wide array of nanocarriers developed for RNA delivery, only a limited number of platforms have been employed for intracellular delivery of mAbs. Surprisingly, despite the elevated status of mAbs in the realm of protein therapeutics, minor attention has been directed to the intracellular delivery of mAbs. This field has been hampered by the limited accessibility to these kinds of Abs. Under the assumption that mAbs cannot enter the cells, companies have diverted their attention away from them, somehow ignoring the possibilities offered by nanotechnology to overcome this hurdle.

The concept of intracellular mAb delivery via rationally designed nanomedicines was first introduced by our group.[255] Through the engineering of HA-based NCs for the delivery of antigasdermin B mAb, we were able to address the intracellular oncoprotein gasdermin B. Our technology efficiently facilitated endosomal escape and delivered the mAb intracellularly. This led to a mismatched cell migration in vitro, which was correlated with a reduction of tumor growth and diminished lung metastasis in an orthotopic mammary fat pad tumor-bearing mouse model. The success of this technology encouraged us to extend our efforts and develop the proprietary Multifunctional Polymeric NC (MPN) Technology, to successfully engage undruggable targets through mAb delivery. The versatility of this technology has been proven for the encapsulation of small molecules. Notably, recent advanced efficacy studies in animal models have reported successful outcomes in pancreatic and colorectal mutated KRAS-bearing tumor models.[256] Later, other authors addressed RAS mutations by entrapping an antiKRAS mAb in Pluronic F127 micelles. Following administration of this formulation in a subcutaneous colon G13D-mutant KRAS-bearing mouse model, both tumor size reduction and diminished KRAS and ERK protein expression, was observed.[257] A different approach (no mAb) was adopted by Haley et al., which consisted in the administration of a designed ankyrin repeat protein targeting RAS-loaded LNPs. Although intracellular delivery of the protein was confirmed by luminescence signal triggered by the formation of protein–protein complexes in a hepatocellular carcinoma model after the NPs accumulation in liver, the therapeutic response was comparable to that of the free protein in terms of tumor counts.[258]

The mAb that has garnered the most attention with regards to delivery carriers is bevacizumab (BVZ). Although the VEGF target of this mAb is mainly located in the tumor stroma, some authors suggested that targeting the intracellular VEGF pool might yield more robust therapeutic outcomes. This hypothesis underpinned the investigations conducted by Tangutoori et al., who proposed intravenous administration of liposomes carrying the small molecule benzoporphyrin and BVZ. A minor tumor regrowth and extended necrosis was found in a subcutaneous pancreatic ductal adenocarcinoma mouse model.[112] Similar results were observed in an orthotopic murine pancreatic model when BVZ was entrapped into benzoporphyrin derivative-loaded PLGA–PEG NPs.[259] Phototherapy boosted the production of VEGF intracellular levels, a premise that has been exploited for the administration of combination therapy. Nevertheless, similar efficacy was ascribed to both the administration of the free BVZ and after its encapsulation, casting doubt on this assertion.

MAbs related to the metastatic spreading of cancer cells have also been explored exemplified by the antiS100A4 mAb. In this approach, so-called fusogenic PEG-liposomes were engineered to deshell upon encountering the acidic pH of the TME, thereby exposing four arginines intended to maximize cell penetration capacity. Upon administration of liposomes carrying doxorubicin and the antiS100A4 mAb, a synergistic effect was observed in a breast cancer-bearing mouse model. This combination inhibits liver metastasis, which was comparable to the efficacy observed for antiS100A4 mAb-loaded liposomes.[116]

The utilization of antiBCR/ABL mAbs has been proposed as an alternative strategy to the tyrosine kinase inhibitors (TKI), currently facing challenges due to their resistance mechanisms.[260] Consequently, transferrin-targeted PLGA-NPs have been developed for the entrapment of antiBCR/ABL mAbs. Their efficacy was demonstrated in a chronic myeloid leukemia-mouse model in terms of white blood cell levels and bone marrow $CD45^+$ cells, suggesting their capacity to control leukemia progression. When compared to the efficacy of imatinib (first line TKI), only mAb-NPs could degrade the oncoprotein in an imatinib-resistant cell line. Indeed, after recognition, several downstream molecules such as pSTAT5, pERK, and pCRKL were downregulated.[174]

A particularly attractive Ab is anti-MYC mAb, which was encapsulated within pH-responsive PEG-polylysine and PEG-*b*-poly{*N*-[*N*′-(2-amino-ethyl)-2-aminoethyl]aspartamide [PEG-pAsp-(DET)] micelles (**Figure 10**). Notably, the anti-MYC mAb-loaded nanocarrier induced significant tumor reduction as compared to the free mAb in a colon adenocarcinoma mouse model.[121]

In summary, leveraging nanotechnology for intracellular delivery of mAbs certainly represents a novel approach. Among the extensively investigated strategies for mAb delivery, liposomes, micelles, and polymeric NPs (e.g., PLGA) have been the most prominent. They predominantly relied on physical entrapment or the exploitation of ionic interactions to facilitate mAb loading. While these methods have demonstrated significant association efficiencies, reports on their loading capacity have been scarce. Notably, the field has understood the importance of elucidating the underlying mechanism, therefore, establishing correlations between the mAb-loaded NPs ability to engage the intended target[121,174] and the in vivo outcome. Although monotherapy with mAbs has exhibited promising therapeutic opportunities, a notable trend toward their combination with small anticancer drugs driven by the occurrence of resistance mechanisms is observed. Despite this progress, we believe that there are several aspects that need to be addressed to facilitate the in vitro–in vivo correlation and aid their clinical translation. This is the case for the implementation of pharmacokinetic studies, the further exploration of active targeting possibilities and the consideration of alternative combination strategies to maximize mAbs' outcomes. Although further advancements are needed, these nanotechnologies would open novel avenues for the clinical exploitation of mAbs.

## 5. Challenges and Future Perspectives

Nanotechnology has been harnessed to address inherent delivery challenges of biologicals. Successful application however demands the nanosystems' ability to overcome multiple biological barriers encountered to reach the target tissues and, notably, the intracellular compartments. During this intricate journey toward the target site, the first challenge involves modulating the interaction with plasma proteins, while preventing premature degradation of the carrier and drug release. In this context, the pursuit of a "the ideal stealth material" has given rise to a new generation of materials, such as zwitterionic polymers and cell membranes, which appear to diminish the formation of a protein corona. However, it remains to be elucidated whether the quest for the ideal stealth material versus the deliberate attraction of favorable proteins will lead to higher success rates in terms of reaching the target.

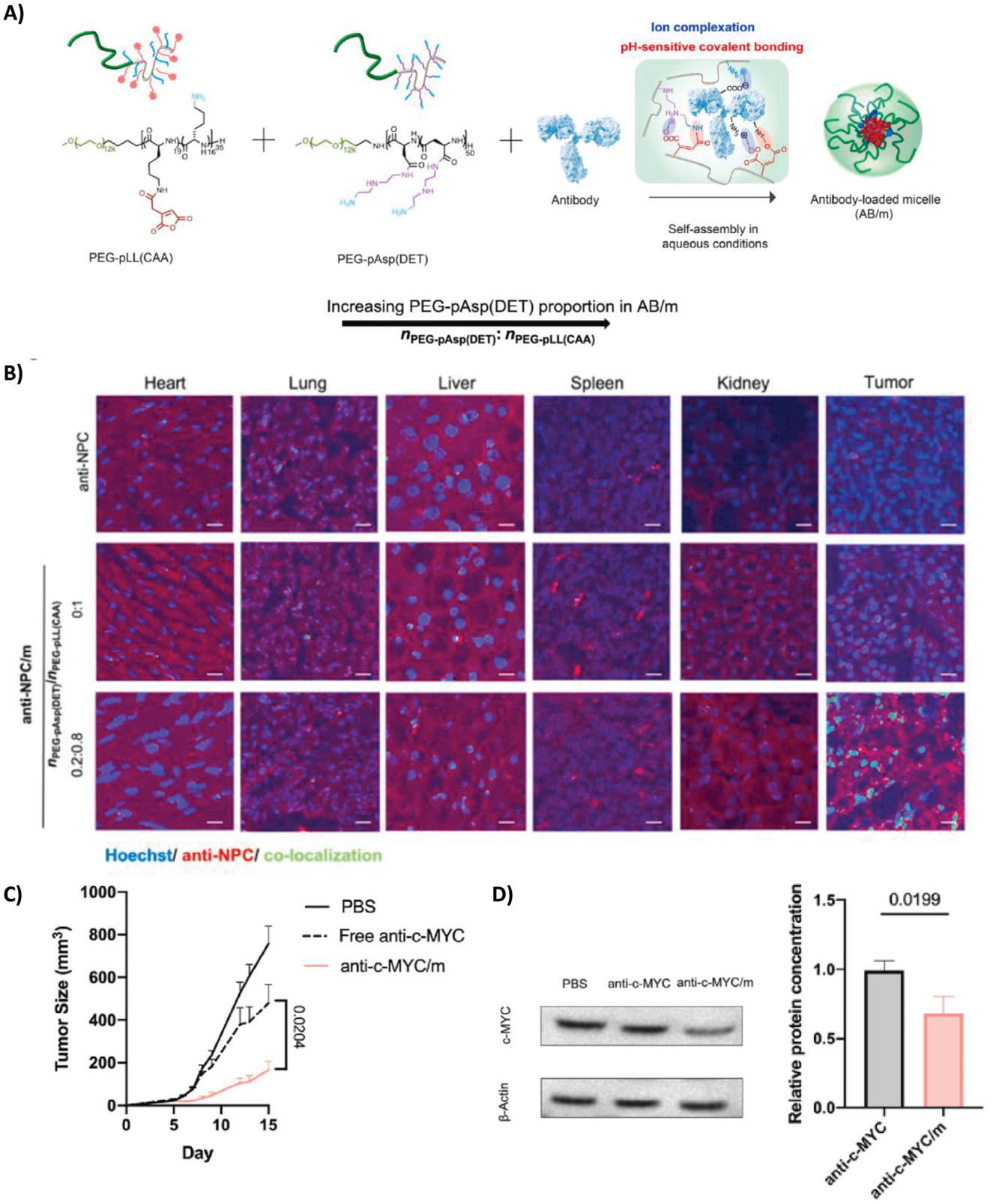


**Figure 10.** Structural organization and in vivo outcomes associated with polymeric micelles designed for the intracellular delivery of mAbs. A) By simple mixing the polymers and the mAb, the entrapment of the mAb trough pH-sensitive amide bonds and electrostatic interactions was successfully achieved. B) After the intravenous administration of anti-nuclear pore complex mAb-loaded NPs, the intracellular location of the mAb was suggested, as exhibited by the tissue sections. C) Anti-MYC mAb-loaded micelles impaired the tumor growth in a colon adenocarcinoma-bearing mice model as a consequence of the D) MYC protein expression reduction. Reproduced with permission.[121] Copyright 2022, Elsevier Ltd.

Presently, a greater body of knowledge exists regarding the avoidance of protein adsorption for enhanced circulation and target accumulation, currently holding more promise for success, while the field of endogenous targeting is still in its infancy. This scenario however might change in the near future, driven by an increased number of studies around deeper analysis of NP–protein corona correlation along with the elucidation of functional roles of corona constituents, which could allow for controlled biodistribution. As highlighted earlier, in vitro–in vivo correlations of marketed strategies such as Doxil[40] have failed, underscoring the necessity to apply modern approaches like machine learning to enhance the predictability of the functional protein corona composition of nanocarriers.[267] While we acknowledge the potential of machine learning and artificial intelligence, it is crucial to avoid overestimating their it, recognizing that these approaches heavily rely on the quality of input data. Enhancements to the current methodology are imperative to ensure the generation of reliable predictions.

Given that the abundance and profile of proteins in the surrounding medium significantly influence the composition of the protein corona, it is imperative to delve into investigating protein corona formation in disease contexts, as alterations in plasma protein profile might affect the outcome of endogenous targeting strategies.

Although the field has provided NPs that efficiently accumulate in the tumor tissue, achieving satisfactory percentages of NPs reaching the tumor remains a challenge. In this regard, an optimized rational design that combines stealth-granting properties resulting in prolonged circulation time seems to be crucial for surpassing the liver barrier and efficiently accumulating into the tumor site. In the process of creating new vehicles for carrying cancer therapeutics, the utilization of design of experiments (DoE) methodology has proven to be exceptionally helpful. The DoE approach facilitates comprehensive exploration of a range of different parameters in a large multidimensional design space and encompasses the principal outcomes and second-order effects, eliminating the necessity for extensive screenings.

The subsequent step involves tissue targeting, which may transpire passively, based on the prolonged circulation of the nanocarrier, or by reaching specific targets in the tumor vasculature or tumor stroma through active targeting. Tumor penetration is an additional critical barrier prior to reaching the target cells, which has thus far been inadequately addressed. Advances in multiplex cellular identification as well as the implementation of nuclear imaging with high spatial resolution are anticipated to deliver critical information about this biological barrier. The challenges linked to replicating tumor complexity and heterogeneity as well as interpatient immune status cannot be overlooked. Consequently, we encourage the field to introduce alternative workflows by systematically assaying the pharmacokinetic profile of a panel of NPs and redirect their application based on their preferential tissue accumulation. Similarly, the implementation of human translatability models would facilitate the generation of technologies with enhanced clinical potential.

The success of active targeting remains a topic of ongoing discussion. The lack of targets exclusively expressed in the desired tissue and specific ligands thereof is currently the foremost hurdle of active targeting. Moreover, a persistent challenge involves the comprehension of ligand valency, a function of both ligand and receptor density, and the correct orientation of the recognition segment. Avoidance of ligand screening by the protein corona is a further impediment that needs to be resolved. Hence, more systematic studies exploring these relations would enhance predictability and rational design of actively targeted NPs. Considering the multitude of biological barriers that need to be overcome for successful intracellular delivery, dual- or multitargeting approaches have proven advantageous, albeit posing specific NP design challenges. In response to these, artificial intelligence presents great potential for the design of novel ligands that tackle multiple biological needs. Indeed, its utility can be expanded by constructing models that allow prediction of the protein corona formed based on the exposed nanomaterial.

Once the nanocarriers reach the target cells, the acquired surface modification until reaching the target location may affect their intracellular trafficking. A common observation in gene delivery is the discrepancy between biodistribution and functional delivery, which applies to other cargos like mAbs. However, there exist only a limited number of hypotheses trying to explain these observations as for example the increasing interest for the resulting NPs' physicochemical properties, e.g., their $pK_a$.[77] Target-specific preferences regarding NP properties granting successful endosomal escape or cell-dependent intracellular trafficking might also contribute to the observed phenomenon. Furthermore, more detailed mechanistic analysis is needed to address the variances in tumor heterogenicity, which inevitably involves differences in receptor expression and therefore, difficulties in predicting intracellular trafficking.

Overall, in this review our aim has been to illuminate the pivotal challenges that necessitate meticulous consideration during the rational design of nanomedicines based on mAbs or RNA molecules. We are now transitioning into the era of RNA delivery, initially boosted by the success of Onpattro and the COVID-19 vaccines, allowing to translate the generated knowledge to cancer treatment. Simultaneously, a new path toward maximization of the usefulness of mAbs is being paved. Although there are still barriers to be overcome, we hold a positive outlook on the prospects of the engineering of mAbs and nanobodies against undruggable targets, ensuring prosperity of this field. In essence, we anticipate the understanding of the biological barriers–NPs interaction to be crucial for the clinical translation of biologicals.

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## Conflict of Interest

M. J. Alonso is founder and shareholder of LiberaBio. The rest of the authors declare no conflict of interest.

## Keywords

cancer, intracellular, monoclonal antibody, nanotechnology, RNA

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