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Recent advances in nano/micro systems for improved circulation stability, enhanced tumor targeting, penetration, and intracellular drug delivery: a review

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Abstract

In recent years, nanoparticles (NPs) have been extensively developed as drug carriers to overcome the limitations of cancer therapeutics. However, there are several biological barriers to nanomedicines, which include the lack of stability in circulation, limited target specificity, low penetration into tumors and insufficient cellular uptake, restricting the active targeting toward tumors of nanomedicines. To address these challenges, a variety of promising strategies were developed recently, as they can be designed to improve NP accumulation and penetration in tumor tissues, circulation stability, tumor targeting, and intracellular uptake. In this Review, we summarized nanomaterials developed in recent three years that could be utilized to improve drug delivery for cancer treatments.

Introduction

Cancer is a major contributor to mortality and represents a significant global health challenge. Recent data has indicated that over 1.9 million new cancer cases were diagnosed in the United States, resulting in approximately 609,000 reported deaths in 2023 [1]. Nanotechnology holds great promise in enhancing cancer treatment outcomes and improving diagnosis. The unique characteristics of nanoparticles (NPs) could overcome the limitations of conventional therapeutics. NPs have shown potential to enhance solubility and stability of loaded cargos, prolong circulation time, and increase drug safety [2–5]. Due to high loading capacity, NPs can reduce dosing frequency and improve patient compliance [6]. Based on these features, a great number of NPs were engineered for cancer research, generating positive results in animal models [7–10]. Some nanoscale formulations exhibiting promising outcomes in clinical trials have been approved for clinical cancer treatments. For example, liposomal doxorubicin (Doxil) showed improved pharmacokinetic properties and reduced cardiotoxicity compared to free drug in lung cancer [11, 12]. Moreover, albumin-bound formulation of paclitaxel (Abraxane) has been shown to be superior over an equivalent dose of free drug with significantly reduced toxicity for pancreatic cancer treatment in a large randomized phase III trial (ClinicalTrials.gov Identifier: NCT00844649) [13, 14]. To accelerate the clinical translation of nano-based technologies, the National Nanotechnology Initiative (NNI) was launched in 2000 [15]. These well-defined initiatives motivated investigators to study nanotechnology and create significant advancement in the last two decades. Despite extensive research of innovative NPs, the number of nano-formulations approved by Food and Drug Administration (FDA) is dramatically below projections [16, 17]. This gap comes from the inability of early NPs to overcome biological barriers as shown in figure 1.

NPs encounter both biological and physical barriers in circulation. For example, the formation of a plasma protein corona on the surface of NPs can have a significant impact on how these NPs interact with biological systems [18, 19]. Several factors contribute to NP stability in circulation including size, surface properties and compositions[20–24]. To minimize clearance, many NPs can be functionalized with polyethylene glycol (PEG) as a stealth coating [25]. PEGylation improves the half-life of NPs in blood by altering their solubility and shielding their surface charge, which helps NPs escape from the recognition by mononuclear phagocytic system (MPS) cells [26, 27].

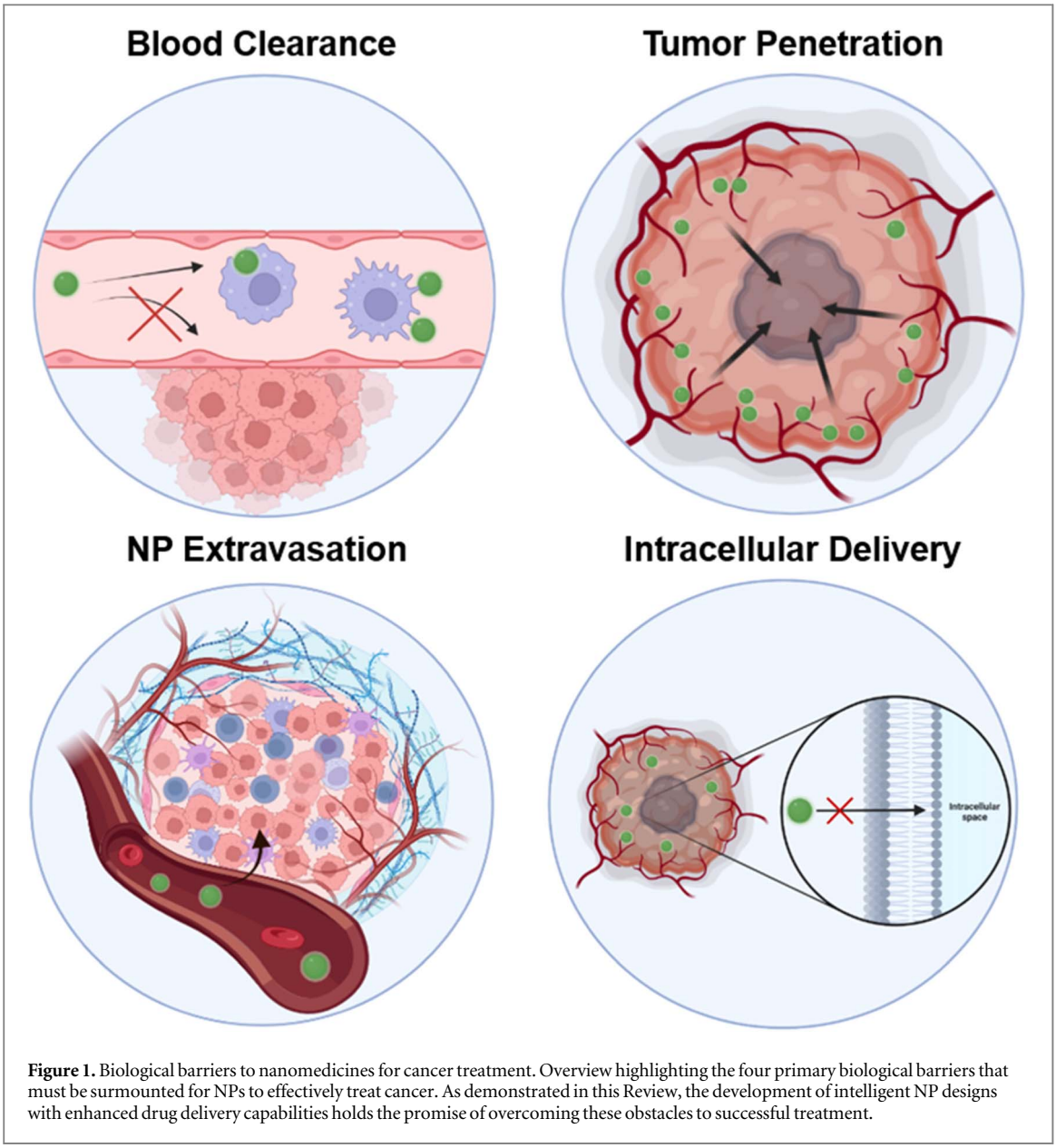

Figure 1. Biological barriers to nanomedicines for cancer treatment. Overview highlighting the four primary biological barriers that must be surmounted for NPs to effectively treat cancer. As demonstrated in this Review, the development of intelligent NP designs with enhanced drug delivery capabilities holds the promise of overcoming these obstacles to successful treatment.

However, this strategy does not completely avoid blood clearance resulted from MPS. Additionally, systemic exposure to PEG induces the production of anti-PEG antibodies, resulting in rapid clearance of PEGylated NPs [28, 29]. Overall, due to these immune responses to the surface properties and architectures of PEGylated NPs, novel NP design is required to overcome these delivery barriers and escape immune recognition.

The tumor microenvironment play a critical role in determining the fate of nanomedicines [30]. The leaky vasculatures within tumors enable the extravasation of NPs, facilitating their accumulation in tumors. This phenomenon is referred to as the enhanced permeability and retention (EPR) effect [31–33]. NP accumulation in tumors based on EPR effect has been highly debated [34, 35]. For example, a clinical report revealed that less than 3.5% of injected NPs accumulated into tumors in patients with head and neck, breast, and lung cancers, and two out of 17 patients were not shown to have experienced the EPR effect at all [36]. This conclusion could be supported by a meta-analysis study reviewing 232 data sets. The study found that the median of NPs accumulated in tumors was only 0.7% of the total intravenously injected dose, de-emphasizing the importance of the EPR effect [37]. Once NPs reach tumor tissues, their penetration ability is essential for further prolonging the retention of NPs and enhancing NP accumulation. Poor NP penetration into tumors leads to low accumulation and further reduces therapeutic efficacy [38–41]. Cells often overexpress extracellular matrix (ECM) within tumor microenvironment, generating a dense ECM that physically prevents penetration of NPs [42–45]. Moreover, the absence of lymphatic vasculature in tumors leads to decreased interstitial fluid drainage that increases intertumoral interstitial pressure and reduces NPs permeability [46–48]. In short, low NPs

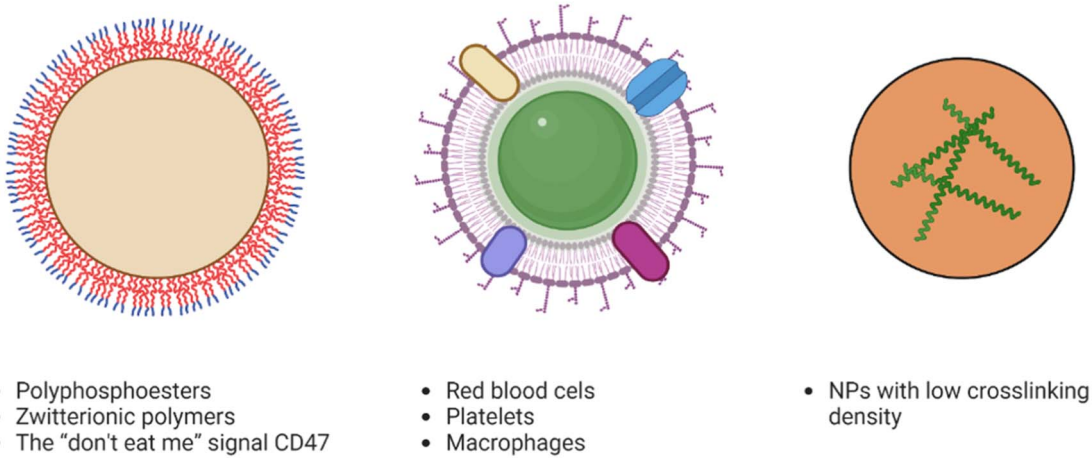

Figure 2. Various strategies have been devised to enhance the *in vivo* stability of NPs. These include surface modification of bioinert materials, cell membrane coating, and utilization of soft NPs, all of which are intended to mitigate recognition by immune system and to extend the blood half-life of NPs.

extravasation, thick ECM, elevated interstitial pressure are barriers to achieve higher accumulation and penetration of NPs in tumors. Therefore, it is challenging to improve the delivery efficiency of NPs due to the properties of tumor microenvironment.

In addition to the general issues described above, NPs must overcome cellular and intracellular barriers because the interaction of therapeutic agents with cellular components is critical for most anti-cancer treatments [49, 50]. Because only small and uncharged molecules can cross cell membrane via passive diffusion, most NPs rely on active transport such as clathrin-mediated endocytosis to cross the cell membrane [51, 52]. Thus, surface modification of a targeting ligand has become a common strategy to facilitate NP uptake. During endocytic processes, the stability of NPs and their cargo may be affected by endosomal environment, which features proteolytic enzymes, low pH and high ionic strength [53–55]. As such, pH responsive materials that can induce a proton sponge effect have been studied to trigger endosomal escape, and to prevent NP degradation [56–59]. However, the NPs or cargos may still need to cross additional intracellular membranes to reach certain compartments. For example, for successful genome editing, therapeutic DNA must cross the nuclear membrane [60, 61]. To summarize, diverse barriers created from cellular uptake to internal trafficking are obstacles to NP delivery, while intelligent NP designs may help overcome these challenges.

Recently, second-generation nanomedicines, such as stimuli-responsive NPs, targeted NPs and cell-based nano-systems, have been designed with new functions, such as the incorporation of cell-derived components, the combination of multiple therapies or therapeutic agents, active targeting and stimuli-responsive drug release [62–69]. Many of them are in preclinical studies to further investigate their capability of improving therapeutic outcomes of cancer therapy. The modifiable features of NP based platforms, including surface properties [70, 71], physicochemical characteristics [72, 73], controllable drug release [74, 75], aid these newly developed nanomaterials to overcome systemic, microenvironmental, and cellular barriers [76–78]. This review focused on recent advances in nanomedicines that aimed to overcome obstacles derived from numerous biological barriers and further improve therapeutic responses. These emerging strategies have explored new opportunities for clinical translation of NP-based therapies.

NPs to improve circulation stability

In circulation, factors including phagocytic cells, blood flow and coronas can reduce NP stability [18, 79–82]. Thus, effective drug delivery and desired biodistribution of NPs are difficult to achieve. This section will discuss the nanomaterials engineered to prevent recognition by macrophages or other immune cells in order to overcome the abovementioned challenges (figure 2).

Surface functionalization of NPs

In the last decades, PEG has been widely recognized as the gold standard in stealth coating for small molecules, protein-based therapeutics and nanocarriers [83–87]. However, several of its limitations, such as the accelerated clearance induced by anti-PEG antibodies and the reduced biodegradability, have been reported [88–90]. To address these issues, PEG-free alterations including polyphosphoesters (PPEs) and zwitterionic polymers have been actively studied.

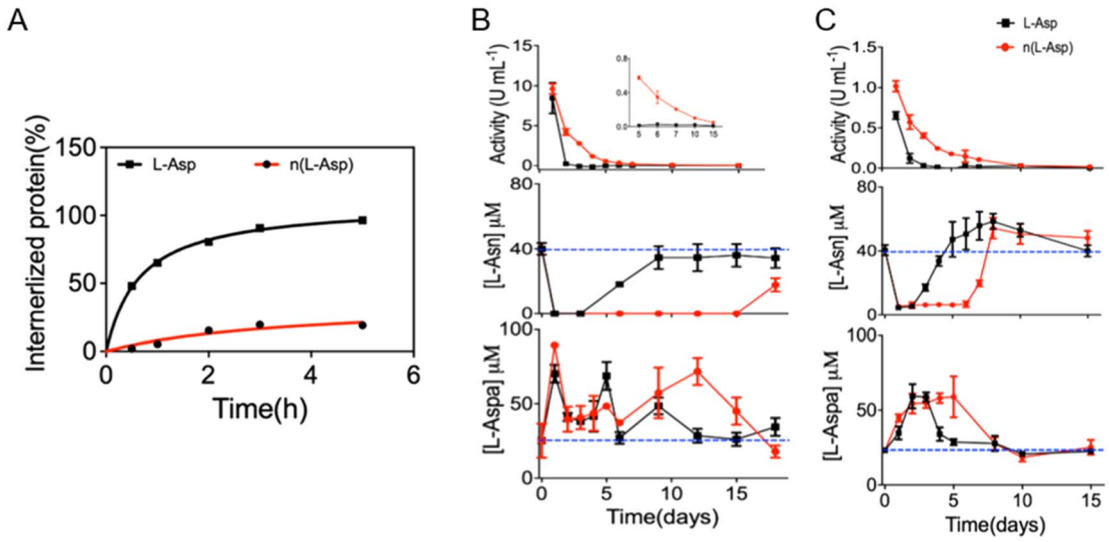

Figure 3. (A) The uptake of native L-Asp and n(L-Asp) by macrophages at various time intervals by using fluorescence-activated cell sorting (FACS) with a concentration of 0.1 mg ml^{-1}. Pharmacokinetic profiles of L-Asp and n(L-Asp) were evaluated after individual intravenous injections in mice at doses of either (B) 1000 or (C) 100 U/kg. Enzymatic activity, L-Asn concentrations, and L-Aspa concentrations were monitored over specific time intervals. The blue dashed lines represent the baseline levels of L-Asn (~40 μM) and L-Aspa (~25 μM) in untreated mice. The data presented are the mean ± standard deviation (SD) from three independent experiments [98] Copyright 2022, American Chemical Society.

PPEs are a type of polymers consisting of repeating phosphoester structures. They have been showing great promise because of their biodegradability, high chemical modifiability and stealth-inducing ability [91, 92]. To illustrate the efficacy of this material, Wang *et al* designed a series of NPs composed of a poly (ε-caprolactone) (PCL) core and a PPEylated shell with a well-controlled surface hydrophilicity [93]. PPEylated NPs were stable in PBS-10% FBS for 2 days and had half-life of 4 h in blood circulation in MDA-MB-231 breast tumor-bearing BALB/c nude mice. Notably, they demonstrated that NPs with high surface hydrophilicity had longer half-life *in vivo*, lower protein binding, and higher accumulation in tumors compared to their more hydrophobic counterparts.

Zwitterionic polymers have demonstrated promising characteristics to improve NP stability such as robust hydration and resistance to fouling [94]. There was evidence that the coating of zwitterionic polymers for NPs could prevent nonspecific protein binding of NPs, protect NPs from fast clearance by the MPS, and prolong the blood circulation time [95–97]. For example, a study developed a zwitterionic nanocapsule by *in situ* polymerization of 2-methacryloyloxyethyl phosphorylcholine (MPC) on the surface of asparaginase, forming a zwitterion-coated enzyme nanocapsule [98]. *In vitro* experiments showed that all the macrophage internalized the native asparaginase after 5 h. On the other hand, only approximately 20% of macrophage internalized zwitterionic nanocapsules after coincubation for 5 h under the same conditions, showing the ability of zwitterionic nanocapsule to escape from macrophage uptake (figure 3(A)). Moreover, they demonstrated that native enzyme was rapidly eliminated from the mice with the evidence that its enzymatic activity became undetectable at 24 h after intravenous administration. In contrast, zwitterionic nanocapsule enabled the enzyme to circulate in the blood for at least 15 days (figures 3(B) and (C)).

Compared with PEG, the peptide alternatives are non-toxic, biodegradable and non-immunogenic [99]. The most common studied strategy is the use of CD47-derived molecules [100]. The CD47 molecule is referred as a widely expressed cellular surface receptor that can activate the transduction of the 'don't-eat-me' signal [101]. By taking advantage of this anti-phagocytic signal, NP stability could be improved by surface functionalization of CD47 or its biomimicry peptides. However, CD47 is a protein with a molecule weigh of 70 kDa, which is difficult to be conjugated to the surface of NPs. As a result, CD47 mimicry peptides have been developed. They are one type of self-peptides and have been used as an appropriate alternative to CD47 molecules [100]. Gheibihayat *et al* prepared doxorubicin loading liposomes with surface modification of self peptide (SP-LD) and studied their blood half-life compared to PEG-functionalized LD (PLD) [102]. The circulation time of SP-LD was 1.5 folds higher than the PEGylated formulation. Given that anti-phagocytic effects of SP, reduced NP accumulation in the spleen, liver, heart and kidney tissues was observed.

In summary, both PPEs and zwitterionic formulations can significantly prolong blood half-life of loaded cargos in NPs through their significantly higher hydrophilicity compared with PEG formulations [91, 92, 103]. Although stealth strategies aim to improve NP stability, they may also reduce cellular uptake. The addition of a shielding layer on NP surface

drastically reduce cell–NP interactions, thereby decreasing cellular uptake. For example, in vitro data showed that cellular uptake of Au NPs reduced at least 50% in PEGylated NPs compared to non-PEGylated ones in human umbilical vein endothelial cells (HUVECs), murine C17.2 neural progenitor cells, and rat PC12 pheochromocytoma cells [104]. Thus, the balance between NP stability and effective intracellular delivery, needs to be considered.

Cell membrane coating

Cell membrane-coated NPs are a type of bio-inspired materials composed of a synthetic core camouflaged by a natural cell membrane [105]. The source of cell membrane could be red blood cells [33], leukocytes [81], stem cells [106] and bacteria [32]. The various receptors or ligands presenting on the membrane can interact with other cells *in vivo*, enabling cellular recognition. Therefore, cell membrane-camouflaged NPs exhibit enhanced ability to escape the recognition of the immune system, which may prolong blood circulation time and improve targeting abilities of cell membrane-coated NPs compared with conventional NP-based systems [107]. Among the various types of cell membranes, red blood cell membranes (RBCMs) are the most abundant and well-studied for NP delivery. Their unique membrane molecules such as CD47 could help NPs escape MPS in circulation and extend the half-life of NPs to as long as 120 days in the human body [108, 109]. For example, one study engineered a RBCM-coated nanoformulation (FRCS NPs) for targeted delivery of paclitaxel (PTX) for the treatment of epithelial malignancies [110]. Their membrane-cloaked NPs remarkably accumulated within the tumors for at least 24 h in a murine model. Additionally, the fluorescence signals of FRCS NPs were observed in the lungs, while being nearly unmeasurable in other organs, such as the liver, indicating that the coating of RBCM contributed to the escape from the hepatic RES system, prolonging the NP circulating time. Platelets are another extensively studied cell type in designing biomimetic NPs because of their physiological functions, hemostasis and immune escape ability [111]. Pei *et al* developed platelet membrane-cloaked nanoparticles (PM-NPs) to improve photothermal therapy (PTT) treatment of breast cancer [112]. In this study, they found that the half-life of the PM-NPs was 30.8 h, which was 2.4-fold longer than the NPs without platelets membrane coating. In addition, approximately 75% of the uncoated NPs were cleared from the blood at 72 h, while 43% of the PM-NPs remained in the blood, demonstrating the long-circulation properties of the PM-NPs.

However, this approach encounters various challenges. Among them, batch-to-batch inconsistencies and the intricate tasks in scalable mass production are crucial. In this context, meticulous quality control measures are imperative to ensure the reliability of membrane-cloaked NPs. Moreover, in the preparation of NPs coated with cell membranes, meticulous attention is warranted. Specifically, stringent measures must be taken to prevent the introduction of viral or pyrogenic contaminants into the cell membranes. Furthermore, the proactive removal of denatured proteins is essential to preempt any inadvertent immune reactions after administration.

NP elasticity

The influence of NP properties including size, shape, surface charge, and hydrophilicity/hydrophobicity on *in vivo* stability have been well investigated [113, 114]. However, the role of NP elasticity in biological responses has recently gained attention due to its key role in circulation time, MPS evasion, renal clearance, and cellular uptake [115]. In general, softer NPs possess a longer circulation time compared with stiffer NPs [116]. Tao *et al* constructed hyaluronic acid modified mesoporous organo-silica nanoparticles (MONs-HA) with a wide range of elasticity and studied the influence of their elasticity on blood circulation *in vivo* [117] (table 1). The terminal phase half-life of elastic MONs-HA was 51.39 h, which was 2.50 folds higher compared with rigid NPs, demonstrating prolonged circulation time of MONs-HA with a lower Young's modulus. Another study showed that nanogels (NGs) with a Young's modulus of 37 kPa could evade MPS better compared to NGs with 93 kPa modulus [118] (table 1). *In vitro* results showed that rigid NGs had a significantly higher cellular uptake by macrophages compared with soft NGs. A longer circulation time was also observed in mice injected with soft NGs. By using SDS-PAGE/phosphor imager analysis, the authors found that soft NGs could pass through membrane with labeled molecular weight (MW) 5-fold lower than the MW of their own due to their deformable nature. Thus, it was hypothesized soft NGs were able to squeeze through the glomerular filtration, reducing toxicity. As discussed above, cell membrane-coated NGs have drawn significant attention in recent years. The impact of NP elasticity on the membrane-coating process has been explored recently by Zou *et al* In their work, two mesenchymal stem cell membrane-coated silica NPs (MCSNs) with similar sizes but distinctly different modulus values (44 MPa and 2.3 GPa) were synthesized to study their bio–nano interactions [119] (table 1). They studied the immune evasion effects by measuring cellular uptake of fluorescent labeled MCSNs in RAW264.7 macrophages through flow cytometry. The soft MCSNs showed the lowest uptake, which was approximately 10 times lower compared with the hard MCSNs, indicating that the combination of the soft NPs with MSC membrane coating could minimize macrophage uptake. Notably, they found that the soft MCSNs had a higher cancer cell uptake than hard ones due to the high density of

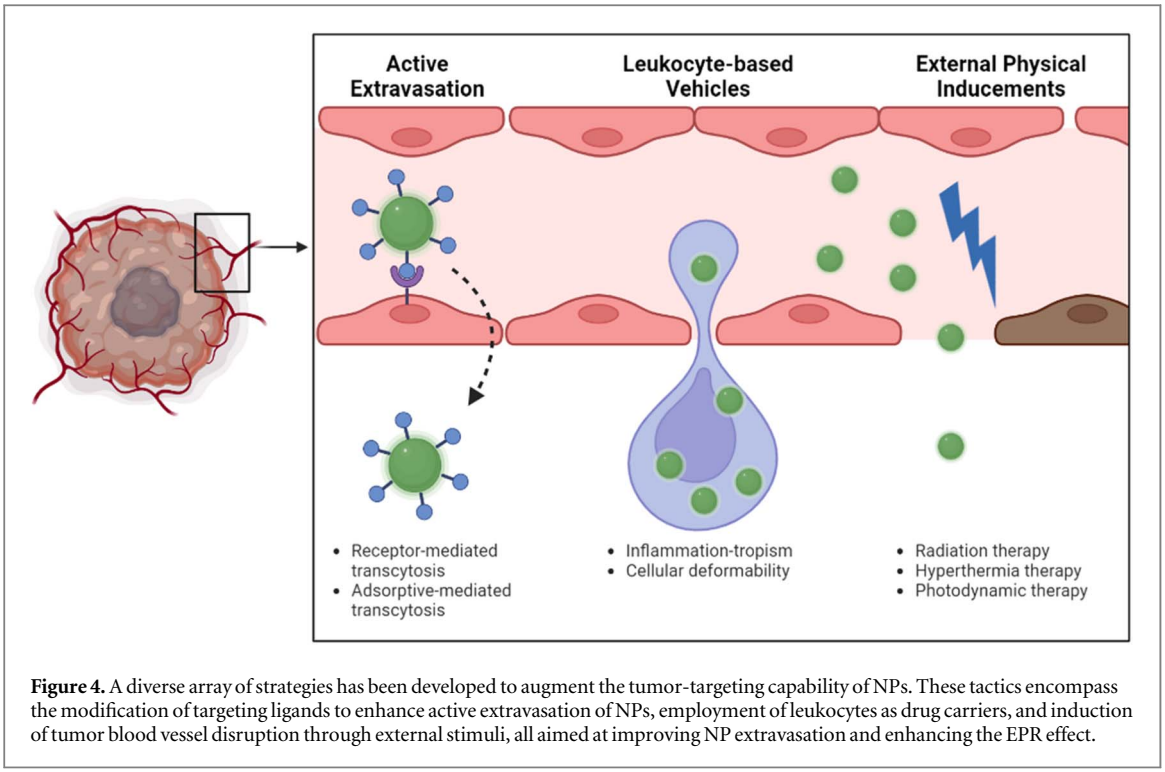

Figure 4. A diverse array of strategies has been developed to augment the tumor-targeting capability of NPs. These tactics encompass the modification of targeting ligands to enhance active extravasation of NPs, employment of leukocytes as drug carriers, and induction of tumor blood vessel disruption through external stimuli, all aimed at improving NP extravasation and enhancing the EPR effect.

CXCR4 and CD90 receptors, which contributed to tumor targeting, on the soft MCSN surface. In short, the combination of elastic NPs and membrane coating could not only enhance blood circulation time, but also facilitate the uptake of NPs by cancer cell.

Despite recent advancements in the engineering of NP elasticity for improved drug delivery, there are still significant discrepancies in the results reported by various studies. For example, a study compiled diverse *in vivo* datasets, revealing a non-monotonic correlation that delineates into three discernible zones based on nanoparticle (NP) elasticity. To be precise, these three distinct zones are classified as Region I, characterized by NP elasticity <15 kPa; Region II, marked by NP elasticity ranging from 15 to 75 kPa; and Region III, encompassing NP elasticity exceeding 75 kPa. While, within each region, a lower NP elasticity aligns with prolonged blood clearance durations, as corroborated by prior research, it's noteworthy that across these regions, particles in Region II consistently demonstrated the shortest clearance half-lives (<8 h) [120]. This variability has been attributed to inconsistencies in the physicochemical parameters of NPs, including size, surface charge, shape, and the significant differences in defining the magnitude of elasticity. To address these issues, future studies should employ drug delivery systems with a broad range of elastic moduli values spanning from Pa to GPa (10^9 Pa), while maintaining similar physicochemical properties of NPs. By adopting this approach, it may be possible to acquire comparable results across multiple studies, leading to more consistent findings in this area of research.

NPs to improve tumor targeting capacity

The first step for NPs in circulation to reach tumor tissues is extravasation, which can be altered by the properties of NPs [121]. For example, small NPs tend to cross capillary barriers more easily than large NPs. As a result, NPs generally exhibit size-dependent distribution across organs. Non-specific distribution of NPs presents a challenge for therapeutic applications [122, 123]. As shown in figure 4, several strategies have been developed to improve tumor targeting ability of NPs.

Active extravasation of NPs through transcytosis

Although the EPR effect has been validated in preclinical studies, accumulating evidence demonstrating the high heterogenicity of EPR effect and limited presentation in clinical solid tumors, resulting in inefficient extravasation of nanomedicines, has been found [124–127]. Unlike the EPR effect accumulating NP in tumors in a passive manner, transcellular transcytosis is an active process that various macromolecules are transported across the cellular barriers [128]. Transcytosis is an intrinsic cellular process and is less affected by cell-to-cell variation [129, 130]. Currently, one study demonstrated that up to 97% of NPs enter tumors using active processes through endothelial cells based on the analysis from multiple types of mouse models recapitulating human tumors, mathematical simulation and imaging techniques, casting doubts on the significance of the EPR effect [131]. As an alternative, the transcytosis pathway may be utilized as an effective delivery strategy to overcome the limitations of EPR effect.

Table 1. Summary of elastic NPs for prolonged blood half-life.

Nano-systems	Results	References
MONs-HA	MONs-HA with 0.29 GPa modulus has a 2.5-fold higher blood half-life compared to rigid counterpart (1.64 Gpa)	[117]
NGs	NGs with a Young's modulus of 37 kPa could evade MPS better compared to NGs with 93 kPa modulus	[118]
MCSNs	10-fold lower macrophages uptake was observed in MCSNs with 44 MPa modulus compared to MCSNs with 2.3 GPa	[119]

Overexpression of a variety of surface receptors is crucial for survival and proliferation of tumor cells [132]. Thus, functionalizing the surface of NPs with targeting ligands can actively transport NPs in tumors through receptor-mediated transcytosis (RMT) and reduce off-target effects (table 2) [133–141]. Among the various surface receptors identified so far, integrins are one of the most widely used receptors for triggering RMT to target solid tumors [142]. Integrins play a key role in regulating metabolic processes, cell growth, proliferation and metastasis [143]. The overexpression of $\alpha v\beta 3$ integrin receptor has been identified in various types of cancer including colon, melanoma, prostate, breast, glioblastoma, lung and ovarian cancers [144]. The tumor penetrating peptides (TPPs) are well-established ligands for integrin $\alpha v\beta 3$, but several challenges such as instability and low selectivity have limited their use [145]. Recently, several strategies such as N-methylation, cyclization, the incorporation of d-amino acids and the masking of charged residues have been developed to enhance the stability and selectivity of TPP by preventing enzymatic degradation and their recognition by the immune system [146–148]. Corti *et al* replaced glycine with N-methylglycine (sarcosine) in a head-to-tail cyclized peptide (c [CGNGRG]) to prevent asparagine deamidation *in vivo* [149]. They demonstrated that glycine N-methylation in NGR peptide, denoted as MeN1, could prevent asparagine deamidation, and improve peptide stability. They also studied the potential of MeN1 to deliver nanocarriers such as TNF-bearing nanogold and liposomal doxorubicin to tumors. *In vivo* studies using WEHI 164 fibrosarcoma bearing mice showed a significant reduction of tumor volume (50% reduction) at 48–72 h after administration of MeN1-modified TNF-bearing nanogold, as measured by contrast-enhanced ultrasound (CEUS) imaging technique. Meanwhile, treatment with MeN1-modified liposomal doxorubicin significantly prolonged the survival time of mice compared to liposomal doxorubicin tagged with a negative control peptide (ARA-Lipo[doxo]), There was no evidence of increased toxicity of the MeN1-moified liposomal doxorubicin as well, as demonstrated by monitoring of body weight.

In addition to RMT, adsorptive-mediated transcytosis (AMT) has been extensively explored for the delivery of nanocarriers to solid tumors [150]. In spite of some limitations like non-specific uptake, AMT has shown a high transcytosis efficiency because of the low binding affinity required [53]. AMT can be induced by cationic nanocarriers which also possess improved tumor extravasation ability compared with neutral or anionic NPs [53]. However, cationic NPs can induce opsonization and further trigger rapid MPS clearance [151, 152]. Thus, the ideal NP must be neutral or slightly anionic in blood circulation, while the cationization of NP only occurs at the luminal tumor endothelial cell surface to specifically induce AMT for active tumor extravasation. For instance, Wang *et al* developed a dendrimer–camptothecin (CPT) conjugate that was actively transported into pancreatic ductal adenocarcinoma (PDA) through γ-glutamyl transpeptidase (GGT)- triggered AMT [153]. The design principle of this enzyme-responsive system was that the overexpressed GGT on the vascular endothelial cell or tumor cell induces the γ-glutamyl transfer reactions of glutathione to generate primary amines on NP surface. The positively charged dendrimer would bind to the negatively charged endothelial membrane, inducing caveolae-mediated endocytosis followed by AMT. This enzyme-responsive process occurred when it was delivered to the PDA tumor periphery, preventing rapid MPS clearance in the circulation. This study suggested that NPs utilizing cationization-initiated endocytosis and AMT had a great potential for cancer targeting.

The density of charge also assumes a pivotal role within the processes of AMT. In this regard, Chen and colleagues conducted an exploration into how the charge of a cationic polymer influences AMT, utilizing *in vitro* multi-layered tumor spheroids (MTSs) [154]. The cationic polymer, namely polyethylenimine (PEI), underwent amidization with acetic anhydride, resulting in acetylated PEIs (AcPEIs) with varying cationic charge densities. Notably, due to its high charge density, PEI adheres strongly to the cell membrane but lacks efficiency in triggering endocytosis. Conversely, AcPEI with an 87% acetylation rate displays no interaction with tumor cells. Strikingly, PEI with 24% acetylation emerges as possessing the most efficient transcytosis rate, attributed to its well-balanced cell-binding affinity, which expedites AMT.

Despite the progress made in understanding the transcytosis process, the knowledge of its basic cell biology remains limited. Several questions still need to be answered such as the mechanisms responsible for the selective transcytosis of nanomedicines from tumor blood vessels compared to healthy tissue.

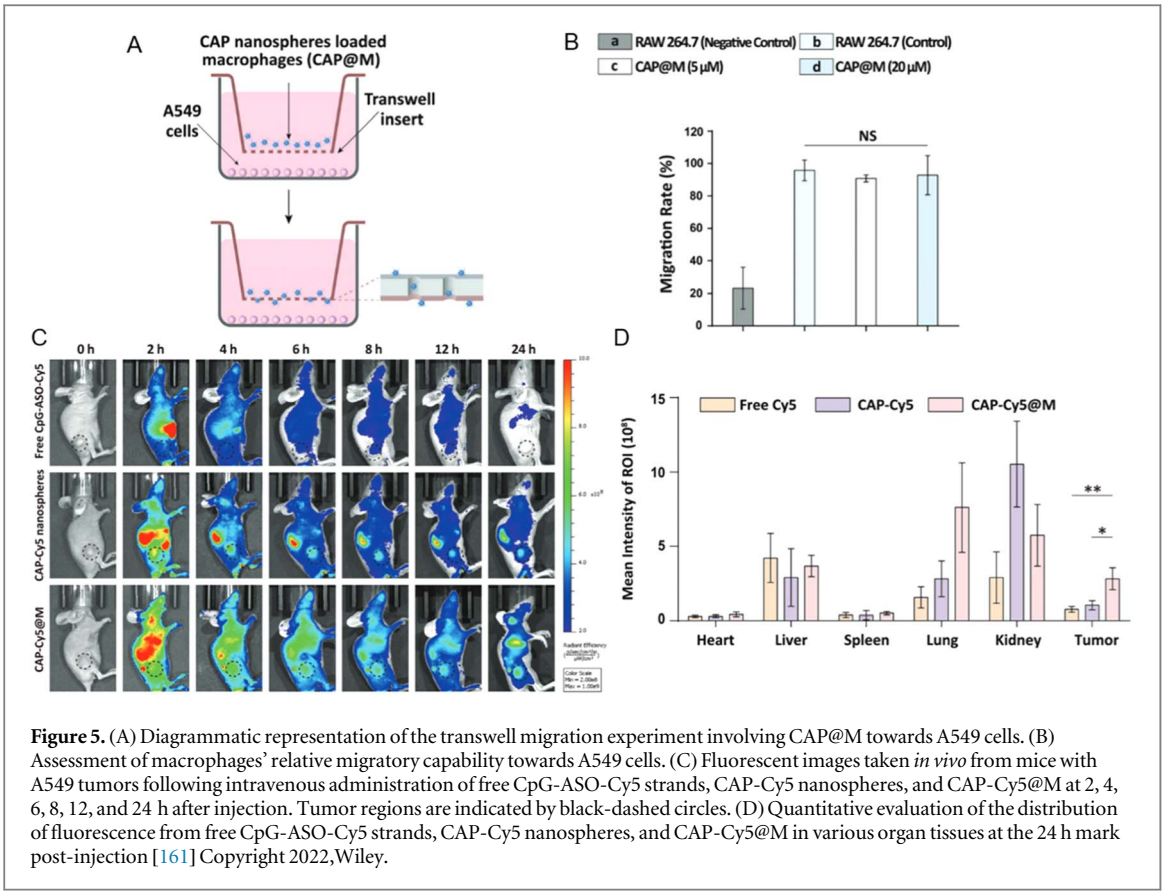

Figure 5. (A) Diagrammatic representation of the transwell migration experiment involving CAP@M towards A549 cells. (B) Assessment of macrophages' relative migratory capability towards A549 cells. (C) Fluorescent images taken *in vivo* from mice with A549 tumors following intravenous administration of free CpG-ASO-Cy5 strands, CAP-Cy5 nanospheres, and CAP-Cy5@M at 2, 4, 6, 8, 12, and 24 h after injection. Tumor regions are indicated by black-dashed circles. (D) Quantitative evaluation of the distribution of fluorescence from free CpG-ASO-Cy5 strands, CAP-Cy5 nanospheres, and CAP-Cy5@M in various organ tissues at the 24 h mark post-injection [161] Copyright 2022,Wiley.

Addressing these issues is crucial for a better understanding of transcytosis and the development of highly effective and clinically translational nanomedicines.

Leukocyte-based vehicles

Leukocytes, also known as white blood cells, are cells involved in immune responses to protect the body against infectious pathogens and diseases [155]. Leukocytes exhibit remarkable tropism to the damaged or inflammatory sites, and extravasate effectively to inflamed tissues due to their high deformability [155]. Notably, cancer has been widely identified as a chronic inflammatory disease [156]. It generates a chemokine gradient to recruit various leukocytes such as macrophages, myeloid-derived suppressor cells, dendritic cells, neutrophils, natural killer cells, mast cells, T and B lymphocytes [157, 158]. By taking advantage of excellent inflammation-tropism and cellular deformability, leukocytes have been engineered as EPR-independent delivery vehicles for anticancer applications [159]. Because of their phagocytotic activity, macrophages can engulf therapeutic nanocarriers, so that a NP-loaded living macrophage can be fabricated by *in vitro* incubation with therapeutic agents [160]. For example, Wang *et al* engineered a macrophage-mediated drug delivery system loaded with a nanosphere (CpG-ASO-Pt) (CAP) consisted of nucleic acid therapeutic (CpG-ASO) and chemotherapeutic drug cisplatin for lung cancer treatment [161]. In their study, CpG motif was shown to induce immunostimulatory effects and M1 polarization, resulting in anti-tumor effects. Anti-P-gp ASO was designed to downregulate the expression of P-glycoprotein to prevent excretion of chemo-drugs from tumors and consequently maintain the effectiveness of cisplatin. They demonstrated that CAP nanospheres loaded macrophages (CAP@M) could maintain the tumor tropic property of macrophages by transwell migration assay (figures 5(A) and (B)). Also, they studied the *in vivo* biodistribution of CAP@M and its cargos by labelling them with fluorescence (figure 5(C)). Free CpG-ASO-Cy5 strands, CAP-Cy5 nanospheres, and CAP-Cy5 loaded macrophages (CAP-Cy5@M) were intravenously injected into nude mice bearing subcutaneous A549 tumors, respectively. CAP-Cy5@M group exhibited the highest fluorescence intensity at the tumor site, supporting the hypothesis that the use of macrophage could improve the tumor targeting via the tumor homing capability of macrophages (figure 5(D)). Due to the complicated preparation process to load NPs into leukocytes, the relatively facile *in vivo* loading strategy, which is known as leukocytes hitchhiking, has also attracted much attention [162]. For example, L-selectin and Siglec-1, two surface receptors for sialic acid (SA), are overexpressed in circulating leukocytes. Thus, SA modification can be deployed to hijack leukocytes *in vivo* [163]. Besides the above-mentioned strategies, bacterial-membrane coated cargos can be exploited for *in vivo* drug-loading as well, because of the intrinsic

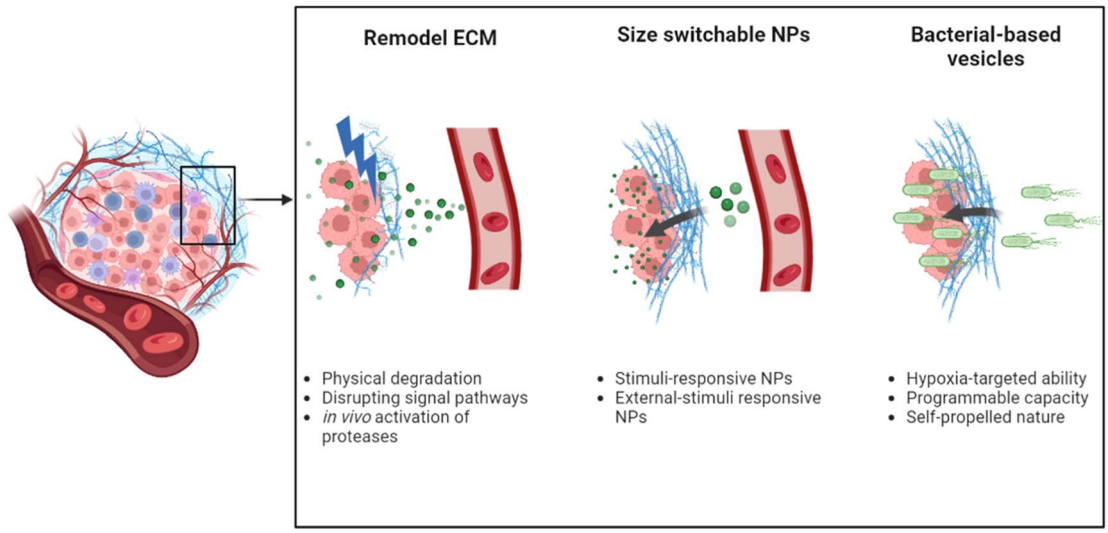

Figure 6. Strategies have been devised to enhance the penetration of NPs in tumor tissues. These techniques include the restructuring of the ECM in the tumor microenvironment, the development of size-changing NPs, and the use of bacteria as drug carriers, all of which are intended to improve NP penetration and to further elevate therapeutic effectiveness.

phagocytic capacity of it to uptake foreign pathogens [164].

Although preclinical studies have shown promising results, the development of innovative delivery vehicles using carrier leukocytes is still in its infancy and faces significant challenges. Firstly, the interaction between the loaded cargos and carrier leukocytes needs to be thoroughly investigated. The stability of leukocyte-based vehicles may depend on how nanomedicines interact with the numerous enzymes inside carrier leukocytes. Secondly, there is an urgent need for facile and benign cargo loading technologies to produce robust leukocyte-based vehicles with high loading efficiency. To achieve these goals, the development of novel biorthogonal chemistry and safer nanocarriers, as well as optimization of loading methods, are required. Thirdly, cargo-releasing models of leukocyte-based vehicles remains to be studied. Therefore, it is crucial to address these challenges and improve the leukocyte-based vehicles for effective drug delivery.

NPs to increase vascular permeability

In addition to EPR-adaptive delivery strategies, external physical inducements including radiation, hyperthermia and photodynamic therapy can increase tumor vascular permeability and thus enhance NP extravasation [165]. Radiotherapy can increase the extravasation of NPs within tumors by decreasing IFP through generating cytotoxic radicals resulting in a decrease in cell density within tumors [166]. In hyperthermia therapy, it has been found that once tumors are heated to 43 ℃, tumor vascular permeability increases significantly, improving EPR effect [167]. For instance, after hyperthermic therapy, the size range of extravasated liposomes in ovarian tumors changed from 7–100 to 7–400 nm and the increased accumulation of liposomes was observed in mouse model [168]. Recently, near infrared photo-immunotherapy (NIR-PIT) has been developed. NIR-PIT can selectively damage membrane of cancer cells through NIR induced photoreactions [169]. This novel strategy can improve NP extravasation for up to 24 folds compared with conventional EPR effect [170]. Notably, NIR-PIT has entered a global Phase 3 clinical trial for patients with recurrent head and neck cancer (ClinicalTrials.gov Identifier: NCT03769506) [171].

To effectively implement vessel modulation strategies and combined therapies for cancer treatment, it is necessary to identify the appropriate patient population. To achieve this, methods must be developed to accurately determine which patients are likely to benefit from specific vessel modulation strategies. Once identified, suitable vessel modulation strategies can be selected for individual patient based on their specific tumor characteristics. Thus, future research should focus on developing these methods for patient stratification to maximize the efficacy of vessel modulation strategies and combined therapies.

NPs to improve tumor penetration

Several characteristics of tumor microenvironment such as interstitial fluid pressure (IFP), integrity of vasculatures, and extracellular matrix (ECM) density leads to the limited penetration and permeation of NPs [172]. In this section, the strategies developed to improve NP penetration are discussed (figure 6).

Remodel ECM

Various barriers in the tumor microenvironment limit NP penetration and further reduce NP accumulation in tumor tissues. These barriers are composed of dense

ECM, increased IFP, unmatured lymphatic system, uncontrolled cell proliferation and leaky vasculature [30, 173, 174]. Among these components, ECM is the one most responsible for limiting NP penetration. ECM represents more than 60% of the total mass of the tumors and includes various macromolecules such as fibronectins, elastin, collagens and hyaluronic acid (HA) [175]. Thus, as one of the main barriers in the tumor microenvironment, ECM has attracted much attention in current studies aiming to enhance NP penetration [176]. To achieve this goal, a variety of strategies have been developed to degrade tumor ECM. The principle was either to physically breakdown ECM using ultrasound and hyperthermia, or to degrade ECM biochemically by collagenase or hyaluronidase [42, 177, 178]. Recently, several novel engineered NPs have been developed to remodel the ECM. Disrupting signal pathways in tumor tissues can modulate the stiffness of the ECM, thereby increasing tissue penetration [179]. For example, one study designed a multiplexed dendrimer lipid nanoparticle (LNP) loaded with focal adhesion kinase (FAK) siRNA, Cas9 mRNA and sgRNA (siFAK + CRISPR-LNPs) to target tumor mechanics, enabling efficient LNP delivery to tumors and enhance gene-editing efficacy [180]. Cancer and stromal cells can exert actomyosin-generated forces on the ECM, resulting in increased ECM stiffness. These contractile forces are mainly mediated by a process involving FAK activation. Thus, targeting FAK in tumors could reduce ECM stiffness. In this study, it was demonstrated that FAK inhibition reduced the membrane tension properties and the contractile force of tumor cells and ECM stiffness, leading to significantly improved CRISPR gene editing efficacy in tumor cells both *in vitro* and *in vivo*. Moreover, co-delivery of siFAK and sgPD-L1 could reduce FAK and PD-L1 expression. Ovarian cancer was chosen as a typical example of a cancer prone to metastasis, while liver cancer was selected as a representative case involving fibrosis and barriers within the ECM. Deep LNP penetration and improved mRNA translation to protein expression in tumor tissues were observed as well. The proposed NP enhanced overall gene editing efficacy by 10 times, if not more, indicating that this approach could edit enough cells to reverse disease symptoms. The siFAK +CRISPR-PD-L1-LNP therapy was also evaluated in mouse models of human liver cancer in their study, which demonstrated the potential of such approach to improve tumor immunotherapy and to inhibit metastasis.

Nevertheless, the delivery of ECM-degrading enzymes in solid tumors is still facing several challenges, such as the low stability of enzymes in the circulation and poor tumor penetration [172]. Currently, *in vivo* activation of proteases in the tumor microenvironment has been studied to effectively modulate tumor stiffness. For instance, Chen *et al* developed a tumor-targeting nanogenerator of peroxynitrite (ONOO−) by loading cisplatin and sodium nitroprusside (SNP) into poly(D,L-lactide-co-glycolide) NP, designated as PMCS NPs, for improved tumor penetration and chemotherapy [181]. The nanogenerator could generate ONOO− in the tumor via a cascade of nicotinamide adenine dinucleotide phosphate oxidases catalysis and glutathione reduction. The generated ONOO− had several functions. First, it could enhance tumor penetration of PMCS NPs by activating matrix metalloproteinases (MMP)-mediated degradation of the ECM. Second, the generated ONOO− would strengthen vascular permeability remarkably. Third, ONOO− was able to upregulate copper transporter 1 (CTR1), which is an important plasma membrane transporter for cisplatin transport and can thus amplify chemotherapeutic efficacy. *In vivo* results in CT26 colorectal tumor-bearing mice showed that 54% upregulation of MMP-2 protein activity and 36% upregulation of MMP-9 protein activity in tumors after intravenous injection of PMCS NPs compared with the control groups, which were only cisplatin-loading and SNP-loading NPs. Their data suggested that PMCS NPs could trigger ECM degradation via MMPs activation. Also, the vascular permeability of tumors was evaluated via Evans blue assay and approximately 6-fold Evans blue in tumors was found in the PMCS groups compared with that of the PBS group. Additionally, the deep tumor penetration of PMCS NPs was observed. It was preliminarily shown that such approach could improve NP penetration, vascular permeability and chemotherapeutic efficacy.

There are several obstacles that need to be overcome during the implementation of nano-based therapies for enhanced NP penetration by remodeling TME. Firstly, the safety of nanomaterials is a crucial concern that limits their clinical application. To overcome this issue, biodegradable nanomaterials that do not induce biological aggregation or resistance are preferred. Secondly, due to the heterogeneity of TME and diversity in tumor structures, the therapeutic response to the same therapy can vary among different types of tumors, leading to individual differences in the outcome of nanomedicine therapy. Thirdly, regulatory strategies that alter the tumor microenvironment structure through immunoregulation pose a potential risk of promoting tumor metastasis. Therefore, further investigation is required to assess the long-term effects of TME regulation strategies. In future studies, more clinically relevant *in vivo* and *in vitro* models should be developed to accurately simulate the complex interactions between NPs and the tumor microenvironment.

Size switchable NPs

The size of NPs could determine the tumor penetration capability of NPs. In general, NPs with larger sizes have a long blood half-time and are less subjective to

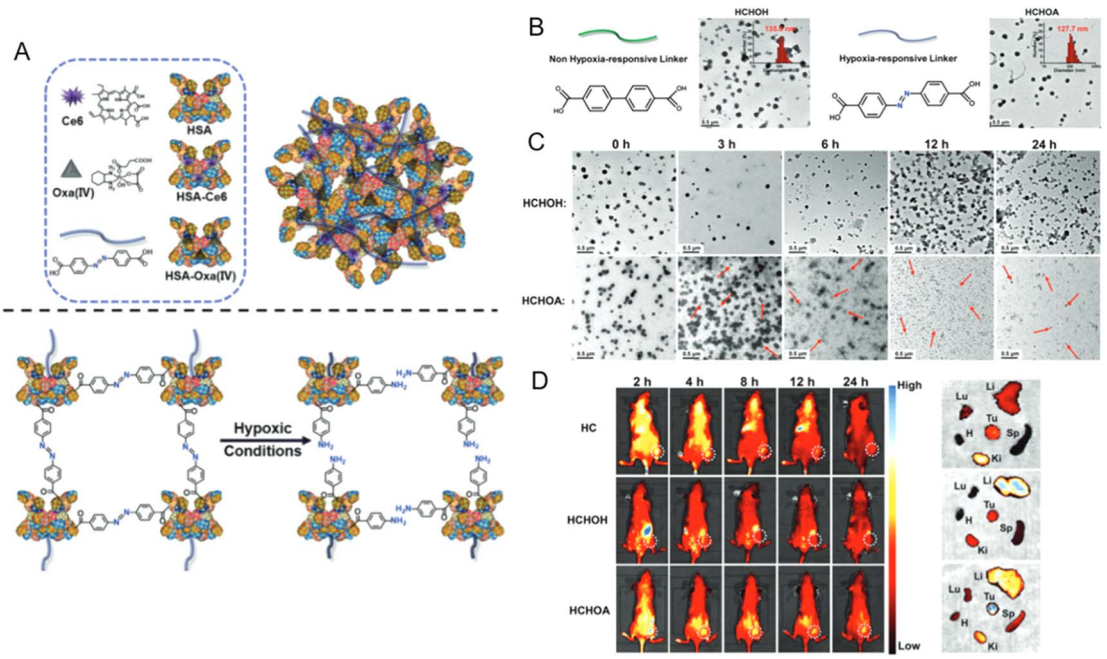

Figure 7. (A) Diagrammatic representation detailing the components of HCHOA. (B) Depiction of the structure of the non-hypoxia-responsive linker HCHOH and the hypoxia-responsive azo-linker HCHOA, accompanied by transmission electron microscopy (TEM) imagery and dynamic light scattering (DLS) data of the synthesized nanoparticles derived from them. (C) TEM images of nanoparticles generated from HCHOH and HCHOA following incubation with rat liver microsomes/NADPH under hypoxic conditions for varying time intervals. (D) Fluorescence images acquired over time illustrating the dependence on time for HC, HCHOH, or HCHOA in mouse models. Additionally, fluorescence images of major organs and tumors obtained ex vivo at the 24 h mark post-intravenous injection of nanoparticles HC, HCHOH, or HCHOA are also displayed. In the abbreviations Li, Sp, Ki, H, Lu, and Tu, 'Li' stands for liver, 'Sp' stands for spleen, 'Ki' stands for kidney, 'H' stands for heart, 'Lu' stands for lung, and 'Tu' stands for tumor [183] Copyright 2019,Wiley.

clearance by kidney filtration, while smaller NPs could penetrate deeper into tumor tissues [182]. As such, theoretically, NPs with switchable sizes could simultaneously achieve deep penetration into tumors and a long half-life in blood circulation. Yang *et al* recently developed hypoxia-responsive human serum albumin (HSA)-based NP (HCHOA) by crosslinking the hypoxia-sensitive azobenzene group with photosensitizer chlorin e6 (Ce6)-conjugated HSA (HC) and oxaliplatin prodrug-conjugated HSA (HO) (figure 7(A)) [183]. In order to demonstrate the tumor penetration properties of HCHOA NPs, the authors synthesized NPs by covalently conjugating with HC and HO via 4′4-biphenyldicaboxylic acid (H2BPDC), which was selected as the control group owing to its stability under hypoxic conditions (figure 7(B)). HCHOA NPs exhibited a size of 100–150 nm under normal oxygen pressure. In contrast, under hypoxia, these NPs could quickly dissociate into ultrasmall HC and HO NPs with a diameter of less than 10 nm triggered by the reductase-induced cleavage of the azobenzene moiety in HCHOA NPs, enabling deep tumor penetration (figure 7(C)). *In vivo* results in 4T1 breast tumor-bearing mice showed that HCHOA NPs exhibited a long circulation time and high tumor accumulation, while ultrasmall HC nanoparticles were rapidly cleared by in mice. After intravenously injection of HCHOA NPs in 4T1 breast tumor-bearing mice, Ce6 florescence of HCHOA NPs was detected away from blood vessels, achieving deep penetration. In contrast, the fluorescence of Ce6 of control group was mostly detected near tumor blood vessels in the control group (figure 7(D)).

In addition to endogenous tumor microenvironments, size transformation can be induced by external stimulations [184–187]. Recently, one study engineered a TNBC-targeting photothermal-responsive size-switchable albumin nanocluster (ICG@HSAAzo-HP) by crosslinking indocyanine green-laded human serum albumin (ICG@HSA) through a thermally labile azo linker (VA057) and then modified with a tumor homing tLyP-1 peptide (HP) [188]. Once treated with the mild irradiation of 808 nm laser, ca. 149 nm nanoclusters can disintegrate into 11 nm albumin fractions with improved intratumoral diffusion ability. At 30 min post-injection, the remaining ICG, ICG@HSAAzo, and ICG@HSA-Azo-HP in the blood of mice was 27.33%, 49.56%, and 82.61% of the injected dose, respectively, indicating that albumin nanocluster had a significantly higher the blood half-life than free ICG. Notably, because of the EPR effect, selective binding, and enhanced penetration, ICG@HSA-Azo-HP showed strong fluorescence at tumor tissues 2 h post-injection, exhibiting prolonged retention and improved accumulation in tumor.

Specifically, achieving structural stability of nanocarriers in physiological media and instability at tumor sites, despite advances, remains a difficult task.

Additionally, the intricate chemical modifications often lead to high batch-to-batch variances, impeding the translation of experimental findings to clinical applications.

Bacterial-based delivery systems

Bacteria are capable of self-directed motion in response to physical and chemical stimuli, a phenomenon that enables their successful infiltration into dense tissues [189]. Notably, the tumor microenvironment with hypoxia property serves as an ideal habitat for numerous facultative and anaerobic anaerobes such as *Clostridium tetani spores*, *Bifidobacterium*, *Escherichia coli* and *Salmonella* [106, 190–193]. Therefore, these bacterial strains have been engineered as hypoxia-targeting therapeutics for cancer therapies. Currently, Zheng *et al* developed living photosynthetic bacteria (PSB) as hypoxia-targeting carriers for cancer treatment by taking advantage of their near infrared (NIR) chemotaxis and anaerobic characteristics [194]. Due to their significant photo-absorption in the NIR region, PSB can serve as a photothermal agent to generate heat via non-radiative relaxation pathways. Moreover, PSB can induce immune response as natural bacteria and thus increase the infiltration of cytotoxic T lymphocyte. Thus, PSB could be an all-in-one agent for hypoxia targeting, photothermal therapy and immune stimulation. Regardless of NIR irradiation, PSB could migrate from the injection site toward tumor hypoxic cores, whereas NIR induced higher PSB accumulation in tumor tissues due to its phototactic capacity. After the injection of PSB, the temperature in the center of tumor reached up to 45.4 °C within 5 min of irradiation in MCF-7 breast tumor-bearing mice, demonstrating PSB penetration into the peritumor and strong photothermal effects induced. They also showed that both PSB and PSB + NIR could induce T-lymphocyte infiltration.

Bacteria can be utilized as drug carriers to improve tumor penetration because of their targeting ability and self-propelled nature. In a recent study, covalently conjugated mesoporous silica NPs on *Escherichia coli* bacteria (Bac-MSN) via click chemistry was fabricated [195]. To evaluate the penetration ability of Bac-MSN, a tumoral matrix-mimic model composed of 3D collagen gel with HT1080 human fibrosarcome cells embedded within an organic matrix has been employed. Bac-MSN and fluorescent MSN were placed at the top of tumoral matrix-mimic gels incubated for 3 h. The results showed that free particles were not able to penetrate the matrix, while Bac-MSN were distributed in the whole gel, indicating that bacterial-based system had the potential to penetrate tumor tissues. This enhanced penetration ability is a result of the bacteria's ability to target hypoxic areas and their inherent motility, which allows them to transport NPs across the ECM.

Despite the promising potential of bacterial-based delivery systems, this therapeutic approach still has several issues. Firstly, one notable challenge pertains to the elevated levels of anaerobic toxicity inherent in such systems. Anaerobic toxicity refers to the harmful by-products caused by bacterial activities such as hydrogen sulfide. Unfortunately, existing methods for detoxification often fall short of producing satisfactory outcomes. This discordance between the toxicity levels and the efficacy of detoxification presents a notable hurdle that must be addressed. Secondly, the process of introducing therapeutic agents into bacteria through intricate genetic modification pathways presents a substantial and intricate obstacle. This procedure demands meticulous precision and in-depth understanding to ensure the successful integration of therapeutic payloads within the bacterial carriers. The intricate nature of this genetic manipulation process necessitates extensive research and optimization. Thirdly, the integration of therapy genes into bacterial vectors frequently gives rise to genetic instability concerns. This instability can have far-reaching implications, affecting the overall performance and safety of the delivery system. It underscores the requirement for stringent quality control measures and thorough investigation into the stability of these engineered bacterial carriers.

NPs to enhance intracellular drug delivery

While engineered NPs can be a potent cancer treatment, there are still several barriers to intracellular trafficking affecting the therapeutic efficacy [196–198]. To overcome these barriers, this part will explore the potential nanomaterial designs to improve intracellular drug delivery (figure 8).

Fluorinated NPs

Due to their distinctive properties of excellent self-assembling ability and biological inertness, research interest in fluorinated materials has been on the rise for the intracellular delivery of biomacromolecules and nanocarriers [199–202]. The lipophobicity and hydrophobicity characteristics of fluorinated molecules can prevent non-specific adsorption of serum proteins and improve the physiological stability of NPs [203]. More importantly, with hydrophobic and lipophobic nature, fluorocarbons can be easily fused with membrane lipids through hydrophobic interactions and lipophobic property can achieve a rapid diffusion of the fluorocarbons across the cell membranes, significantly facilitating endocytosis [204, 205]. Therefore, fluorinated NPs hold great potential to enhance cytosolic delivery. One group constructed a personalized nanovaccine (F-PEI/OVA NPs) prepared by mixing the fluoroalkane-grafted polyethyleneimines (F-PEIs) with a model antigen ovalbumin (OVA) [206]. When incubating F-PEI/OVA NPs

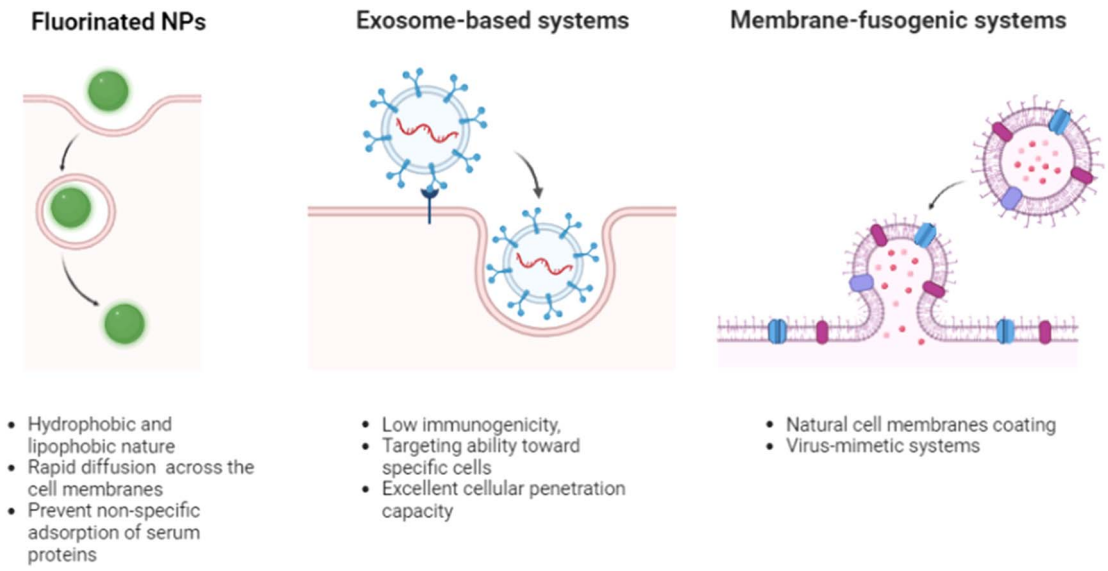

Figure 8. Several strategies have been developed to improve intracellular drug delivery, including fluorination functionalization, employment of exosome and membrane-fusogenic systems, all of which aim to overcome the biological barriers associated with intracellular drug delivery.

with mouse bone marrow-derived dendritic cells (BMDCs), F-PEI/OVA NPs showed a significantly higher cellular uptake and endosomal escape compared with PEI/OVA NPs and bare OVA. They also found that F-PEI/OVA NPs could dissociate to release the loaded OVA upon entering the cell, which was favorable for antigen presentation by the DCs. Zhang *et al* designed an efficient protein delivery system fabricated by co-assembly of proteins and fluoroamphiphiles into the NPs [207]. Thanks to the bioinert property of fluoroalkane, proteins loaded in fluorinated nanocomplexes showed minimal denaturation. In contrast, significant changes in secondary structures were observed in proteins entrapped in NPs formulated with non-fluorinated components. Notably, fluorescein isothiocyanate (FITC)-labelled fluoroamphiphiles exhibited remarkably higher internalization in MCF-7 breast cancer cells than the hydrogenated controls. It was also found that the fusion of hydrogenated amphiphiles leads to long-term retention and membrane disruption, whereas fluoroamphiphiles showed very limited toxicity.

Despite recent progress, there are still several issues remaining to be addressed in the field of fluorinated materials for cytosolic biomolecule delivery. Firstly, the detailed interactions between fluorinated materials and cell membranes, as well as their intracellular trafficking after endocytosis, are still poorly understood. Secondly, the inherent stability of fluorinated materials, owing to their stable backbone and/or excellent assembly behavior, results in limited degradation or metabolism under physiological conditions, and therefore inducing safety issues. For example, fluoroacetic acid, an especially detrimental metabolite found in certain drugs containing fluorine, exhibits a median lethal dose (LD50) of 10 mg kg^{-1} in humans. Fluoroacetic acid has the capability to disturb the Krebs cycle by reacting with acetyl coenzyme A [208]. Moreover, fluoride released from fluorinated materials interferes with enzyme activities, induces oxidative stress, and causes hormonal disturbances, and neurotoxicity [209, 210]. To avoid potential toxicity, it is necessary to reduce the dose of fluorinated NPs. Finally, the use of fluorous tags for the delivery of longer peptides remains a significant challenge. Once these issues are resolved, we anticipate that fluorination will play a pivotal role in cytosolic biomolecule delivery, and that the development of novel fluorinated smart materials will continue to expand in the future.

Exosomes

Exosomes are a subclass of extracellular vesicles that are nanosized, composed of a lipid bilayer membrane, and secreted by most cells [211]. They can transport a variety of biomolecules such as signal proteins, nucleic acids and lipids through the interactions between the proteins on the lipid membrane and the receptors on the target cell [212]. As endogenous nanovesicles secreted by cells, they possess a long half-life, low immunogenicity, potent targeting ability toward specific cells and excellent cellular penetration capability [152, 213–215]. Thus, exosomes have been extensively studied for applications in drug delivery in recent years (table 3) [151, 216–220]. For example, Li and colleagues used exosomes (Exos) derived from macrophages to encapsulate boron-containing carbon dots (BCDs), resulting in the formation of BCD-loaded exosomes (BCD-Exos) for boron neutron capture therapy (BNCT) as shown in figure 9(A) [221]. By utilizing exosomes from circulating macrophages, BCDs could traverse the blood–brain barrier (BBB) without

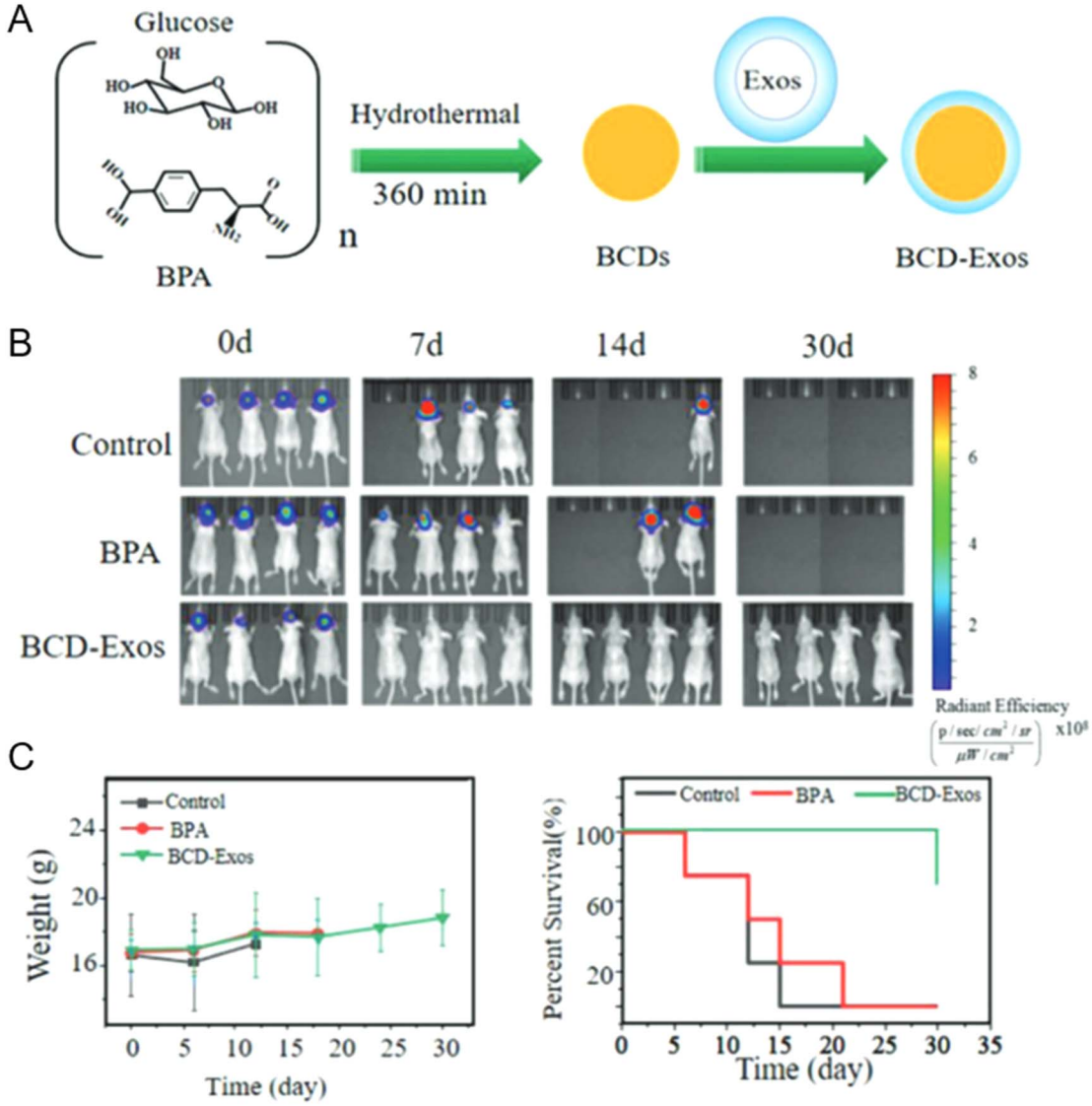

Figure 9. (A) Preparation of BCD–Exos. (B) *In vivo* IVIS spectral images of mice models with transplanted U87-Luc cells, subjected to treatment with saline as the control, BPA, and BCD–Exos post BNCT. (C) Alterations in body weight and survival curves following BNCT [221] Copyright 2021,Wiley.

compromising the integrity of the membrane. The tumor/normal (T/N) ratio was used as a measure of the concentration of boron in tumors relative to normal organs. In this study, the T/N ratios were determined 4 h after intravenous injection of both BCD-Exos. The T/N ratio in mice treated with BCD-Exos was found to be 5.28 ± 0.29, whereas those for BPA and BCDs alone were 2.03 ± 0.08 and 2.91 ± 0.13, respectively. These results suggested that the use of exosomes as a drug carrier enhanced the accumulation of boron at the target sites (figure 9(B)). Notably, when BCD-Exos were used in combination with single neutron exposure to treat brain tumors in a mouse model, the survival rates of mice treated was 100% at day 30, demonstrating a substantial improvement in treatment efficacy. In contrast, the survival rates of mice treated with BCDs and BPA were only 50% at day 21 and 0% at day 15, respectively (figure 9(C)).

The cell–cell communication performance of exosomes can be heavily influenced by the local cellular environment in which they are secreted [222, 223]. Considering this, reprogramming exosomes presents a novel approach for intelligent drug delivery and potentially personalized therapy against specific tumors. Gong *et al* discovered that exposing cells to conditions that mimic the tumor microenvironment (such as low pH and hypoxia) led to a considerable rise in tumor exosome uptake [224]. In the study, exosomes were collected from MGC803 human gastric cancer cells treated with various conditions, including normal conditions, ultraviolet irradiation, low pH, high temperature, H_2O_2 treatment, and hypoxia. The exosomes stimulated by low-pH and hypoxia (LP-Exos and Hyp-Exos, respectively) demonstrated greater uptake efficiency in MGC803 cells, suggesting that the uptake properties of exosomes could be

Table 2. A summary of surface receptors for actively transporting NPs in tumors.

Nano-systems	Targeting ligand	Surface receptor	Cancer type	Specificity	References
Zein NPs	iRGD or RGD	α_v integrins	Pancreatic cancer	Tumor and angiogenic endothelium	[135]
Albumin NP	albumin	GP60 and Fc receptor	Lung cancer	Specific for albumin across the endothelium	[138]
Hydroxyethyl starch-folic acid NPs	Folic acid	Folate receptor α	Breast cancer	Epithelial malignancies and tumor cells	[141]
PEGylated magnetic NPs	Insulin	Insulin receptor	Brain cancer	Brain endothelial cells	[133]
Self-assembled photosensitizer nanostructure	IgG	Neonatal Fc receptor	Colon and breast cancer	Epithelial and endothelial cells	[139]
Polymeric NPs	Leptin30	Leptin receptor	Brain cancer	Brain endothelial cells	[140]
Lumazine synthase protein cage	Epidermal growth factor (EGF)	EGFR	Epidermoid carcinoma	Endothelial, epithelial cells, and tumor cells	[137]
Super paramagnetic iron oxide NPs	Anti-VCAM-1	Vascular cell adhesion molecule	Breast cancer	Tumor endothelial cells	[134]
Mesoporous organosilica	mAnnA1	Annexin A1	Breast and lung cancer	Lung, mammary, prostate and lung tumors	[136]

Table 3. A summary of the potential of exosomes as carriers for cancer therapy drugs.

Source of exosomes	Cargo	Cancer type	Target strategy	Type of study	Therapeutic potential	References
Fibroblasts	CD-47, GM-CSF and docetaxel	Metastatic Peritoneal Cancer	SIRPα of macrophage	In vitro and *in vivo*	Inhibit tumor growth	[218]
Mesenchymal stem cell line	CPP and TNF-α	melanoma subcutaneous cancer	Magnetic directing	In vitro and *in vivo*	suppressed tumor growth with mitigating toxicity.	[220]
B16F10 cells	DOX	melanoma subcutaneous cancer	Integrin	In vitro and *in vivo*	Increased DOX concentration in tumors and inhibit tumor growth	[216]
293 T cells	Antisense miRNA oligonucleotides	Glioblastoma	Transferrin receptor	In vitro and *in vivo*	reduced the miR-21 level in the glioblastoma and tumor size	[217]
induced pluripotent stem cells (iPSCs)	PD-1 antibody and DOX	esophageal gastric cancer	CXCR4/SDR1 axis	*in vivo*	amplified the anti-tumor immune effect	[151]
colostrum powder	siRNA or pDNA	Lung cancer	folic acid receptor	In vitro and *in vivo*	enhanced gene silencing and tumor growth inhibition	[219]

enhanced under by manipulate external environment of cells.

By leveraging the role of lipids in facilitating the interaction between tumor exosomes and tumor cells, the engineering of exosome membrane lipids has become an efficient approach to enhance tumor cell uptake [225]. Zhan *et al* inserted phosphatidylcholine (PC) molecules into the membrane lipid layer of reticulocyte-derived exosomes (Exos) to construct PC-engineered exosomes (PC-Exos) [226]. They conducted in vitro experiments to study cellular uptake of PC-Exos. The results in both glioblastoma U87 and MDA-MB-231 breast cancer cell lines showed that PC-Exos had a significantly higher cell internalization compared to native Exos, with an increase of up to two folds. After loading the Exos with therapeutic agent, PC-Exos demonstrated a significant improvement in intracellular accumulation of DOX and RNA in tumor cells. As a result, PC-Exos achieved higher *in vitro* anticancer activity.

However, current strategies for loading cargo onto exosomes do not meet the loading efficiency required for clinical applications. Specifically, the simple incubation method is limited in terms of the type of cargo that can be loaded and its efficiency is insufficient for clinical use. Moreover, a major obstacle to the clinical application of exosomes is their low yield. Although the yield of exosomal protein may vary based on donor cells, it generally falls below 1 μg per ml of culture, thereby requiring a large number of cells to be cultured for clinical trials.

Membrane fusion-mediated delivery systems

The fusion of membrane composition between cells, known as membrane fusion, is a crucial biological process that facilitates cell-to-cell communication and cargo transport [227]. This process involves blending foreign substances with the cell membrane, resulting in the transportation of inner content to the cellular cytosol [228]. Membrane fusion has served as inspiration for the latest advancements in membrane fusion-mediated NPs, providing new strategies for intracellular drug delivery. This approach offers a unique cytoplasmic delivery method to evade endosomal entrapment and improve transportation efficiency [229]. In natural cell–cell fusion, the fusogenic proteins expressed on cell membranes is crucial in bringing together membranes for fusion [230]. Several surface proteins have been identified to have the ability to promote cell–cell membrane fusion. These include highly conserved, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) and proteins containing immunoglobulin (Ig)-like domains such as CD9 and CD47 [228]. This method allows the preservation of authentic fusogenic proteins present on natural cell membranes. A straightforward and effective approach for constructing biomimic membrane-fusogenic systems is to coat natural cell membranes onto particle surfaces. Nie *et al* utilized a yolk–shell-structured nanoparticle coated with cancer cell membranes to facilitate fusion-based delivery of therapeutic agents for cancer treatment [231]. With natural membranous fusogens present on the particle surface, these nanoparticles could target homologous sites and induce direct cellular fusion, which resulted in efficient internalization and a 23.3-fold increase in tumor penetration observed in MCF-7 breast tumor-bearing mice. Furthermore, co-encapsulation of DOX and the poly (ADPribose) polymerase inhibitor in the nanoparticle yolks displayed significant anti-tumor activity, underscoring the potential of cancer-cell-membrane-coated particles as a promising strategy for cancer therapy.

The membrane fusion between viruses and host cells is a common pathophysiological process, typically facilitated by viral glycoproteins such as spike vesicular stomatitis virus G-protein (VSVG) and hemagglutinin (HA). It has been experimentally demonstrated that these glycoproteins are capable of initiating membrane fusion under acidic pH [232]. Inspired by this process, Kim *et al* utilized VSVG-engineered exosomes to attain tumor xenogenization, promoting enhanced antitumor immunity [233]. The VSVG glycoproteins enabled the fusion of exosomes with tumor cells, resulting in their presentation on the tumor surfaces as pathogen-associated molecular pattern molecules, thus facilitating recognition and engulfment by dendritic cells for immune activation. The tumor xenogenization approach, mediated by VSVG-modified exosomes, elicited effective immunogenic reactions that inhibited tumor growth, as evidenced by multiple tumor mouse models.

Recently, rationally designed polymers have emerged as promising candidates in fusion-based delivery for efficient cargo release as well. Through elaborate engineering, polymer materials are capable of mimicking viral behavior by adhering to the cell membrane and initiating a fusion pore to deliver encapsulated cargos to the cytoplasm. Shen *et al* have developed virus-mimic polyplexes as a novel approach for gene delivery [234]. The polyplexes consist of quaternized linear polyethyleneimine decorated with N-(p-acyloxy benzyloxycarbonyl) ethyl groups on its ammonium moieties. The DNA was condensed by electrostatic interactions and hydrophobic blocks to form nanoparticles. Upon binding to the cell membrane, esterase hydrolyzes the phenol ester bonds in the cytoplasm, transforming the polymer from a cation to a zwitterion, leading to the release of DNA into the cytoplasmic membrane. The zwitterion blocks offered protein resistance to adsorption and a long acyl chain to facilitate membrane insertion, resulting in retention of the residual polymer on the cell membrane. This pore-mediated fusion approach enabled direct delivery of DNA into the cytoplasm. Besides, polyplex-based nanoparticles coated with a poly(γ-glutamic acid) layer could prolong their blood

retention. This polymer-mediated membrane fusion approach displayed promising potential for the design of fusogenic particles for various applications.

Membrane-fusogenic systems have been identified as promising tools for advanced biomedicine. However, several limitations have been found as well. Firstly, a notable hurdle is the limited comprehension of the intricate mechanisms governing the behavior of these membrane-fusogenic systems within the dynamic and intricate milieu of complex biological environments. While these systems may exhibit promising behavior in controlled laboratory settings, their behavior in the context of living organisms is a complex interplay of various factors that requires further exploration. Another significant concern revolves around the safety aspects associated with membrane-fusogenic systems. As these systems often involve interactions at the cellular and molecular levels, it's crucial to thoroughly understand any potential unintended consequences, immune responses, or adverse reactions that could arise upon their application. Furthermore, achieving optimal efficiency in terms of cargo delivery, fusion processes, and overall therapeutic efficacy remains a challenge. The intricacies of how these systems interact with target cells, trigger fusion events, and successfully deliver payloads need to be finely tuned for optimal performance. Scalability for clinical translation is yet another pivotal aspect that needs attention. While promising results may be obtained on a small scale, transitioning these systems to a clinical setting demands the ability to produce them at a larger scale without compromising their integrity, functionality, or safety.

Conclusion

This review presented a comprehensive overview of recent developments in NP designs and strategies for therapeutic drug delivery, with a focus on overcoming various biological barriers that impede effective cancer treatment. NP platforms offer versatile features such as modifiable surface properties and responsiveness, enabling the selection of optimal drug delivery approaches. The engineered properties of NP have led to the development of numerous NP delivery systems that improve drug accumulation, tumor penetration, and intracellular uptake for cancer therapies.

While research in the field of cancer therapy is rapidly advancing, the design of NP delivery technologies is still in its early stages. Thus, the importance of foundational research has been increasingly recognized, particularly for the interactions between innate biological barriers and NPs. For instance, studies have focused on validating the EPR effect and exploring the mechanisms of NP extravasation in tumors to achieve a better understanding of nano-bio interactions. Such fundamental research will be essential for future innovations in cancer therapy by enabling more comprehensive and curative drug delivery approaches. The delivery platforms discussed in this review provided promising strategies for improving drug delivery efficiency and overcoming innate delivery barriers in cancer therapy, and summarized their potential for widespread implementation. Therefore, continued research efforts, both fundamental and applied, are crucial for advancing the field and realizing the full potential of cancer drug delivery.

In recent years, numerous novel NP systems have been developed to overcome biological barriers for cancer treatment. However, as previously discussed, each type of NP designed to address specific biological barriers has its own limitations, including a lack of understanding of nano-bio interactions, scalability issues, and safety concerns. Therefore, researchers should focus on studying bio-nano interactions to develop a deeper understanding of how NPs interact with various biological systems at different levels. Furthermore, many of these novel drug delivery systems are overdesigned, leading to batch-to-batch variability, low yield, and poor quality control, hindering clinical translation. Consequently, further studies are required to improve fabrication processes to meet clinical requirements. Lastly, novel delivery systems, such as bacterial-based NPs and fluorinated NPs, have potential safety concerns, requiring further investigation of their *in vivo* toxicity. In summary, to advance NP-based drug delivery for cancer treatment, we must investigate the mechanisms of NP-based drug delivery in complex biological environments, optimize NP fabrication methods to reduce batch variability and increase production scale, and design NP platforms that integrate different design principles to overcome biological barriers from the intracellular level to systemic conditions.

Data availability statement

No new data were created or analysed in this study.

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