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Review article



# Harnessing cells to improve transport of nanomedicines

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ABSTRACT

Efficient tumour treatment is hampered by the poor selectivity of anticancer drugs, resulting in scarce tumour accumulation and undesired off-target effects. Nano-sized drug-delivery systems in the form of nanoparticles (NPs) have been proposed to improve drug distribution to solid tumours, by virtue of their ability of passive and active tumour targeting. Despite these advantages, literature studies indicated that less than 1% of the administered NPs can successfully reach the tumour mass, highlighting the necessity for more efficient drug transporters in cancer treatment. Living cells, such as blood cells, circulating immune cells, platelets, and stem cells, are often found as an infiltrating component in most solid tumours, because of their ability to naturally circumvent immune recognition, bypass biological barriers, and reach inaccessible tissues through innate tropism and active motility. Therefore, the tumour-homing ability of these cells can be harnessed to design living cell carriers able to improve the transport of drugs and NPs to tumours. Albeit promising, this approach is still in its beginnings and suffers from difficult scalability, high cost, and poor reproducibility. In this review, we present an overview of the most common cell transporters of drugs and NPs, and we discuss how different cell types interact with biological barriers to deliver cargoes of various natures to tumours. Finally, we analyse the different techniques used to load drugs or NPs in living cells and discuss their advantages and disadvantages.

## 1. Introduction

Cancer is one of the leading causes of premature death worldwide [1]. Chemotherapy remains the most widely used therapeutic approach, albeit suffering from poor selectivity, undesired off-target effects [2], and multidrug resistance [3–5].

Nanoparticle (NP)-based drug delivery systems have been proposed to improve the selectivity of traditional chemotherapy via e.g., the *enhanced permeability and retention* (EPR) effect [6], or through active targeting [7]. Indeed, small-size NPs have been shown to passively accumulate into solid tumours by leaking through their defective and fenestrated vasculature via the EPR effect; while surface-modified NPs have been demonstrated to actively engage with specific receptors on target cells [8,9]. As a result, NPs have the potential to improve the site-specific accumulation of chemotherapeutic agents and to reduce their side effects.

Despite these advantages, a statistical analysis showed that, on average, only 0.7 % of the administered NPs reach the tumour mass [10]. This is mainly due to obstacles in NPs transport, such as immune clearance, non-specific distribution, and other biological barriers that prevent NPs accumulation at the target site [11,12]. Moreover, the effect of such barriers depends on the tumour characteristics, such as the site of injury and the stage of progression, as well as on the route of administration [13,14]. Following oral or intravenous administration, NPs must bypass the immune system [15,16] and penetrate multiple endothelial and epithelial barriers to reach the target [17,18]. Additionally, many solid tumours, such as pancreatic adenocarcinoma and glioblastoma multiforme [19], are characterized by a heterogeneous and aggressive microenvironment with high interstitial fluid pressure and stiff stroma, which hamper drug and NPs penetration [20].

Therefore, different attempts have been made to improve NPs transport through these obstacles. As summarized in Fig. 1, these strategies include surface modification of NPs, NPs coating with cell membranes, and, recently, NPs loading into living biological transporters [21].

As shown in Fig. 1A, the surface of the NPs can be functionalized with ligands displaying affinity for receptors overexpressed by the target cancer cells, thereby improving cell-specific delivery. For instance, NPs have been modified with epidermal growth factor (EGF[22]) or transferrin (TF[23]) for specific targeting of tumours, such as sarcoma [24],

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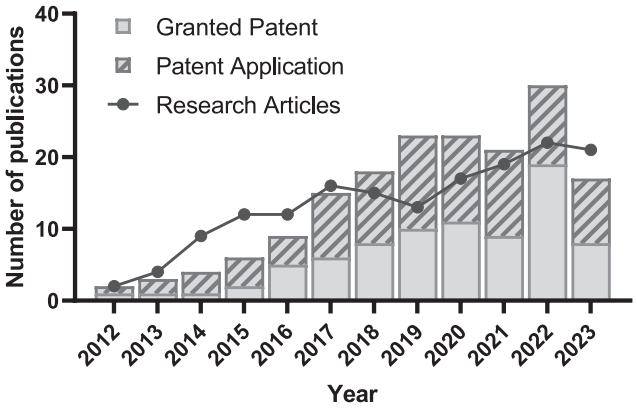
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hepatocellular carcinoma [25,26] and lung cancer [27,28], or to enhance their extravasation [29,30]. However, successful exploitation of these NPs is limited, as surface modification is complex to scale up and the ligand density must be precisely adjusted to allow effective association with receptors. Moreover, ligands may compete with other endogenous molecules to bind to the target receptor, while unspecific coverage of NPs with blood proteins may mask the ligands, hindering their binding to the receptor [31,32].

In cell membrane-coated NPs (CMC-NP) (Fig. 1B) the NPs core is coated with membranes extracted from cancerous or non-cancerous cells to harness their natural ability to extravasate from capillaries and infiltrate the tumour mass [31,33]. Moreover, this camouflage avoids the non-specific coverage by plasma proteins, increasing immune escape [11]. However, depending on the cell source, the process of membrane isolation may display low yield, variable efficiency, and limited reproducibility [34–36], making the manufacturing of CMC-NPs expensive and hardly scalable, with a negative impact on their clinical translation [37].

Loading NPs directly inside living cells (Fig. 1C) takes advantage of the cells' natural tropism towards tumours [38]. Different loading techniques and cell types [39–41], such as stem cells [42,43], neutrophils [44], lymphocyte [45], and macrophages [46] have been used to transport drugs or nanomedicines to brain [47], lung [48,49], and other solid tumours [50,51], as well as to deliver drugs to the CNS [52] achieving significantly improved transport across biological barriers, enhanced tumour accumulation, and reduced toxicity. Therefore, cell-mediated transport represents a safe, virtually patient-specific, and versatile approach to enhance drug delivery to target sites.

The increasing interest in cell-mediated drug delivery is evidenced by the slow but steady rise in publications and patents on the topic in the past decade (Fig. 2). The data show that this field, albeit promising, remains relatively underexplored in comparison to traditional nanomedicine, probably due to the additional complexity in handling living systems. However, new clinical trials (e.g., NCT01523808, NCT01523782, NCT04741984, NCT03815682) on cell-based delivery




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**Fig. 2.** Number of published research articles and patents on cell-based drug delivery from 2012. Data Sourced from PubMed Database (National Library of Medicine; National Institutes of Health; U.S. Department of Health and Human Services) and The Lens (https://www.lens.org/).

with circulating cells are being performed, confirming the promising potential of the approach, supporting further research in this area [53,54].

This review article analyses the use of different cell-based drug delivery systems to bypass biological barriers. The review also provides an overview of the most widely used approaches to load NPs into living transporters, discussing their experimental applications.

## 2. Interaction of cellular transporters with biological barriers

Biological barriers involve a set of obstacles to NPs transport that hinder their site-specific accumulation [11]. Among the obstacles to NPs transport, the mononuclear phagocyte system (MPS) is responsible for the rapid sequestration of circulating NPs [55]. MPS cells, such as

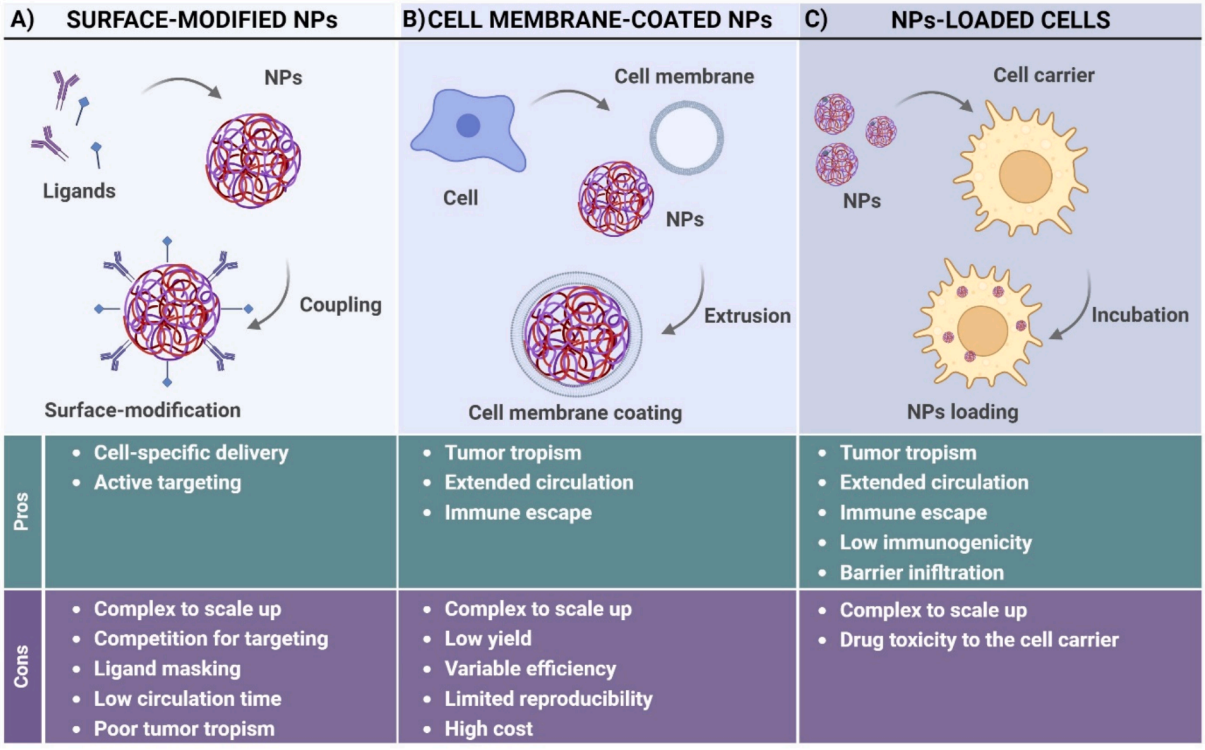



**Fig. 1.** Schematic representation of the main strategies for NPs engineering to improve targeted drug delivery. Created with BioRender.com.

macrophages and Kupfer cells, are known to facilitate the clearance of NPs resulting in their non-specific accumulation in excretory organs [56,57]. Endothelial barriers, namely the vascular endothelium [58], the blood–brain barrier (BBB) [59] and the gastrointestinal epithelium [60], represent another stringent obstacle to NPs extravasation and tissue accumulation. Finally, the tumour microenvironment (TME) with its high interstitial pressure and stiffness reduces treatment penetration, confining them to the tumour margins [61].

Several biological transporters can naturally move across these barriers. For example, cells expressing self-markers (e.g., CD47 on erythrocytes) are known to escape immune clearance [62], while circulating cells like leukocytes [63], macrophages [64], or platelets [65] can spontaneously undergo *trans*-endothelial migration [66] and are often found as an infiltrating component in solid tumours [67,68]. Therefore, these carriers have been used to transport NPs or drugs to tumours across different biological barriers, as summarized in Table 1.

### 2.1. Bypassing the MPS with cell transporters

The MPS is the first biological barrier to the administration of NPs and consists of phagocytic cells located in the blood vessels and in the organs involved in clearance processes (e.g., liver, spleen, kidneys, and lymph nodes[85,86]). As schematized in Fig. 3, circulating NPs are rapidly taken up by macrophages (particularly Kupfer cells), which are activated by the plasma proteins (opsonins[87]) that cover the NPs surface, in a process called opsonization [88]. This phenomenon results in reduced circulation time, poor target selectivity, and altered NPs transport ending in their undesired accumulation in off-target organs, such as the liver and the spleen [11,89].

"Stealth" polymer coatings or coating with membranes isolated from circulating cells (such as erythrocytes and white blood cells) have been proposed to extend the circulation time of NPs with some success [90]. Unfortunately, detachment of the coating or development of coating-specific antibodies have limited the applicability of these approaches [91,92].

Cells expressing "self" markers (e.g., major histocompatibility complex I [93] or CD47[94]) such as erythrocytes [69,95], thrombocytes [70,96], mesenchymal stem cells [71,97] and immune cells [66,75,79,98] have the potential to escape the MPS, representing powerful transporters for NPs and drugs (Fig. 3).

Considering that the lifespan of human red blood cells (RBC) typically exceeds 100 days [99], several authors have proposed RBC-mediated transport of NPs.

For example, Chambers et al. [69] successfully covered RBCs with polystyrene (PS) NPs (200–450 nm), through a passive loading approach, in which living RBCs were incubated with NPs to allow NPs adsorption on the cell membrane. They demonstrated that NPs associated with RBCs were found in circulation for a significantly longer time than free PS NPs (12 h vs 2 min). Using a similar adsorption approach, Anselmo et al. [100] reported a 5-fold increase in lung accumulation and a 50 % decrease in undesired spleen uptake for RBC-bound NPs as compared to free NPs after intravenous injection in mice [101]. The high NPs release in the lung was attributed to NPs detachment from the RBC membrane, due to cell squeezing inside lung capillaries [48]. However, *in vivo* tests demonstrated that the half-life of RBCs heavily depends on the RBC/NPs incubation ratio, with higher ratios resulting in faster clearance. Therefore, the use of RBCs as vehicles to overcome NPs uptake by the MPS remains debated.

Platelets represent a valid alternative to RBCs to achieve evasion of the MPS, as they express different surface proteins such as P-selectin, CD47, and CD44 that allow immune escape and promote specific adhesion to injured sites, such as tumours [96].

Xu et al. [70] designed a platelet-based platform for lymphoma treatment by incubating the cells with DOX to allow drug internalization in cells through the open canalicular system. After intravenous injection in mice, they demonstrated an improved transport for DOX-loaded platelets as compared to either, free DOX or pegylated liposomal DOX. Indeed, after 4 h from administration, the quantification of DOX in the tumour was 3 times higher in the group treated with the cell transporters. Moreover, DOX-loaded platelets significantly reduced cardiac damage and undesired accumulation in liver and spleen as compared to pegylated liposomal DOX.

Mesenchymal stromal cells (MSCs [102]) have also been tested by virtue of their low allogeneic rejection [103]. Xiao et al. [71] investigated MSC-mediated transport of bismuth selenide ($Bi_2Se_3$) NPs for targeted radiotherapy of non-small cell lung cancer (NSCLC). Using fluorescent cell transporters, they showed strong fluorescence in the tumour regions only for the MSCs-treated group, while no fluorescence was detected in tumours after free fluorophore injection. These results suggest the long circulation ability of MSCs as well as their tumour-homing capacity, supporting their use as drug transporters to lung tumours [104].

Immune cells represent another well-studied vector to extend the circulation time of drugs and NPs. Although their half-life is extremely

**Table 1**
Summary of cell-mediated drug delivery systems used to overcome biological barriers.

| Cell Carrier | Cargo | Administration | Barrier | Target | Ref |
|---|---|---|---|---|---|
| Erythrocytes | Polystyrene NPs | Intravenous | MPS | Lung | [69] |
| Thrombocytes | Doxorubicin (DOX) | Intravenous | MPS | Lymphoma | [70] |
| Mesenchymal Stem Cells | $Bi_2Se_3$ NPs | Intravenous | MPS | Lung tumour | [71] |
| | DOX-Silica NPs | Intra tumour | MPS/TME | Glioblastoma | [72] |
| Neutrophils | Paclitaxel (PTX)-liposomes | Intravenous | BBB | Glioma recurrence | [73] |
| | TPCA-1- Cefoperazone- Albumin NPs | Intravenous | Vascular Endothelium | Lung Infection | [74] |
| | Catalase-poly-L-lysine NPs | Intravenous | BBB/MPS | Cerebral Ischemia | [75] |
| | Edaravone-liposomes | Intravenous | BBB | Cerebral Ischemia | [52] |
| Circulating Monocytes | DOX-nanodiamonds | Intravenous | BBB | Glioblastoma | [76] |
| Macrophages | Gold-silica nanoshells | Intravenous | BBB | Brain metastases | [77] |
| | Indinavir-liposomes | Intravenous | BBB | Encephalitis | [78] |
| | DOX- Poly (lactic-co-glycolic acid) (PLGA) NPs | Intravenous | BBB | Glioma | [46] |
| | DOX-silica NPs | Intravenous | BBB | Glioma | [79] |
| | Catalase-Poly (allylamine hydrochloride) NPs | Intravenous | BBB/Vascular Endothelium | Brain Inflammation | [41] |
| | DOX-liposomes | Intravenous | Vascular Endothelium | Lung tumour | [80] |
| Microglia | PTX-Liposomes | Intravenous | MPS/BBB | Glioma | [66] |
| T cells | Polystyrene NPs | Carotid artery | BBB | Brain inflammation | [81] |
| | SCH-liposomes | Intravenous | TME | Adenocarcinoma | [40] |
| Dendritic cells | DOX-nanodiamonds | Intravenous | BBB | Glioblastoma | [47] |
| Bacteria (*E. coli*) | TNF- α gold NPs | Oral | Gastrointestinal epithelium | Breast Tumour | [82] |
| Lyophilized Plant Cells | Coagulation factor IX | Oral | Gastrointestinal epithelium | Haemophilia | [83] |
| | Myelin basic protein | Oral | Gastrointestinal epithelium | Alzheimer's disease | [84] |

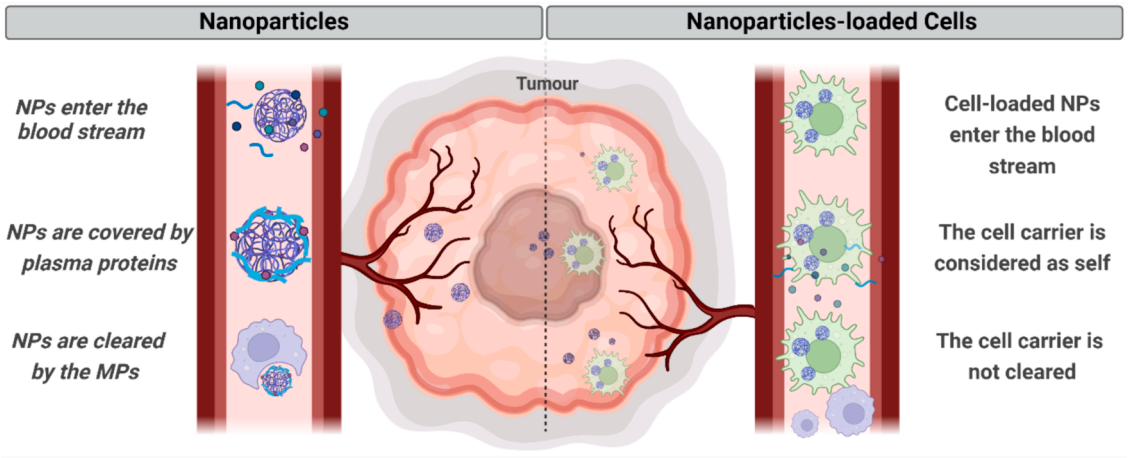



**Fig. 3.** Schematic representation of MPS effect on NPs and NPs-loaded cells. NPs are recognized as foreign and phagocyted by MPS cells (e.g., circulating macrophages). This uptake leads to a nonspecific accumulation in excretory organs, such as the spleen and the liver. Cell-based platforms are considered as "self', thereby avoiding immune detection and consequent capture by MPS cells and clearance. Created with BioRender.com.

variable, ranging from a few hours for neutrophils [105] to a few weeks for activated T-cells [106], their high phagocytic activity (i.e., their high loading capacity) and their natural tumour tropism, prompted their investigation as drug transporters [107]. For example, Du et al. [66] used murine microglia to deliver fluorescent liposomal paclitaxel (PTX-Lip) to brain tumours. They demonstrated that liposomes-loaded microglia accumulated significantly less in liver, spleen, lungs, and kidneys, suggesting their ability to evade MPS uptake. Moreover, after intravenous injection in mice bearing an orthotopic murine glioma model, significantly higher microglia accumulation in the brain and enhanced anti-tumour effect were achieved in comparison with intracranially-administered PTX-Lip.

These results confirm that delivery strategies based on cell-mediated transport have the potential to avoid MPS uptake, thereby reducing clearance and off-target accumulation of their payload.

### 2.2. Bypassing endothelial barriers with cell transporters

Once injected, NPs must leave the circulation stream and extravasate to reach the desired target. The ability of NPs to extravasate depends on the type of junctions between endothelial cells, on the integrity of the endothelial layer, and on the presence of fenestrations [12]. Moreover, NPs extravasation is hindered by their tendency to circulate in the cell-free layer, without interacting with the vessel walls (Fig. 4A) [9,11]. Several cells (e.g., neutrophils, platelets, and monocytes) are found to naturally circulate near the vessel walls, through which they can extravasate following specific stimuli (like chemokines [108] or inflammation-induced selectins [109]). Therefore, these cells are potential candidates for NPs and drug transport across the endothelial vessel barrier.

Leukocytes can easily marginate under the pressure of haemodynamic forces or after collisions with circulating erythrocytes [63]. After margination, leukocytes can interact with the vascular endothelium, e.g., through adhesion molecules activated by inflammatory signals [110]. Leukocytes can also undergo *trans*-endothelial migration to reach tumours in response to chemotactic stimuli released by tumour cells [64].

Several authors exploited the natural endothelial transmigration ability of cells to deliver drugs across the blood vessels. In preclinical research, leukocyte-based delivery systems have been extensively investigated [111,112]. Moreover, their high phagocytic activity facilitates nanomedicine loading via endocytosis, while their natural tropism towards cancer and inflamed organs facilitates their accumulation in the diseased area [52,76–78]. For instance, Chu et al. exploited leukocyte transmigration to transport albumin NPs to inflamed tissues through the blood vessel endothelium in a murine lung inflammation model [74]. Reduction of the lung tumour volume and extension of mice survival was also reported for NPs transported by macrophages [79].

Preliminary evidence suggests that MSCs are a promising alternative to immune cells for enhancing drug delivery across endothelial vessels in various diseases [113]. Wang et al. [49] confirmed the natural lung tropism of MSCs [114–117] by demonstrating significant accumulation in lung tumours in a murine model. MSCs were us7ed to transport docetaxel (DTX)-loaded NPs, achieving significantly reduced tumour volume as compared to free DTX-NPs. However, concerns remain regarding the safety of MSC-based delivery systems, as *in vivo* injection can lead to uncontrolled differentiation [117] and, in some cases, to tumorigenesis [118].

Cell transporters also have the potential to interact with more specialized and less permeable endothelial barriers, such as the blood–brain barrier (BBB) or the intestinal endothelium (Fig. 4B and 4C).

The BBB is a neurovascular unit that tightly controls the transfer of ions and molecules to the brain [119], limiting the access of drugs or NPs to the central nervous system (CNS) [120–122].

Neutrophils have been shown to accumulate in the CNS following specific stimuli. Their ability to cross the BBB was demonstrated in a mouse ischemic model, where neutrophil-mediated transport increased catalase accumulation in ischaemic regions and reduced the infarct volume to 33 % as compared to untreated mice [75]. Moreover, transwell migration assays also demonstrated that the movement of neutrophils through the endothelial barrier was enhanced by pro-inflammatory factors, such as those associated with ischaemia.

Xue et al. [73] used neutrophils to transport PTX-loaded liposomes through the BBB in a mouse resection model of glioblastoma multiforme (GBM). Mice were treated with intravenous injections of Taxol (10 mg $kg^{-1}$ PTX), PTX-liposomes (10 mg $kg^{-1}$ PTX) and neutrophils carrying PTX-liposomes (5 mg $kg^{-1}$ PTX). Neutrophils were able to infiltrate the surgical cavity and the infiltrating margins of the tumour. As a result, neutrophil-mediated delivery resulted in a significantly higher amount of drug in the tumour, and in the extension of mice survival up to 61 days, as compared to a mean survival of 29 days and 38 days, for the Taxol and the PTX-liposomes groups, respectively.

Similarly, T lymphocytes are attractive BBB transporters, as they have been shown to accumulate in the brain parenchyma even in the absence of neuroinflammation [45]. Ayer et al. [81] loaded PS NPs inside $CD4^+$ helper T cells ($CD4^+$ $T_{EM}$ cells) and demonstrated accumulation in the CNS parenchyma after systemic administration in TNF-α

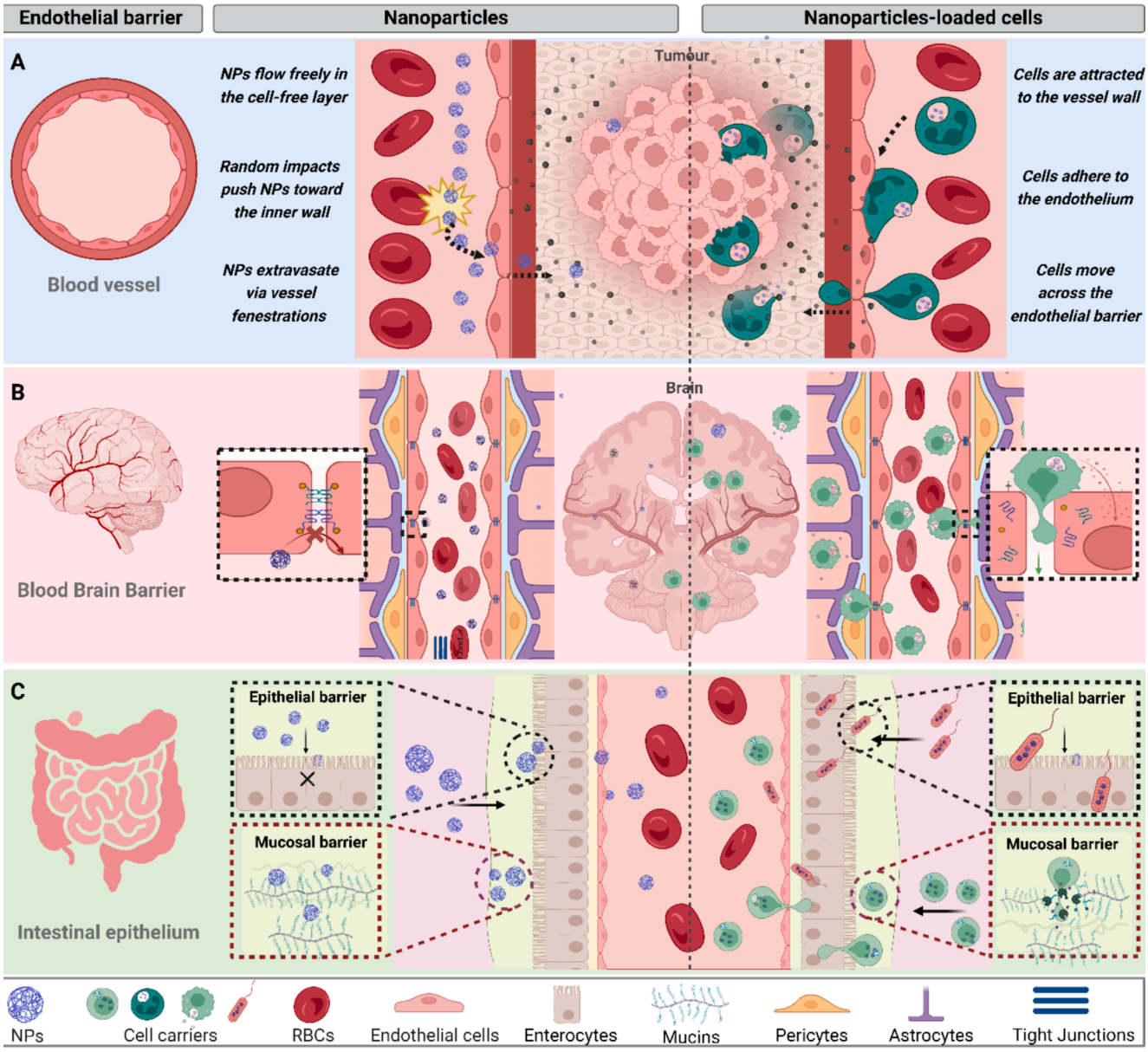



**Fig. 4.** Schematic representation of the endothelial barriers effect on NPs and NPs-loaded cells. A) Blood vessel: NPs are confined to the cell-free layer and, in the absence of functionalization, have limited margination and extravasation due to poor interactions with endothelial cells. In contrast, circulating cells can transport the cargo across the capillary walls as they express receptors for binding to endothelial cells. B) Blood-Brain Barrier: The tight junctions in brain capillaries represent a stringent obstacle to NPs extravasation. Several cells can bypass the BBB and are recruited towards the brain parenchyma in conditions of brain inflammation or tumours. C) Intestinal barrier. NPs poorly interact with the tight intestinal barrier and are not able to bypass the mucosal membrane. Bacteria and immune cells can degrade and infiltrate the mucus barrier thereby bypassing the gastrointestinal membrane, resulting in enhanced NPs transport. Created with BioRender.com.

pre-treated mice. While free NPs did not accumulate in the brain, confocal analysis of the brain sections revealed that 5 % of the administered NPs-loaded T cells crossed the BBB.

The potential of circulating monocytes and monocyte-derived cells (such as macrophages [64] and dendritic cells [47]) to transport drugs across the BBB has also been extensively investigated. The first study of macrophage-mediated drug delivery across the BBB was reported by Dou et al. [78] for the treatment of HIV-associated encephalitis. Murine bone marrow macrophages were loaded with NPs containing the antiretroviral drug Indinavir (IDV-NP), and intravenously injected in a mouse model of HIV-1-related encephalitis. Results proved that macrophages accumulated in the inflamed areas of the brain and were able to transport a significant dose of IDV, leading to a reduction in the number of infected cells as compared to untreated controls.

Pang et al. [46] used murine M1 macrophages from bone marrow to transport DOX-loaded poly(lactide-co-glycolide) (PLGA) NPs to the CNS. They showed that cell-mediated transport significantly improved the mean survival of glioma-bearing mice as compared to mice treated with intracranial injection of free DOX or DOX-loaded NPs (38.5 vs 21–27 days). Similarly, Wang et al. [76] demonstrated that macrophage-mediated transport increased DOX accumulation in GBM, in comparison to free DOX or bare DOX-NPs, while Choi et al. [77] demonstrated that activated macrophages were able to transport gold NPs to brain metastases. Du et al. [66] used resident brain macrophages (microglia) to enhance the delivery of PTX-liposomes to GBM. When administered intravenously, microglia were able to cross the BBB and to efficiently transport PTX, achieving a comparable reduction of the tumour volume as observed for PTX-liposomes with an 8-fold lower drug dose. If administered intracranially, microglia also demonstrated a fourfold increase in the apoptotic index of tumour cells as compared to PTX-

liposomes.

These results demonstrate that the delivery of drugs or NPs to the CNS can be significantly improved through the use of cell transporters previously loaded with the selected cargo.

Other cell types, such as platelets and MSCs could also be used for BBB transport. For instance, platelets have been found to accumulate in brain tissue during neuroinflammation [123] while MSCs have been shown to infiltrate the BBB following their intravenous/intra-arterial injection [124]. However, the efficacy of platelets/MSCs-mediated transport across the BBB following systemic injection has not yet been investigated.

An alternative approach, proposed by Hou et al. [52], is based on the *post-administration* targeting of the selected cell carrier. Briefly, NPs are first surface modified to selectively target the cell carrier in circulation, allowed to be internalized by the selected cell transporter, and transported by it to the desired target. To this aim, they functionalized liposomes with the cRGD peptide sequence to trigger their accumulation in monocytes after intra-nasal administration, followed by monocyte-mediated transport to the CNS. In a rat ischaemic/reperfusion (I/R) injury model, they demonstrated a significantly higher accumulation of cRGD-liposomes in the ischaemic areas and their co-localization with infiltrating macrophages (in comparison to plain liposomes). After administration of edaravone-loaded cRGD-liposomes, a significant reduction of the infarct volume was reported, demonstrating the therapeutic efficacy of this approach.

An interesting, yet poorly exploited, application of cell transporters is the delivery of drugs across the intestinal barrier following oral administration.

Over 80 % of drugs are administered orally, being the oral route safe and straightforward [125]. Unfortunately, proteins and nucleic acids, as well as most anti-cancer drugs are unsuitable for oral administration [126]. This is primarily attributed to the barriers posed by the gastrointestinal tract (GIT), including acidic degradation and enzymatic breakdown, and to the significant side effects of anti-cancer drugs after adsorption through the GIT [127,128]. Consequently, there is a crucial need for effective carriers designed for tumour targeting upon oral administration [82]. While different promising carriers derived from human cells have been identified for most endothelial barriers, only a few are suitable for delivery through the GIT. This is due to the high selectivity of the intestinal mucosal barrier and the challenge of overcoming the stomach's acidic environment.

Bacteria can resist the harsh stomach environment and have been shown to cross the GIT [129]. Fan et al. developed an anti-cancer oral delivery system using an *E. coli* strain (MG1655) [82] previously transfected to produce tumour necrosis factor α (TNF-α). The authors reported selective accumulation of bacteria in the mouse intestinal epithelium 30 min after oral administration and bacteria extravasation into the systemic circulation 120 min from administration. Moreover, the authors showed extended accumulation and significant reduction of the tumour volume in a 4 T1 tumour model, with negligible infiltration in healthy organs.

Recently, Xiao et al. demonstrated the efficacy of plant cell-based drug delivery systems by investigating the oral administration of lyophilized plant cells expressing Green Fluorescent Protein (GFP) in mice. The study focused on the cellular targeting and biodistribution of a tag-fused GFP to enhance GIT endothelium penetration. Three different TAGs were tested: the protein transduction domain (PTD), the non-toxic B subunit of cholera toxin (CTB), and a Dendritic Cell peptide (DCpep). The presence of plant cells expressing GFP in the spaces between the villi of the ileum provided the first direct evidence of the protection of plant cells from the digestive system and their accumulation in the intestinal mucosa. The penetration capability across the mucosa was strongly influenced by the grafted tag. PTD-GFP produced the highest systemic GFP levels, followed by CTB-GFP and DCpep-GFP. Biodistribution showed notably higher GFP levels in the liver and lungs with PTD fusion, while CTB facilitated broader delivery to epithelial cells and DCpep enabled systemic delivery via M cells. Subsequent studies have expanded this approach to the treatment of various diseases (such as Gaucher's disease [130], pulmonary hypertension[131], hepatitis [132,133], and haemophilia[83]) by modifying the therapeutic molecule and targeting tag according to the specific target of interest. In all instances, oral administration through plant cells has demonstrated the ability to maintain the structural and functional integrity of the encapsulated therapeutic agent, effectively protecting it from gastric juices and degradative enzymes [134]. The plant cells can be also employed to deliver conjugated PD/fusion protein through the BBB after oral administration. For instance, Kohli et al.[84]investigated plant cells for the production, bioencapsulation, and oral delivery of myelin basic protein (MBP) and CTB to treat Alzheimer's disease. Oral administration of bioencapsulated CTB-MBP in mice showed successful MBP deposits in the hippocampus as confirmed by immunofluorescence imaging of brain slices. Moreover, the administration of CTB-MBP through plant cells successfully reduced amyloid-β level up to 67.3 % and 33.4 % in the hippocampus and cortex, compared to untreated controls, confirming that the delivery preserved the functionality of the therapeutic agent.

Only few human cells have been proposed to bypass the GIT. Several studies indicated that certain leukocytes (e.g., neutrophils [135] and T-cells [136]) can migrate through the intestinal epithelium and mucosa under inflammatory conditions, suggesting their possible use to enhance chemotaxis-mediated drug transport across the GIT barrier. Despite their use as drug carriers in other contexts (as discussed above), oral drug delivery with leukocytes has never been explored, and their ability to survive the acidic stomach environment remains to be clarified. One potential approach is to protect these cell-based carriers within capsules [137] or hydrogels [138], which are already utilized to preserve peptides and other molecules during oral administration, offering protection from harsh gastric conditions. Nonetheless, such approaches have still to be thoroughly investigated.

### 2.3. Bypassing the TME with cell transporters

Drug penetration and its even distribution inside solid tumours is made difficult by the stiff tumour ECM, resulting in suboptimal exposure of the tumour mass to treatments [139]. Abnormal deposition of collagen, elastin, and proteoglycans contributes to the increase in ECM stiffness and to the generation of high interstitial pressure, hindering the accumulation of drugs and NPs inside tumours [10,102].

Several circulating cells, such as immune cells, leucocytes [111] or platelets [65] can enter the dense tumour ECM despite the high pressure [110] and are found in the heterogeneous TME of several hard-to-treat tumours, including GBM [76,140], pancreatic ductal adenocarcinoma (PDAC) [141,142] and mesothelioma [143], suggesting their potential use as transporters to enhance drug accumulation in these tumours, even in the absence of the EPR effect [40]. For instance, macrophages are attracted by tumour-secreted cytokines [144–146], thereby infiltrating the TME [147,148], and interact with tumour-expressed adhesion molecules [149,150]. Using a tumour-microenvironment-on-a-chip (TMOC), Wang et al. [151] showed that macrophages were able to infiltrate rigid ECM models and transport PTX-NPs deep inside tumour spheroids.

Siriwon et al. [40] conjugated multilamellar liposomes (cMLV) to CAR T-cells prior to their systemic administration in mice bearing an ovarian cancer xenograft. The liposomes were loaded with SCH-58261 (SCH), a compound that enhances the cytotoxic effect of T-cells against tumour cells. They found a 2-fold increase in the tumour concentration of SCH after T-cell mediated delivery, in comparison to free SCH-liposomes. They also reported a significant (50 %) reduction in tumour volume for mice treated with the cell transporter, as compared to coadministration of CAR T-cells and SCH-liposomes, or CAR T-cells alone.

Other tumour-infiltrating cells, such as lymphocytes can be utilized to enhance drug accumulation in tumours. For instance, lymphocytes,

along with T cells and macrophages [152], are part of the heterogenous infiltrating cell population in PDAC and may represent a promising strategy to facilitate drug or NPs transport to this tumour, overcoming its dense stromal barrier.

## 3. Strategies to prepare cell transporters

Several methods have been proposed to load drugs or NPs inside cells, as summarized in Fig. 5. Among them, surface-loading methods rely on the passive adsorption or the chemical attachment of drugs or NPs to the cell membrane, while intracellular-loading methods require the internalization of the cargo inside the cell and its on-site release.

A good loading method should guarantee an adequate drug level within the carrier without compromising the drug or changing the viability and metabolism of the cell carrier. For free drugs, intracellular loading provides protection of the drug from degradation and/or clearance and reduces its systemic side effects. However, the internalized drug may be cytotoxic to the cell carrier [153]. Therefore, the optimal drug loading method should balance the need to deliver a therapeutic dose with the necessity to maintain the viability of the cell carrier. Additionally, the mechanism of drug release from the carrier is still not well understood and, therefore, far from being controllable. Surface loading of drugs on the cell membrane may simplify its release. Nevertheless, the drug adsorption/linking process may alter the membrane elasticity or shield critical cell membrane proteins [154]. Moreover, many works have shown that surface adsorption of NPs on cells can significantly enhance the rate of phagocytosis [155–157].

Loading NPs instead of free drugs in cells may limit the toxic effects on the cell carrier, since NPs reduce exposure of the cell to the cytotoxic drug. NPs shape, size, surface charge, and composition influence the process of loading into and releasing from the chosen cell carriers [158]. The nature of the cell carrier also influences the loading process and its efficiency (see Table 2). For instance, RBCs, which are readily available and easy to manipulate, can be straightforwardly loaded by surface modification or by forcing drug internalization [159]. while cells with relevant phagocytic activity (e.g., neutrophils [73,75] and microglia [66]) or covercytic activity (internalization mediated by the open canalicular system, e.g., platelets[70,160]) are prone to spontaneous uptake of the cargo [161].

Previous works highlighted that most loading techniques rely on extensive *ex vivo* cell manipulation, which may be an obstacle to clinical translation, or may be limited by the scarce availability of the selected cells [162,163]. A possible solution may be the targeting of the cell carrier after *in vivo* administration of the cargo, for instance by using surface functionalized NPs [64,164].

Only few works provide direct information on the loading efficiency of therapeutic agents in cells, while some studies report the amount of therapeutic agent per single cell [73,165]. The following section provides a more detailed overview of the loading approaches used to prepare cell carriers and discusses their characteristics and main applications.

### 3.1. Surface loading techniques.

#### 3.1.1. Passive surface absorption

Passive absorption approaches are based on non-covalent interactions between the cargo and the cell membrane, achieved by incubating the cargo with the selected cells. This technique has rarely been employed to adsorb naked drugs directly on the cell membrane, with only few examples available in the literature [51,175,182]. Indeed, the net negative charge of the cell membrane does not allow stable electrostatic interactions with bare drugs in physiological solutions, resulting in weak bonds, poor stability, and uncontrolled release [183]. Cationic polymeric NPs, such as polyethyleneimine NPs [184] or chitosan [184] NPs, have been proposed to potentiate the cargo-cell membrane interactions. Nevertheless, a strong positive charge is not recommended, since experimental evidence suggests potential cytotoxicity [185].

Some studies showed that various pathogens can non-specifically adsorb on the erythrocyte membranes to avoid MPS detection [186–188]. This evidence inspired a biomimetic strategy known as *red blood cell (RBC) hitchhiking*: a combination of van der Waals forces, hydrogen bonding, and hydrophobic interactions exploited to assemble NPs onto the RBC surface. Chambers et al. [69] proposed the first

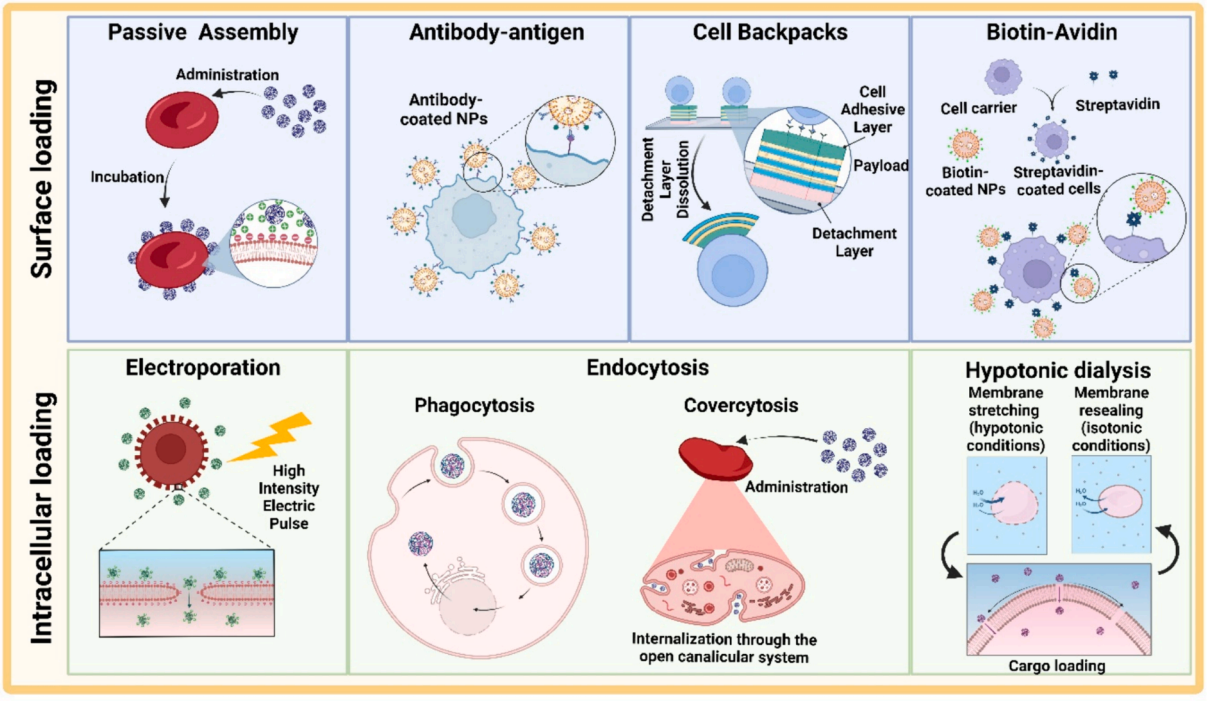



**Fig. 5.** Schematic representation of surface and intracellular loading strategies for the preparation of cell-based carriers. Created with BioRender.com.

**Table 2**
Loading efficacy of drugs or NPs achieved in different cell-based systems with different loading methods. Loading efficiency refers to the percentage of drug or NPs encapsulated in the carrier relative to the amount of drug or NPs initially supplied.

| **Surface Loading** | | | | |
|---|---|---|---|---|
| **Technique** | **Cell-Carrier** | **Cargo** | **Loading Efficacy** | **Ref** |
| Passive Surface Assembly | RBCs | DOX-PLGA NPs | 38.7 ÷ 45.7 % | [166] |
| | RBCs | PS NPs | N/A | [69] |
| | RBCs | Nanogels | N/A | [167] |
| Antibody-antigen interaction | MSCs | DOX-Silica Nanorattles | 22 % | [72] |
| | NK cells | TRAIL- liposomes | N/A | [168,169] |
| | Platelet | Fucoidan-functionalized DOX-loaded micelle | N/A | [164] |
| Cell Backpacks | Lymphocytes | Magnetic NPs-patches | N/A | [170] |
| | Macrophages | FITC-BS-patches | N/A | [171] |
| | Monocytes | DOX-liposomes-patches | ≈22 pmol/cm$^2$ | [172] |
| | Macrophages | Catalase-patches | N/A | [41] |
| Biotin-Avidin Interaction | Macrophages | DOX-liposomes | 52 % | [173] |
| | Neural stem cells | DTX-NPs | N/A | [174] |
| **Intracellular Loading** | | | | |
| **Technique** | **Cell-Carrier** | **Cargo** | **Loading Efficacy** | **Ref** |
| Hypotonic dialysis encapsulation | RBCs | DOX | 17.0 % | [175] |
| | RBCs | Glucocerebrosidase | 40.0 % | [176] |
| Endocytosis | Neutrophil | PTX-liposomes | 18 μg PTX/10$^6$ cells | [73] |
| | MSCs | DTX-NPs | 36 μg DTX/10$^6$ cells | [49] |
| | Microglia | PTX-Liposomes | 0.25 μg PTX/10$^5$ cells | [66] |
| | RBCs | Pravastatin | 94.0 % | [177] |
| | MSCs | DOX-PLGA NPs | 20.98 ± 4.02 pg/cell | [50] |
| | Platelet | DOX | 86.6 % | [70] |
| Electroporation | RBCs (human) | alcohol dehydrogenase | 23.8 % | [178] |
| | RBCs (mice) | alcohol dehydrogenase | 11.04 % | [179] |
| | RBCs | DOX | 0.275 DOX mg / 100 μL of RBC | [180] |
| | RBCs | DOX | 2–3 109 molecules/cell | [181] |

pioneering example of hitchhiking NPs by binding fluorescently labelled 220 nm PS NPs to RBCs through simple mixing in saline solution. The results confirmed that most erythrocytes (99 ± 11 %) adsorbed the NPs without changing their circulation lifetime, resulting in reduced NPs clearance.

From this evidence, RBC hitchhiking has been tested extensively. For example, the natural tropism of RBCs for alveolar capillaries has been exploited to deliver drugs to inflamed lungs [48,184,189]. Once the cell carrier reached the target, the high shear stress in the narrow pulmonary capillaries induced localized particle detachment. Brenner et al. [167] designed an RBC-based system to deliver NPs containing a thrombolytic enzyme (reteplase) to treat pulmonary embolism. They showed that hitchhiking resulted in lung accumulation of the drug, which reached 41 % of the injected dose. Moreover, hitchhiking enhanced the lung-to-liver ratio of the carrier by 17-fold as compared to free NPs, without clogging or aggregation. Adsorption did not compromise the efficacy of the enzyme, which was able to fully dissolve an artificial blood clot. Furthermore, RBC-based systems were able to remove pulmonary emboli *in vivo* without exhibiting off-target side effects, such as internal haemorrhage, ventricular arrhythmia, and cholesterol embolization, commonly associated with reteplase administration [190].

Zhao et al. [166] developed an RBC hitchhiking-based treatment for lung metastases called *Erythrocyte leveraged chemotherapy* (ELeCt). DOX-loaded PLGA NPs (DOX-NPs) were mixed with murine or human RBCs at different NPs:RBCs ratios. Increasing the ratio improved the percentage of particle-carrying cells (up to more than 96 %) with a DOX-loading efficiency ranging from 38.7 % to 45.7 %. *In vivo*, the ELeCt platform enhanced local drug accumulation, enhancing the anti-metastatic effect (up to 300-fold) and the overall survival to 61 days, in comparison to 29 and 32 days for untreated and free NPs-treated control groups.

This drug delivery method has been tested for other applications, including the treatment of COVID-19-related lung inflammation [189] and intra-arterial injection for brain delivery [185]. Moreover, preclinical evidence confirms that RBC hitchhiking results in safe delivery systems able to effectively minimize off-target accumulation and its associated side effects [191,192]. Despite its large application, the assembly efficacy is highly non-specific and poorly reproducible. Lenders et al. [193] demonstrated that the outcomes vary with the erythrocyte source and with NPs properties, in some cases resulting in aggregation or haemolysis. For instance, a higher lactic/glycolic acid ratio in PLGA-NPs was shown to facilitate haemolysis due to the hydrophobic nature of the polymer. The authors also reported that RBCs of rabbit origin were more prone to aggregation once loaded with NPs as compared to human and mouse RBCs, probably due to the lower content of sialic acid on their cell membranes. In addition, the method is not easily extendible to other cell types, as it exploits specific surface properties of erythrocytes, such as high membrane plasticity [194] and lack of endocytic activity [193].

### 3.1.2. *Ligand–receptor interaction*

This loading method requires the specific attachment of the cargo to the cell surface. Monoclonal antibodies, which associate with antigens on the cell carrier, can be used for this purpose. For instance, Li et al. [72] used a monoclonal antibody (mAb) that specifically binds to CD90, a glycoprotein expressed on MSCs, to link DOX-loaded silica nanorattles (SNs) to MSCs. To achieve cell loading, they covalently linked the anti-CD90 mAb to the activated carboxyl groups on SNs. They achieved a 22 % MSCs-conjugation efficiency of CD90-SNs after 1 h of incubation, while only 1 % loading was obtained with un-functionalized SNs. MSC-mediated DOX delivery resulted in improved tumour retention and a higher apoptotic index in comparison to free DOX or DOX-loaded SNs in a mouse model of subcutaneous GBM (U251 xenograft).

P-selectin has also been targeted to attach NPs to the surface of platelets [195,196]. Modery et al. [197] demonstrated the possibility of grafting fluorescent liposomes, on activated platelets by adding a combination of two peptides (i.e., RGD and DAEWVDVS) able to recognize the P-selectin receptors on platelets. Quantitative analysis on the binding stability under different flow conditions demonstrated that the dual targeting strategy produced a 3-fold increased liposome retention on platelet surface as compared to single-targeted liposomes [198]. Activated platelets are extensively present in the tumour microenvironment

and are also able to associate with circulating tumour cells (CTCs) [199]. Therefore, Guo et al. [164] proposed a platelet-based system to deliver DOX-micelles to a metastatic breast cancer model. To this aim, the micelles were functionalized with fucoidan, a polysaccharide with a high affinity for P-selectin on platelets, to promote hitchhiking. A two-fold increase in platelet association of DOX was achieved with functionalized micelles in comparison to unmodified micelles. Moreover, the half-life of platelet-associated micelles was significantly higher than that of free DOX (6.35 h vs. 0.932 h) and of unmodified micelles (6.35 h vs. 4.434 h). In a murine model of breast cancer, functionalized micelles were able to deliver a two-fold higher amount of DOX to the tumour (200 ng/g of tissue) in comparison to unmodified micelles, resulting in a significantly lower tumour volume.

The ligand-receptor interaction approach can be exploited for cell targeting before or after the *in vivo* injection of NPs, therefore allowing association with the transporter cell directly in circulation with reduced *ex vivo* cell manipulation.

Chandrasekaran et al. [168] developed natural killer (NK) cells platforms to treat lymph node micro metastases. To this aim, they functionalized TRAIL-loaded liposomes with an anti-CD57 mAb that recognizes the HNK-1 epitope [200], on NK cells *in vivo* [169]. When injected in mice, TRAIL-loaded CD57-liposomes were successfully associated with NK cells and were able to reduce the metastatic potential of the tumour in comparison to treatment with free TRAIL or with liposomes functionalized with a nonspecific antibody.

In another study, TRAIL-loaded liposomes, functionalized with E-selectin to recognize leukocytes *in vivo*, were proposed for the treatment of prostate cancer metastases [201]. Results demonstrated that association with leukocytes increased the half-life of TRAIL from only 30 min, for the free drug, up to 35 h. TRAIL-liposomes attached to leukocytes significantly reduced the size of the primary tumour in a mouse orthotopic model of prostate cancer. Moreover, a single dose of TRAIL-liposome attached to leukocytes produced a 50 % reduction in the count of circulating tumour cells as compared to untreated control, suggesting a potential application of this system in metastasis prevention.

Ligand-receptor interactions have been exploited for the loading of different NPs on the membranes of different cells, either before or after administration, with promising results. Unfortunately, poor stability of the cargo-cell complex and detachment of the cargo from the cell carrier have been reported. Moreover, the association between the ligand on the NPs surface and the receptor on the target cell may trigger undesired cell response, such as phagocytosis of the NPs or modifications of the cell membrane. For instance, antigen–antibody binding on RBCs may reduce the cell membrane deformability, causing haemolysis [202], while ligand-receptor interactions cannot be applied to cells with high phagocytic activity, such as macrophages [203], since this will rapidly result in the engulfment of the cargo within phagosomes [171], increasing the risk of degradation of the therapeutic molecule or alteration of the cell carrier function [38].

#### 3.1.3. Cell backpacks

The term *Cell Backpack* was introduced to indicate phagocytosis-resistant multilayer polymeric patches capable of stably associating with the membrane of cells with high phagocytic activity, such as macrophages, monocytes, and other circulating cells, without being phagocyted [203].

Swiston et al. [170] presented the first example of a cell backpack based on multilayer polyelectrolyte (PEM) patches, assembled by combining lift-off photolithography and layer-by-layer (LbL) technique. PEMs were obtained through electrostatic interactions between oppositely charged polymers, with an external cell-adhesive layer composed of hyaluronic acid (HA) and chitosan (CS). HA allowed association with B- and T-lymphocytes expressing CD44 receptors. The authors reported a 1:1 association ratio between cells and patches, without cytotoxicity and preserving the chemotaxis-driven migratory ability of the cell carrier.

A different study [171] demonstrated that also macrophages can be loaded with backpacks without affecting their viability and activity. The authors showed that the backpacks adhered firmly to the cell membrane with a low rate of phagocytosis, which was 14-fold lower when compared to free microspheres.

Other authors [41,172] proposed cell backpacks containing NPs within the layers. For example, Polak et al. [172] incorporated DOX-loaded echogenic liposomes (ELIP-DOX) in the polymer backpacks, which were attached to the membrane of monocytes through an IgG antibody. IgG-mediated association with monocytes was stable, but DOX-related toxicity caused a 50 % reduction in the viability of the cell carrier.

A later study by the same group [41] developed catalase-loaded backpacks modified with the CD11 antibody for attachment to macrophages. When administered in mice with brain inflammation, backpack-loaded macrophages were able to deliver catalase across the BBB, consequently attenuating oxidative stress and reducing microglia activation.

Despite the promising results, the internalization of cell backpacks by the cell carrier remains possible [204,205]. Increasing the size of cell backpacks may minimize phagocytosis [206]. However, larger backpacks have a greater likelihood of masking membrane proteins needed for targeting and signalling, therefore reducing the ability of the cell carrier to home to the desired location [207]. Moreover, larger backpacks also increase the risk of cell aggregation [208].

#### 3.1.4. Biotin/Avidin-mediated attachment

The biotin/avidin interaction is among the strongest non-covalent interactions in nature [209,210], representing an extremely specific, non-cytotoxic, straightforward, and inexpensive loading technique [211–213].

Yang et al [173] used biotin-avidin interaction to attach DOX-loaded liposomes to macrophages and exploit their natural tumour-homing and TME infiltration capacity [144].

Biotin was conjugated to the liposome surface, while streptavidin was integrated into the cell membrane of macrophages by incubating the cells with DSPE-PEG3400-streptavidin (STA) for 2 h at 37 °C. Macrophage viability during the entire procedure was monitored and no signs of toxicity were detected. Moreover, DOX loading in macrophages was quantified at 0.52 ng of DOX/cell. The injection of macrophages modified with DOX-NPs in mice bearing an orthotopic breast cancer model, resulted in smaller tumours, lower proliferation rate, higher apoptotic potential, and extended survival, in comparison to mice treated with free drug or with bare DOX-liposomes.

Using a similar conjugation strategy, Mooney et al. [174] linked DTX-loaded NPs to neural stem cells (NSCs), to exploit their natural tropism toward tumours, such as glioma, neuroblastoma, and metastatic breast carcinoma [214–216]. NSC-NP conjugates were obtained by oxidizing the sialic acid moieties on NSCs and by reacting them with biotin hydrazide, to obtain biotin-NSC through the formation of a covalent hydrazone bond [217]. Then, biotinylated NPs were attached to the biotin moieties exposed on modified NSCs through an avidin linker, achieving nearly 100 % conjugation to cells. When administered intratumour, DTX-NPs-NSCs produced a significant reduction of tumour blood vessels and tumour proliferation index as compared to the group treated with free DTX-NPs.

The above studies confirm that the anchoring of nanocarriers through biotin-avidin interaction does not impact cell viability and function. The primary limitation of this loading technique is the potential immunogenic response caused by avidin or streptavidin [218].

### 3.2. Intracellular loading techniques.

#### 3.2.1. Hypotonic dialysis

This technique is based on hypotonic cell lysis, in which fluid

exchange between intracellular liquids and a hypotonic drug solution is induced, followed by resealing of the cell membrane [169]. Briefly, cell swelling, and membrane stretching are induced through hypotonic shock (achieved by e.g., incubation in 30 mM phosphate buffer [219]), leading to the formation of pores in the membrane. Substances, such as drugs or enzymes can enter the cells through these openings, which are then resealed by restoring the isotonic conditions to allow the cells to regain their normal shape and size [176].

Using this approach, Gao et al.[175]achieved DOX encapsulation within RBCs with a loading efficiency of nearly 17 %. DOX-loaded RBCs were then decorated with the photosensitizer chlorin e6 (Ce6) to achieve photo-dynamic cell disruption, and consequent drug release, at the target site. When irradiated for 10 min (660 nm at 5 $mW/cm^2$), the RBC membrane was disrupted, leading to instant DOX release. To assess the efficacy of this light-controllable drug release, 4 T1 cells were incubated with Ce6-DOX-loaded RBCs and irradiated. After 6 h, the intracellular DOX fluorescence in the target 4 T1 cells was significantly higher in comparison to treatment with DOX-loaded RBC without photosensitized and to free DOX. After 24 h, viability assays showed that treatment with Ce6-DOX-loaded RBCs and light irradiation produced a significant reduction of cancer cell viability (<30 % residual viability). Conversely, cells treated with DOX-loaded RBC without light irradiation displayed a residual viability above 50 %.

Protasov et al. developed circulating bioreactors based on human erythrocytes for treating hyperammonaemia, by loading two enzymes involved in ammonium removal from the blood (glutamate dehydrogenase, GDH, and alanine aminotransferase, AAT) using a reversible hypoosmotic dialysis and resealing method. [220] They found that enzyme encapsulation did not affect the haematological indexes of RBCs. Despite the low encapsulation efficiency of GDH and AAT inside RBCs (around 2 % and 11 % respectively), an almost two-fold reduction of ammonium was observed in mouse hyperammonemia models in comparison to untreated controls.

Other research groups developed industrial-scale drug delivery platforms based on osmotic encapsulation [221], which prompted the initiation of clinical trials on *Drug-Loaded Red Blood Cell Technologies,* as highlighted in a comprehensive review by Rossi et al. [222] and by Berikkhanova et al. [54].

For instance, Levene et al. used hypotonic dialysis to load thymidine phosphorylase (TP) into erythrocytes as a treatment for mitochondrial neuro-gastrointestinal encephalomyopathy (MNGIE) [223]. Experiments with allogeneic erythrocytes in mouse and dog models showed that administration of TP-loaded RBCs was safe and allowed significantly higher TP dose than the anticipated clinical dose, without long-term side effects. Subsequent clinical trials [224] on three adult MNGIE patients showed the TP-loaded RBCs had a mean lifespan of 108 days, minimal intravascular haemolysis, and improved sensory ataxia, balance, gait, and distal sensation. Additionally, plasma creatine kinase levels, a marker of muscle damage, decreased, confirming the reduction of the toxic effect on skeletal muscle related to mitochondrial dysfunction. Further investigations were approved for a Phase 2 clinical trial (NCT03866954), although the study was recently withdrawn due to economic reasons.

Hypotonic dialysis is currently one of the most investigated and promising technique for cell-based delivery due to its reproducibility and simplicity, allowing the encapsulation of a wide range of drugs and enzymes [225]. However, to date, the technique has been primarily adapted and optimized for the development of erythrocyte-based drug delivery systems, with only a few successful applications to other cell types, such as monocytes [222]. Early preclinical trials have shown encouraging results with RBCs, leading to the development of several commercial systems [222]. Additionally, some of these systems have been validated in clinical trials that have progressed to phase 3 [54].

Although homologous RBCs from donors have been deemed compatible with drug loading and delivery through hypotonic dialysis [226], some pre-clinical tests in mice treated with allogeneic erythrocytes revealed an increased incidence of thrombi and emboli in the lungs [223]. Moreover, hypotonic dialysis can cause RBC loss and reduce cellular haemoglobin concentration, inducing haemorrhagic-type anaemia and increasing the presence of reticulocytes [219]. The technique can also alter the biomechanical properties of RBCs by changing the structure of the cell membrane, reducing their lifespan and causing premature drug release [191].

### 3.2.2. Endocytosis

The term “endocytosis” refers to a set of mechanisms which allow cells to internalize macromolecules and particles through transport vesicles derived from their membrane [227,228]. Among the various endocytic pathways, phagocytosis—defined as the engulfment of particles, bacteria, or other cells into vacuoles (phagosomes) via the cell membrane—is undoubtedly the most extensively studied mechanism [235,230,86]. Free drugs or NPs have been loaded with this approach mainly in immune cells. Despite the high phagocytic activity, the loading performance of immune cells with naked drugs is often low, with encapsulation efficiency typically below 1 % [231]. Moreover, the cargo may cause toxic effects on the carrier or reduce its migration capacity towards the target site [232,233]. Using drug-loaded NPs has been shown to bypass this issue, as NPs have the potential to preserve the activity of therapeutic agents while reducing their side effects on the cell transporter [75]. For instance, Xue et al. [73] proved that PTX-loaded cationic liposome could be incubated with neutrophils for 50 min to obtain a loading capacity of 18 μg PTX/10 [6] cells without any significant alteration in cell morphology, physiological activity, or chemotactic migration. A similar approach was presented by Du et al. [66] to load PTX-liposomes inside microglia (BV2) for glioma treatment. Microglia were able to deliver their cargo across the BBB, increasing the antitumoral effect of PTX. Indeed, intravenous administration of drug-loaded cells displayed comparable antitumor effects as intracranially administered PTX-Liposomes. Moreover, intracranial administration of drug-loaded cells resulted in a superior antitumor effect in comparison to PTX-Liposomes, as evidenced by a fourfold higher apoptotic index.

Zhao et al. [50] exploited endocytosis to encapsulate DOX-loaded PLGA NPs or free DOX into MSCs. HPLC analysis revealed that the DOX loading efficiency in the NPs group was 90 %, corresponding to a DOX amount of 20.98 ± 4.02 pg/cell. When treating the cells with a DOX concentration in NPs equivalent to 50 μg/mL, the cell viability was above 90 %, while in the free DOX-treated cells the viability decreased below 80 %. *In vivo* distribution studies demonstrated that MSCs could selectively target lung metastases, where they were able to release nearly 96 % of the drug.

Similarly, Pang et al [46] loaded DOX- PLGA NPs inside M1 Macrophages. Their results showed a DOX encapsulation of approximately 34.0 μg in 5 × 10 [6] cells). Moreover, they demonstrated that DOX encapsulation significantly attenuated drug toxicity against the cellular transporter as compared to the free drug, without affecting the tumour infiltration capacity.

Erythrocytes have also been loaded by endocytosis. For instance, El-Din et al exploited RBCs to reduce the side effects and improve the bioavailability of Pravastatin. [177] High drug loading (94 %) was achieved by incubating the cells with the drug at 37 °C for 120 min. Drug loading did not induce toxicity nor alter the haematological parameters of the RBCs, such as mean corpuscular volume and mean corpuscular haemoglobin.

Phagocytosis is the most exploited endocytosis-based approach to induce the internalization of therapeutic agents within the cell body. Nevertheless, not all cells utilize these internalization processes and instead exhibit unique uptake pathways based on alternative mechanisms [160]. For instance, platelets rely on covercytosis to internalize external agents (such as bacteria [234], plasma proteins [235] or particles [236]) through a unique internal structure called the *open canalicular system* (OCS) [237]. The OCS is a complex interconnected network of membrane channels regulating all endocytic and exocytic

pathways in platelets [238]. Cargoes can be internalised either by direct entry through these channels or by being engulfed in pseudopods and invaginations of the membrane, which form vesicles that converge in the OCS [234,239]. A preliminary study by Sarkar et al. [240] demonstrated that cargo internalized through phagocytosis is ultimately metabolized within cells, while materials engulfed via covercytosis retain their integrity within the platelet body. Additionally, platelet activation in tumours promotes the release of granular matter, including the internalized cargo (or parts of it), thereby increasing the therapeutic effect.

Xu et al. [70] exploited platelets as carriers of DOX for the treatment of lymphoma. In their work, platelets were mixed with a DOX solution in PBS and incubated for 1 h under mild stirring (100 rpm). Using a DOX: platelet volume ratio of 1:2, a 46 % drug loading and an 87 % encapsulation efficiency were achieved, without morphological and functional mutations in the cells. *In vivo* studies evidenced reduced drug clearance and longer circulation time for DOX-platelets in comparison to pegylated DOX liposomes. Moreover, treatment with DOX-platelets produced smaller tumours and higher apoptotic index as compared to free DOX at the same concentration.

Despite being among the most explored techniques for drug loading inside cell carriers, endocytosis-mediated internalization is limited to cells with efficient phagocytic or covercytic activity [241]. Furthermore, following endocytosis, NPs can be degraded within the cell in lysosomes or other cytoplasmic compartments, leading to off-target release of the payload [242]. Lastly, the release mechanism of the therapeutic cargo from the cell carriers at the target site is still poorly understood [229].

### 3.2.3. Electroporation

Electroporation is a physical method extensively adopted to increase the accumulation of molecules in cells [243]. Briefly, short electrical pulses are applied to charge the cell membrane, resulting in a rapid and localized structural rearrangement of the membrane with the formation of small hydrophilic pores, called *aqueous pathways* [244]. These openings are associated with a transient loss of permeability, enhancement of ion leakage, and uptake of hydrophilic molecules (such as DNA or antibodies), ions or NPs [245].

A pioneering study from Lizano et al. [178] explored the use of electroporation to encapsulate alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) into human RBCs. These platforms were explored as potential treatments to prevent liver damage resulting from excessive alcohol consumption. The encapsulation method produced a 23.8 % yield for ADH and 31.8 % yield for ALDH and a cell recovery efficiency above 80 %. The process had minimal impact on RBC properties, since ADH/ALDH-loaded erythrocytes preserved their haemoglobin oxygenation capability and exhibited similar haemoglobin derivative levels as native RBCs. The study also demonstrated that loaded RBCs could effectively release ADH and ALDH in culture medium, inducing the enzymatic degradation of ethanol *in vitro*. Only 25 % of the initial ethanol remained after 58 h of incubation with enzyme-loaded RBCs, while no ethanol degradation was induced by native erythrocytes. These findings suggested the potential use of electroporated ADH- and ALDH-loaded RBCs as circulating bioreactors for ethanol metabolism. To this aim, ADH and ALDH were loaded in murine erythrocytes [179]. The encapsulation efficiency of ADH and ALDH was 12.8 % and 11.2 %, respectively. These values are markedly lower than those reported for human erythrocytes, confirming that the source of the cell carrier is a key determinant of the success of the loading process. After IV injection in mice, approximately 50 % of electroporated RBCs were quickly removed from circulation. This short half-life, of around 1 h, was attributed to the clearance of damaged RBCs. The remaining undamaged modified RBCs showed a similar circulation pattern as native erythrocytes (with a clearance of 4.5 days vs, 5.3 days). Biodistribution analysis revealed that the target organs for electroporated RBCs were the same as those for native RBCs. Native RBCs (control group) or ADH + ALDH-loaded RBCs were intravenously injected in ethanol-intoxicated mice. The evaluation of the ethanol concentration in plasma over time demonstrated that ethanol clearance rate was significantly enhanced by the administration of ADH + ALDH-loaded RBCs (0.39 ml/min), as compared to the control group (0.20 ml/min).

RBC electroporation was also proposed by Lucas et al. [180] for the delivery of DOX. RBCs underwent repeated pulses to establish aqueous pathways, favouring the diffusion of DOX from the medium into the cell body. Subsequently, RBCs were incubated at 4 °C for 5 min and then at 37 °C for 1 h to facilitate membrane resealing. Successful encapsulation of DOX in erythrocytes was achieved, with a drug content of approximately 0.275 mg per 100 μL of RBC suspension. Pharmacokinetic studies following intravenous injection of free DOX or DOX-RBC at an equivalent DOX dose showed that RBC-mediated delivery elevated the area under the concentration curve and the DOX concentration in plasma during the initial 24 h. In mice with tumour xenografts, infiltration into the tumour mass and reduction of the tumour volume were improved in comparison to free DOX. Moreover, measurement of hemodynamic and functional heart parameters, including the maximum and minimum rate of pressure, maximum filling volume rate, and cardiac output, indicated an enhanced cardiac performance for the RBC-DOX group compared to free DOX, suggesting that cell encapsulation mitigated DOX-induced cardiotoxicity.

Electroporation was also proposed by Wang et al. [181] to load DOX into RBCs which were also surface modified with iron oxide NPs (IONPs) coated with Ce6. In comparison to free Ce6 loaded-IONPs, modified RBCs exhibited prolonged blood circulation, reduced off-target accumulation, and enhanced tumour homing after magnetic dragging. In mouse subcutaneous tumour models, the synergistic combination of photodynamic therapy and DOX chemotherapy through RBCs resulted in 90 % tumour inhibition, compared to 38 % and 57 % for treatment with single chemotherapy and single photodynamic therapy, respectively.

Despite the ability to enhance phagocytosis in cells of low phagocytic activity such as erythrocytes, electroporation still presents some relevant drawbacks. The technique may produce irreversible damage to the cell membrane or may permanently alter cell morphology, depending on the electric pulse intensity and duration. Moreover, the method is strongly cell-type dependent and primarily applied to erythrocytes. Some attempts have been made to extend electroporation applications to phagocytic cells, such as macrophages [246] or monocytes [247], for enhancing the internalization of anticancer drugs or messenger RNAs. However, the outcomes were not successful, with unsatisfactory release kinetics and a substantial decline in the viability of the cell transporter [246].

## 4. Conclusions and future perspectives

Effective chemotherapy must overcome several challenges linked to the intricate nature of the TME and to the presence of biological barriers. Despite the advantages of NP-based drug delivery systems in terms of passive or active tumour accumulation, [8,248] only a minimal portion of administered NPs effectively reach the target site [10,11].

Leveraging living cells with their inherent affinity for tumours represents an extremely promising approach to improve transport of NPs to tumours [67,68]. In contrast to conventional delivery systems, NP-loaded cells demonstrated superior performances in improving therapeutic agent accumulation across numerous biological barriers. These advantages include limited immune clearance [62], extended circulation, enhanced extravasation, reduced off-target accumulation [64,75], and higher tumour tropism [249]. This improved transport resulted in enhanced treatment efficacy in comparison to equivalent doses of free drug or particles, further confirming the potential of this delivery method.

Different cell types can be used as carriers, depending on the set of biological barriers that the system should negotiate with. For instance, drug delivery across the GIT requires cells with specific ability to resist the harsh environment of the stomach, while also being able to infiltrate

the mucosal barrier in the intestine. Only few cells have been exploited for this purpose, mainly of non-human origin, leaving space for further investigations in this field.

Many cells, including macrophages and platelets, have undergone extensive investigations as transporters across endothelial barriers, because of their natural ability to infiltrate tumours. However, the available studies have poorly considered their intrinsic anti-tumour efficacy. Immune cells may play an anti-tumoral effect in the early stages of tumour infiltration [112]. For instance, microglia possess a pro-inflammatory and tumour-suppressive role, as demonstrated by their suppressive role in the formation of breast cancer-derived brain metastases [250]. T-cells [251,252] and MSCs [253] have been shown to possess intrinsic tumour suppressive properties and have found application in cell therapies [254,255]. Therefore, the therapeutic effect of empty cell carriers, often neglected, should be considered for a full evaluation of the therapeutic potential of cell-mediated transport.

Moreover, most existing preclinical studies are often based on simplified *in vitro* models, offering only partial validation of the interaction capabilities of loaded cells with biological barriers. A better correlation between the *in vitro* characteristics (e.g., loading efficiency, carrier viability, etc.) and the *in vivo* performances (e.g. tumour homing, clearance, therapeutic effect) of cell-based platforms is also needed to better predict therapeutic outcomes and potential side effects in clinical settings.

The selected drug and the loading method also play a fundamental role in determining the performances of cell-based transporters and their penetration across biological barriers. Different superficial or intracellular loading methods are available, each with advantages and disadvantages. However, only a limited number of studies have specifically examined the effect of the loading process of nanomedicines and drugs on the cell ability to penetrate biological barriers [66,81]. Further systematic studies are necessary to identify, under consistent conditions of cell carriers and therapeutic cargo, the optimal loading process to ensure adequate treatment efficacy without compromising the immune evasion ability of the carriers and their infiltration through endothelial and TME barriers [162]. Moreover, literature studies propose different means of quantification of the loading performances achieved with the different methods, making it difficult to systematically compare the different platforms.

Although currently underexplored, synergistic integration of the superficial and intracellular loading techniques may prove beneficial, albeit poorly investigated. For instance, double loading may be exploited for the simultaneous release of different chemotherapeutics, representing a potential solution to multidrug resistance [5,256], or used for dual incorporation of imaging and therapeutic agents for theranostic applications [181].

To facilitate clinical translation of cell-based drug delivery systems, the design of new devices for their efficient, reproducible, and scalable industrial production is strongly needed [257]. Currently, there is limited availability of manufacturing equipment for drug loading inside cells [221], highlighting the need to engineer new integrated platforms that encompass cell factories, continuous-scale production, automatized cell manipulation, and stable drug-loading [54,258].

Moreover, the definition of clear methodologies and clinical procedures is necessary to address the challenges associated with the manipulation of living cells [222,259,260]. Optimization studies are also required to understand the optimal cell type(s) and to maximize cargo loading while preserving cell plasticity and functionality [193,208].

Despite the need for additional validation to fully exploit the clinical potential of cell-based drug delivery, the insights gained from this literature review clearly suggest the ability of cell carriers to improve nanomedicine and drug transport to tumours or other pathological sites, warranting further investigations and validation of this approach.

## CRediT authorship contribution statement

**Andrea Bezze:** Writing – original draft. **Carlotta Mattioda:** Writing – original draft. **Gianluca Ciardelli:** Writing – review & editing. **Clara Mattu:** Writing – review & editing, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

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

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