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1 **Nano-immunotherapeutic strategies for targeted RNA delivery: Emphasizing the role**
2 **of monocyte/macrophages as nanovehicles to treat glioblastoma multiforme**

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43 **ABSTRACT**

44 Glioblastoma multiforme (GBM) is considered the most aggressive and heterogeneous type of brain malignancies.
45 The substantial invasion of the central nervous system parenchyma is a typical hallmark of all grades of glioma. To
46 improve tumor localization and prevent unanticipated toxicity, anti-tumor drug delivery mechanisms must be
47 upgraded in parallel with pharmacotherapeutics. Monocytes can easily pass the blood-brain barrier, and thus, drugs
48 with difficulty entering the brain can be loaded into monocytes, resulting in the treatment of brain cancers. RNA as a
49 natural and biocompatible polymer has many advantages for biomedical applications, and RNA-based therapies can
50 provide regulated biological functions by highly selective and controlling means. In this context, macrophages are
51 excellent carriers for distributing RNA-based treatments; however, developing an efficient macrophage-targeted RNA
52 delivery has remained challenging. Several approaches have been introduced in the last decade to efficiently deliver
53 RNA-based therapy via macrophages to treat GBM and inflammatory conditions. This review summarizes the most
54 suitable nano-carrier systems to deliver RNA into immunocytes; also, different methods of synthesizing RNA-loaded
55 nanoparticles and their application with an emphasis on targeting GBM are discussed. Furthermore, it focuses
56 specifically on the stability of such nanoformulations and the effect of targeting moieties and adjuvant determining the
57 worth of aroused immune response. Finally, the critical aspects of delivering RNA-lipid hybrid nanoparticles (LNPs)
58 via oral, systemic, and local routes are highlighted. We hope that these findings will pave the way for more effective
59 treatment of solid tumors, such as GBM, in the future.

60 **Keywords:** Glioblastoma multiforme; Immunotherapy; Nanotechnology; Targeted therapy

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71 1. Introduction

72 Glioblastoma multiforme (GBM), as the most frequently occurring brain malignancy in adults, has a median survival
73 of 15 months following diagnosis, and ongoing treatment options are almost palliative in nature [1]. The standard
74 treatment for GBM is surgical treatment accompanied by radiation therapy, which only can extend the survival time
75 up to one year [2]. Delivery of drugs to the brain is difficult since this tissue has an extremely effective protective
76 barrier. The same systems that defend the brain from external chemicals also prevent many potentially therapeutic
77 drugs from entering [3]. This barrier, called the blood brain barrier (BBB), is formed by brain microvessel endothelial
78 cells (BMECs), which exhibit essential morphological features including the presence of tight junctions between the
79 cells, the absence of fenestrations, and a decreased pinocytotic activity, all of which assist to constrain the passing of
80 substances from the blood into the extracellular environment of the brain [4].

81 In embryogenesis, microglia and certain central nervous system (CNS) macrophage populations originate from
82 progenitors in the embryonic yolk sac, whereas monocytes can move into the CNS towards becoming macrophages in
83 adults following neurological damage [5]. Tumor-associated macrophages (TAMs) are important participants in the
84 proliferation and survival of cancer cells; hence, studying their immunological features in GBM patients has many
85 potentials. According to the latest findings, variable immune-globulins and T-cells receptors (TCR) are expressed in
86 subpopulations of monocytes as well as the tumor microenvironment [6]. The immunoglobulins sequences of
87 circulating monocytes and TAMs from GBM patients were studied to see if they could be used as new diagnostic or
88 therapeutic targets. Large numbers of monocytes/macrophages infiltrate into glioma cell growth sites and serve a
89 pivotal role in the tumor-associated immune responses, processing tumor anti-gen and presenting it to T-lymphocytes.
90 Basically, glioblastoma-associated microglia and macrophages (GAMMs) originate from brain-intrinsic microglia that
91 become activated in case of infiltration of systemic monocytes that mature to macrophages, or brain malignancies [5].
92 Monocytes in the systemic blood that leave the cerebral circulation change into microglial cells, whereas
93 macrophages/monocytes may promote the malignant tumor growth through undescribed mechanisms [7, 8].

94 TAMs are renowned as vital biomarkers in the diagnosis and prognosis of cancers and may be considered a potential
95 target in tumor treatment [9]. Direct stimulation of tumor cell proliferation and the formation of immunosuppressive
96 surroundings are two ways TAMs commonly enhance tumor growth [10]. Infiltration of TAMs has been linked to
97 poor clinical outcomes in cancer patients [11]. TAMs, which comprise around 30% of the GBM mass population of
98 cells, may underlie, at least to some extent, the immunosuppressive properties of GBM cells. TAMs attracted to the
99 tumor bulk can indeed be reprogramed by GBM cells, leading to an inefficient anti-tumor cancer response.
100 Surprisingly, possible interactions between TAMs and GBM cells increase tumorigenesis [12]. Both monocyte-
101 derived macrophages and CNS-intrinsic microglia are found in GBM tissues obtained from patients and animal
102 models of the disease. This seems to have implications for GBM treatment, as both CNS-penetrating agents that can
103 get into the brain to target microglia and peripherally acting agents to affect monocytes are needed to affect GAMMs
104 [5]. In this connection, Negai et al. reported that targeting TAMs with a recombinant immunotoxin to folate receptor β
105 could effectively suppress GBM progression [13]. GBM-associated peripheral blood monocyte differs from those
106 found in healthy people. Furthermore, immunosuppression and proliferations are promoted by the glioma-associated

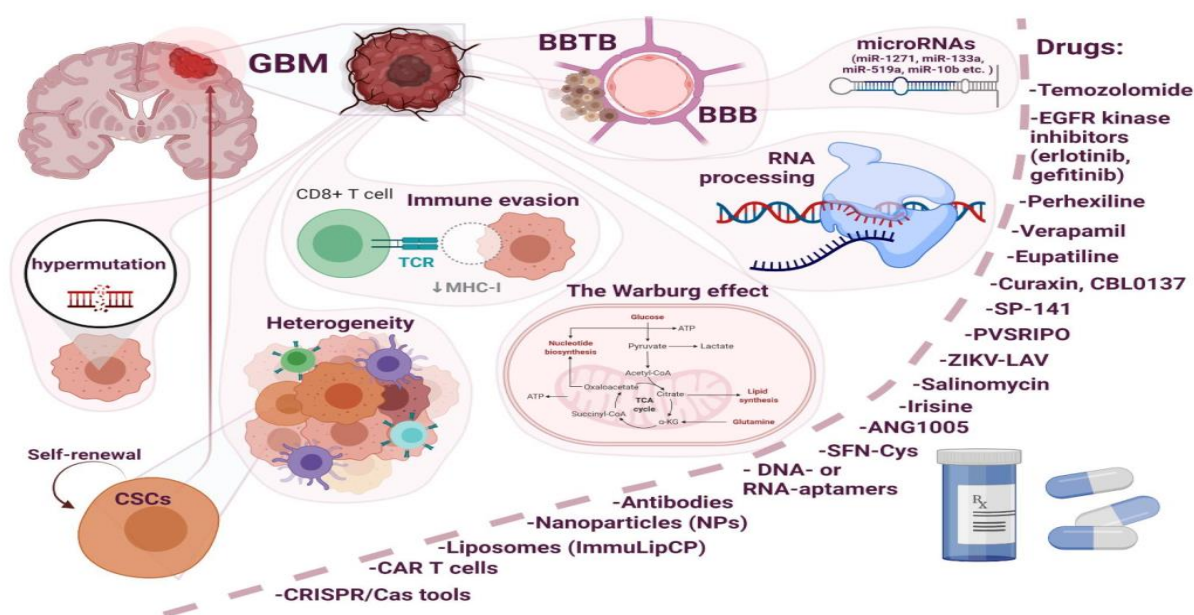
107 blood monocytes by generating exceptionally high quantities of EGF [14]. In addition, using macrophage/monocytes
108 has been envisaged in the clinic for vectorizing therapeutic agents towards GBM treatment [15]. Peripheral
109 monocytes/macrophages can be collected simply from human donor blood as well as the bone marrow, peritoneum, or
110 mouse blood. Cell sorting for the monocyte/macrophage surface protein CD14 is a typical method for obtaining
111 peripheral monocytes/macrophages. Granulocyte-macrophage colony-stimulating factor (GM-CSF) can be used to
112 cultivate isolated monocytes [16-18].

113 Lately, nanomedicine has become a popular term representing the fundamental efforts of the current therapeutic
114 modalities and novel technologies to fight different diseases [19-27]. In this regard, nanoghosts are considered
115 innovative natural nonviral gene delivery platforms that can safely target tumor cells [28, 29]. Employing cells as
116 delivery vehicles allows for focused drug distribution, longer circulation durations, lower tissue, and cell toxicity.
117 Although the design of nano-carriers for cell-mediated drug delivery may not be similar to that of traditional drug
118 delivery systems, exploiting distinct defensive mechanisms in drug administration could open up new pathways for
119 active drug delivery [30].

120 Immunocytes loaded with nanoparticles/mRNA/anti-neoplastic, termed monocyte/macrophage cell membrane-derived
121 nanoghosts, can transport through the blood-tumor barrier or BBB to assist cancer treatments [31]. Because of their
122 capacity for migration and aggregation in the brain, macrophages are appealing carriers for transferring anti-tumor
123 drugs such as nanoparticles. Cancerous cells, epithelial cells, and astrocytes all have a role in controlling
124 macrophage trafficking into the CNS. Another factor is how local releases of BBB permeability-promoting substances
125 play a role. Researchers decided to employ macrophages as nanoparticle delivery vehicles because of the discovery
126 that tumor-infiltrating macrophages aggregated in and around malignant tumors after receiving paramagnetic
127 nanoparticles via intravenous injection (i.v.) [32-34]. In another mechanism, called the "Trojan horse" process,
128 infected macrophages cross-activate BMECs, causing them to colonize in the CNS as infected microglial cells. In
129 GBM, cytokines stimulate the host immune response, and BMECs and immune cells increase the expression of
130 adhesion molecules and their ligands, facilitating the adherence of circulating immune cells to brain vasculature. This
131 type of binding might be the initial stage in passing immune cells through the BBB, known as diapedesis. This
132 closeness may also enhance the passing of small particles between the circulating immune cell and the BMECs,
133 similar to how a virus is transferred between infected immune cells [4]. It has been established that Trojan horse's
134 nanoparticles can be used to deliver nucleic acids to brain tumors [35-37]. A peptide or monoclonal antibody (MAb)
135 can be conjugated to nanoparticles and be used as a molecular Trojan horse. In this scenario, it is important to know
136 which receptors are expressed on the BMECs, so that this Trojan horse can activate the transport of the nanoparticle
137 from the blood into brain cells [36]. It has also been shown that macrophages can pass across the BBB via passive
138 diffusion, which is facilitated by the increased permeability and transport of nanoparticle-containing macrophages that
139 infiltrate these inflammatory organs. Certain functionalized nanoparticles can then enter the CNS under pathological
140 circumstances, particularly in neuroinflammatory disorders, including GBM [38].

141 As a biopolymer, ribonucleic acid (RNA) shares the common characteristics of other polymers and possesses a range
142 of unique properties for applications in nanotechnology and biomedical and material science. RNA molecules have

143 significant roles in biological functions such as gene expression, regulation, and catalysis [39, 40]. RNA molecules are
 144 a new class of therapeutics agents in the recent decade, owing to their structural and functional plasticity, allowing cell
 145 manipulation [41]. Basically, mRNA carries genetic information from DNA to the ribosome [42], and delivery of this
 146 molecule to target sites has recently emerged as a potential therapeutic strategy to combat human diseases [43].
 147 The applications of lipid nanoparticles (LNPs) as mRNA carriers were suggested by the recent improvements in the
 148 use of nano-carriers for RNA interference (RNAi) delivery, particularly the emerging use of the LNPs for this work
 149 [44]. In addition, mRNA transport into malignant cells can be advanced via protecting it from a breakdown in
 150 extracellular compartments and boosting cellular uptake, just like RNAi molecules. Both qualities may be achieved by
 151 encapsulating mRNA in LNPs [30]. Primary issues regarding the development of drug resistance in GBM patients are
 152 shown in Figure 1.



154
 155 **Figure 1.** Main issues related to the development of drug resistance in GBM. Reproduced with permission from ref [45].

156
 157 The current review comprehensively discusses recent achievements in the area of LNPs based RNA delivery, the
 158 significance of innate immunity in the fabrication of RNA delivery systems, and critical aspects of delivering mRNA-
 159 LNPs via different routes of administration. Finally, we elaborated on the effect of targeting moieties and adjuvant that
 160 defines the worth of aroused immune response.

162 2. Contemporary RNA therapeutics for GBM

163 GBM has a poor prognosis despite the presence of typical therapy such as radiation, surgery, and chemotherapy along
 164 with temozolomide. GBM is hard to treat because of its specific biological properties. It is located in the brain and
 165 protected by mechanical barriers such as BBB and blood tumor barrier (BTB) that do not allow therapeutic drug
 166 products to enter into target cancerous cells. Moreover, high heterogeneity, increased diffusion, and the capacity to

167 resist conventional therapies make it difficult to treat [46]. The high heterogeneity of GBM means that it cannot be
168 treated by a single drug, especially biomarker-related therapeutic agents. Diffusive growth, the highest recurrence rate
169 after surgical resection, and its high drug resistance capacity suggest that sustained use of anti-cancer drugs might not
170 be effective against GBM [47]. Some miRNAs are overexpressed in GBM patients [48] or can be detected in
171 cerebrospinal fluid (CSF) presented in the peripheral blood. Therefore, miRNAs can be identified and evaluated
172 noninvasively and contribute to clinical variables of interest [49].

173 Immunotherapeutic strategies focusing on ribonucleic acid (RNA) for targeting malignant cells have advantages over
174 the other treatment strategies.

175 Many peptide vaccines have human leukocyte antigen (HLA) limiting epitopes. However, mRNA deals with multiple
176 tumor-selective epitopes without genomic integration risk. However, through MHC I and II proteins (major
177 histocompatibility complex), the mRNA can be applied across all HLA genotypes [50]. When developing an mRNA
178 vaccine, there are two crucial factors to consider. The first factor is which type of mRNA will be used, and the second
179 is through which route will be administered. The central principle of this approach is to isolate and synthesize tumor-
180 specific mRNA that encodes for antigens specific to tumor cells [51]. The mRNA is translated in the host, and the
181 encoded protein is transferred to lymphocytes with the help of antigen-presenting cells (APCs). This stimulates the
182 host's immune system to initiate an antigen-specific immune response to the resulting peptide, granting the immune
183 system to detect and combat a patient-specific malignancy. Continuous research development necessitates
184 understanding their strengths and potential drawbacks [52].

185 The instability of mRNA and its inability to enter cells properly are its main drawbacks. As a result, the mRNA must
186 be safeguarded, which can be accomplished by loading it into cells *ex vivo*, packaging turned into the virus-like
187 capsid, or encapsulated in a nanoparticle that allows cell internalization. Post-transcriptional gene silencing (PTGS) is
188 a newly discovered, evolutionary conserved, highly efficient, and specific biological mechanism in which the presence
189 of short interfering RNA (siRNA) that shares a homologous sequence with the siRNA for degradation or translation
190 repression [53]. RNA interference (RNAi) is a critical advance in cell biology that has become the method of choice
191 for studying gene function and has opened the door to therapeutic gene silencing for cancer treatment, particularly in
192 drug-resistant tumors. RNAi is a practical approach because only a few siRNA molecules per cell are needed to
193 silence a gene [54].

194 Compared to protein or antibody therapeutics, siRNA molecules are also easily manufactured at a low cost, and RNAi
195 triggers can theoretically be programmed to silence any therapeutically important gene with a known sequence.
196 Furthermore, specific RNAi agents could be used in conjunction with other drugs to improve cancer treatment
197 efficacy. GBM, also known as WHO grade IV glioma, is the most prevalent and deadly type of CNS primary
198 malignancy [55]. GBM is a fast proliferating and aggressive tumor that develops from astrocyte or possibly astrocyte
199 progenitor cells. Although major advancements have been made in brain tumor treatment over the previous decade,
200 including chemotherapy, radiation, and surgical intervention, the disease's prognosis has not improved. These tumors
201 ultimately recur with no effective treatment. The majority of people with this condition die within two years of being
202 diagnosed. As a result, any novel therapeutic technique aimed at this most aggressive tumor will be highly beneficial

[56]. A complicated set of genetic changes have been identified in the formation of GBM, including activation or amplification of oncogenes and growth factors and/or their receptors, because of advancement in cancer molecular biology. Targeted treatments directed against these amplified oncogenes and/or overexpressed proteins implicated in malignant glioma cell proliferation, apoptosis, angiogenesis, or migration could be effective therapy options [57]. RNA interference therapy has been studied in pre-clinical and clinical research to create future clinical treatment methods as a new way to knock down oncogenes or associated proteins in GBM. The use of RNAi in conjunction with ongoing gene-therapy procedures has yielded promising results [58, 59].

Synthetic mRNA is an efficient gene transfection tool with many therapeutic modalities [60]. Because of the instability of mRNA, it was never considered a good option as therapeutic moiety, but recently the interest in clinical utilization of messenger RNA (mRNA) instead of plasmid DNA (pDNA) in gene therapeutic approaches have emerged at a large scale for the last two decades [61]. The biggest weakness of synthetic mRNA gene therapy is its rapid and transient expression, making it unstable but clinically suitable for treating GBM's complexity because of its adaptive convertibility [62]. The most considerable therapeutic application of miRNAs is seen by inhibiting oncogenic miRNAs and replacing tumor-suppressive miRNAs [63]. Some research studies used viral carriers to complete mRNA transfections, but most of the research studies implemented non-viral vehicles. It is proven that mRNA-mediated transfection is an important substitute for gene therapy mediated by pDNA [64]. Various studies have demonstrated that mRNA is capable of resisting the impact of transfection protocols and can translate efficiently. Moreover, mRNA-based gene therapy is safe because of no genomic insertion and no need for the promoter and a terminator [65]. However, nano-scaled target mRNA-bearing lipoplexes, polyplexes, liposomes, and exosomes effectively treat GBM because they can easily cross BBB and BTB in the brain by overcoming natural barriers [66]. In host cells, mRNA-based genes have predominantly improved translational efficiency of foreign mRNA after the discovery of 5'mRNA anti-reverse cap analogs (ARCA), poly(A) tails, and the insertion of additional untranslated regions [67]. Cui and colleagues proposed that the growth and tumorigenesis of GBM stem cells (GSCs) is suppressed by METTL3 and METTL14 due to the down-regulation of ADAM19/EPHA3/KLF4 pathway in GSCs [68]. Methylation of mRNA is an essential mechanism of cellular gene regulation [69]. However, changes in m6A (N6-methyladenosine, m6A) for treating tumors have thoroughly been studied [70].

Visvanathan et al. (2018) described that METTL3 is methyltransferase that promotes tumor growth by targeting the 3' UTR of SOX2 mRNA when highly excessively expressed in malignant GBM with global m6A modification. METTL3 enhanced GBM cell proliferation by targeting the 3' UTR of SOX2 mRNA, while METTL3 silencing was associated with decreased cell proliferation and enhanced tumor radiosensitivity, which can be considered beneficial molecular targets for GBM treatment [71]. In addition, m6A recognition and binding proteins include YTHDFs and YTHDCs, which induce mRNA decay by binding to the methylation site, thus affecting protein synthesis in the development of related tumors. The m6A methylation modification process of mRNA is illustrated in Figure 2.

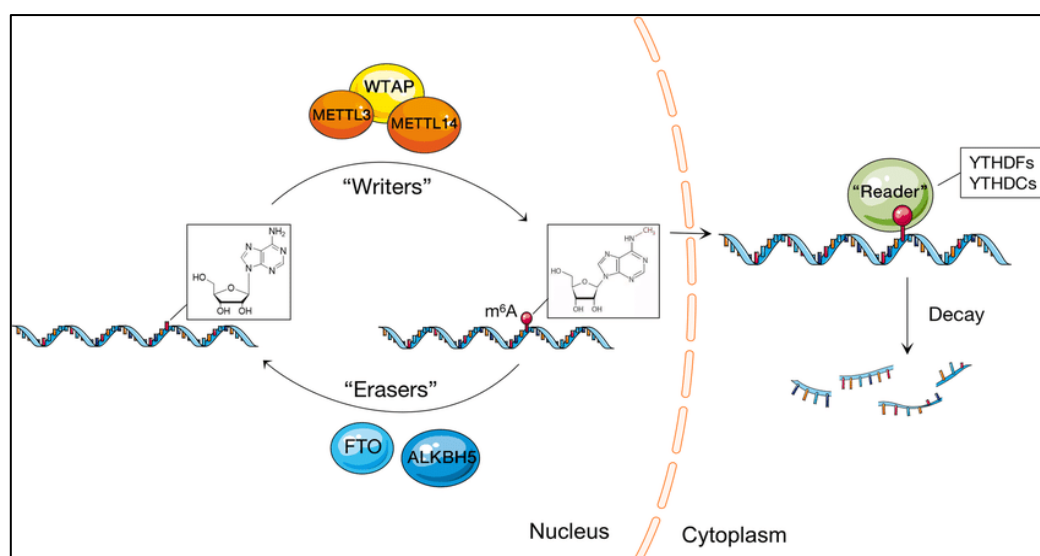


Figure 2. Illustration of the m6A methylation modification process of mRNA. Several methyltransferases, such as WTAP, METTL3, and METTL14, cooperate in methylating the m6A site of mRNA. On the other hand, two esterase enzymes, FTO and ALKBH5, demethylate this site. Other proteins, YTHDFs and YTHDCs, attach to the m6A site of mRNA to induce its decay [72].

m6A is the best-known RNA marker for GBM aggression and progression. However, recent studies have revealed that other RNA markers and modulators, including m6Am (N⁶,2'-O-dimethyladenosine), m1A (N¹-methyladenosine), m5C (5-methylcytosine), hm5C (5-hydroxymethylcytosine), I (inosine), and ψ (pseudouridine) are also associated with GBM progression (Table 1) [73].

Table 1. Role of modifying proteins of RNA in GBM.

Gene Symbol	Gene Description	Role in RNA Modification	Expression	Biological Function	Mechanism	Reference
DCP2	Decapping mRNA 2	m6Am reader	-	DNA damage response and radiosensitivity	-	[74]
ALKBH1	α -Ketoglutarate-dependent dioxygenase alkB homolog 1	m1A eraser	High	Trigger of cell proliferation and tumorigenicity	Induces DNA demethylation on hypoxia response genes	[75]
NSUN5	NOP2/Sun RNA methyltransferase 5	m5C writer	Low	Lead to bioactivate NQO1, the stress-related enzyme, and sensitizes gliomas to their substrates	Causes depletion of protein synthesis	[76, 77]
TET1	Tet methylcytosine dioxygenase 1	hm5C writer	High	-	-	[78]
ADAR1/ADAR	Adenosine deaminase RNA specific 1	I writer	Low	Inhibits cell proliferation	Downregulates A-to-I RNA editing	[79]
DKC1	Dyskerin pseudouridine synthase 1	ψ writer	High	Triggers cell growth	Upregulates N-cadherin, HIF1A, and MMP-2 expression	[80]

A-to-I: adenosine-to-inosine, DCP2: decapping enzyme 2, NSUN5: NOP2/Sun RNA methyltransferase 5, TET1: ten-eleven translocation, ADAR1: adenosine deaminase, DKC1: dyskerin pseudouridine synthase 1, MMP-2, matrix metalloproteinase 2, HIF1A: hypoxia-inducible factor 1 alpha, NQO1: Quaking gene isoform 6.

Alternative splicing patterns in a gene result in various isoforms, which makes the regulatory mechanism of alternative splicing more complex in cancer [81]. Chen and associates reported that prognosis-associated alternative splicing

events are important diagnostic predictor models to predict the clinical prognosis of GBM accurately. Several cancer-specific and prognostic RNA alternative splicing approaches were designed by creating an interaction network to provide potential therapeutic targets for GBM [82]. It has been documented that splicing defects have a higher incidence in tumor cells; hence, small molecules that regulate RNA processing are considered remarkable nanotherapeutics in GBM treatment. Admittedly, mRNA-based gene therapies require few changes in gene expression to become better therapeutic options than present active-targeted strategies, mainly targeting a single protein-coding gene in a signaling pathway associated with oncogenicity. RNA-based gene therapies can develop more radical treatment strategies in the future by an in-depth understanding of RNA biology in GBM treatment.

3. RNA nanocarriers for GBM treatment

Nanoparticles, in general, are subcellular particles with an inorganic or organic composition. Micelles, liposomes, and dendrimers are examples of organic nanoparticles. Silica, gold, quantum dots (QDs), and iron oxide are examples of inorganic nanoparticles [83]. Inorganic nanoparticles can also be used to create RNA-based delivery vehicles *in-vitro* and *in-vivo*. Inorganic nanoparticles' large surface area to volume ratio allows for effective siRNA loading by direct conjugation or non-covalent encapsulation. The ability to modify the surface chemistry of these nanoparticles is a crucial benefit, allowing them to overcome the obstacles of *in-vitro* and *in-vivo* siRNA delivery. Furthermore, inorganic nanoparticles' distinctive physical and optical features can be employed to track siRNA transport to cells or tissues. The limitation of the inorganic nanoparticles is poor degradability, toxicity, poor scale-up, limited efficiency, lack of clinical trials [84].

Various lipids have been utilized for designing lipid-based nanoformulations (lipid nanoparticles or LNPs) in the field of nucleic acid delivery. Delivery systems for nucleic acids have been created using lipoplexes, liposomes, cationic nano-emulsions (CNEs), and nanostructured lipid carriers are all examples of classic liposomes (NLCs) [85]. RNA-based vaccines are comprised of LNPs using sophisticated manufacturing techniques and precise amounts of specific lipid components [86]. LNPs are comprised of ionizable/cationic lipids such as cholesterol, phospholipids, and/or PEGylated lipids, as shown in Table 2.

Table 2. mRNA encapsulated LNP formulation.

Lipid bilayer Structure			Inverted hexagonal structure		References
Cationic/ionizable lipids	Structural helper lipids	Cholesterol	Stealth lipids	PEG forming lipids	[87]
DOTMA, DOTAP/MC3, C12-200	DSPC, DPPC		DSPE-PEG, DMPE-PEG	DOPE	[88]
Nucleic acids complexation Membrane fusion	Bilayer support	Integrity Endosomal release	Hydrophilic surface Steric hindrance	Endosomal destabilization	[89]

*** DOTMA: 1,2-di-O-octadecenyl-3-trimethylammonium propane; DOPE:1,2-dioleoyl-sn-glycero-3-phosphoethanoamine, dioleoyl-3-trimethylammonium-propane; MC3: D-Lin-MC3-DMA; DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane; DSPC: 1,2-distearoyl-sn-glycerol-3-phosphocholine; DSPE-PEG:1,2-distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol).

283 Furthermore, more contemporary LNP delivery methods have been introduced that are more efficient than classic
 284 lipid-based formulations for delivering nucleic acids, such as siRNAs. LNPs have been the most frequently used RNA
 285 delivery system to target a variety of malignancies [30]. Messenger RNAs, small interfering RNAs (siRNAs), and
 286 microRNAs (miRNAs) can all be loaded into LNPs (Figure 3) [90]. A bilayer lipid encapsulating an aqueous core may
 287 not be seen in these advanced LNPs. Instead, they could have a micelle-like shape with small molecules encapsulated
 288 inside a non-aqueous core. Furthermore, LNPs do not participate in electrostatic interactions with the entrapped
 289 nucleic acids [85]. Liposomes offer great biodegradability, efficiency, low toxicity, and ease of production and are
 290 composed of materials with non-polar tail (hydrophobic) and polar head (hydrophilic) groups. [91]. In both infectious
 291 diseases and cancer immunotherapy, liposomes effectively deliver mRNA-based vaccines. One study found that
 292 injecting mRNA–liposomal complexes into the tumor injecting DNA–liposomes to produce in situ tumor transfection
 293 was highly successful and comparable [92]. On the other hand, Lipoplexes are a formulation that arises when cationic
 294 liposomes engage electrostatically with RNAs. As a result of the change from liposomal structure to compact RNA–
 295 lipoplexes, formed lipoplexes have different internal molecular configurations. Since their poor nucleic acid
 296 encapsulation and tolerability, these formulations have been ruled out from clinical trials [93]. Lipoplexes have
 297 remarkable advantages, such as rapid removal from the bloodstream and positively charged composition. However,
 298 they have shown inadequate trapping of RNA molecules while enhanced immunological reaction may occur following
 299 their i.v. injection [94].

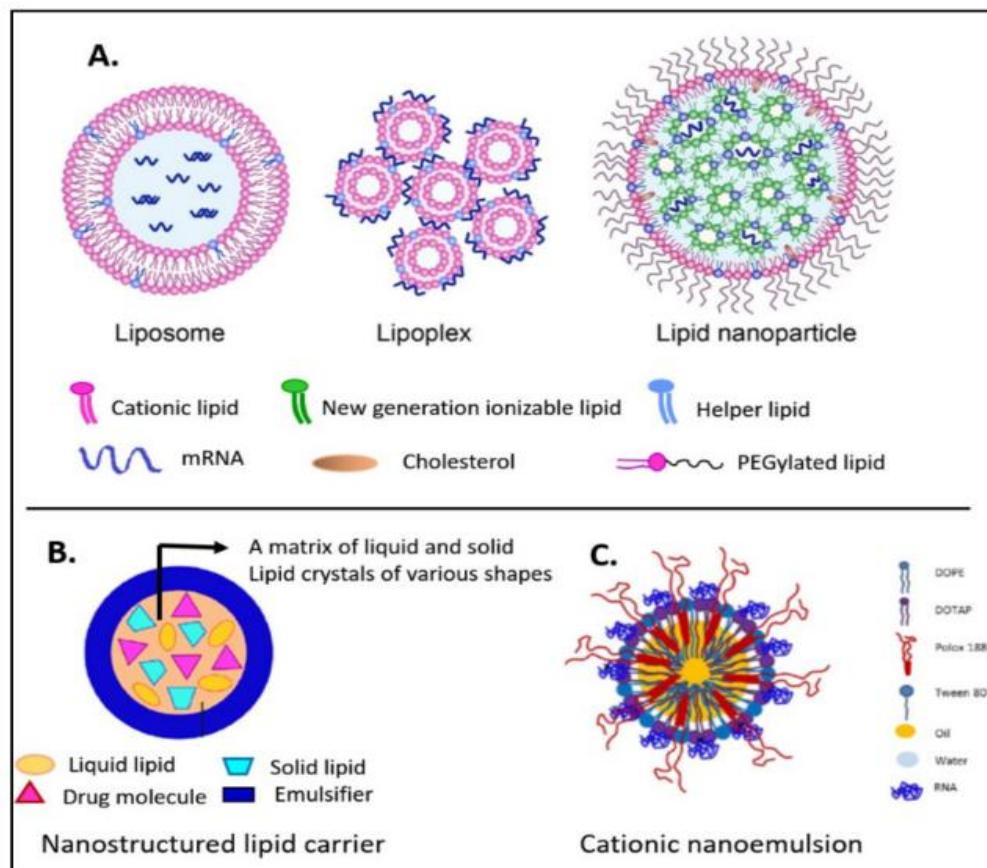


Figure 3. Lipid-based nanovehicles designed for mRNA delivery: (A) liposome, lipoplex, and LNPs; (B) nanostructured lipid carrier; (C) cationic nanomicelles. Reproduced with permission from ref [92].

One of the key advantages of nanomedicine is that it requires less work to produce the vaccine, making it faster and less expensive than other cell-mediated approaches. Moreover, RNA is a toll-like receptor agonist that can facilitate a strong innate anti-cancer response that works in tandem with the adaptive response [95].

Following intramuscular administration, liposomes encapsulating mRNAs trigger a localized inflammatory response, enticing dendritic cells (DCs) to the injection site to engulf the mRNA entrapped particles. DCs that has been activated can be transferred to local lymph nodes to initiate an adaptive T cell response. Moreover, it can be delivered intravenously (i.v.) and spontaneously filtered through lymphoid organs, resulting in DC/myeloid transfection and adaptive T cell immunity induction [96]. Figure 4 illustrates the immunological mechanism involved in the down-regulation of signal and transducer of activation 3 (Stat3) in GBM cells using nanoparticles.

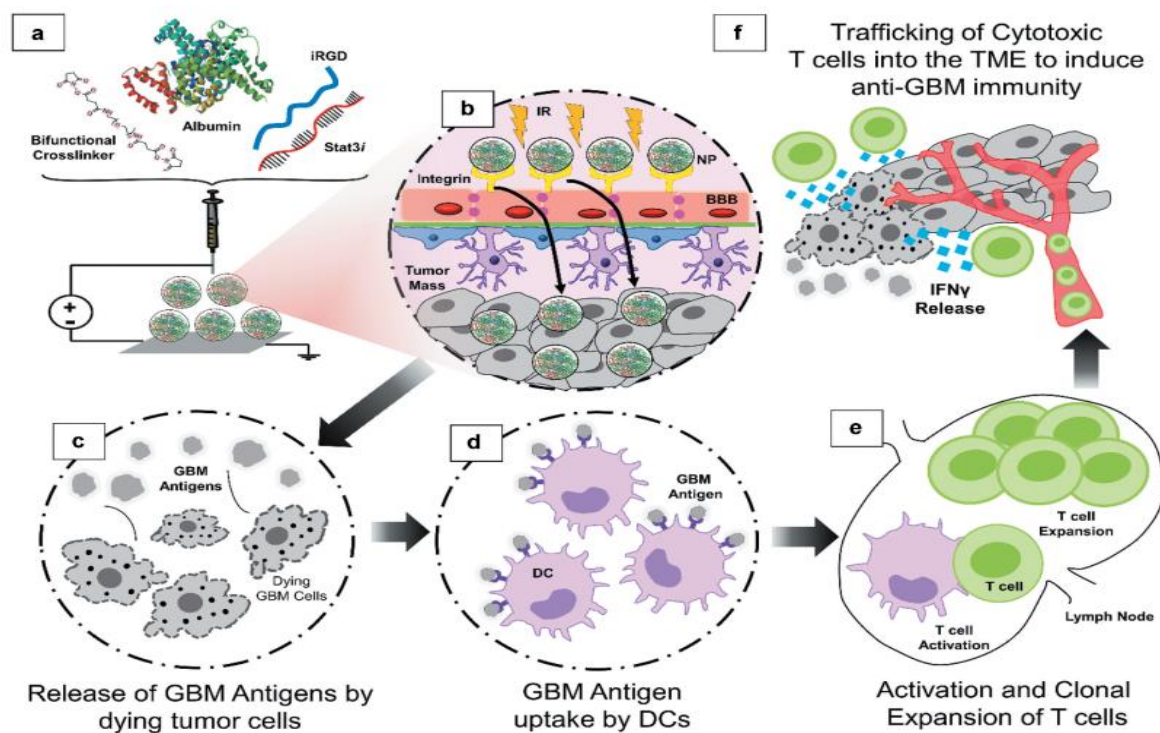


Figure 4. GBM nano-immunotherapeutic mechanism targeting Stat3 (a) Electro-chemically co-jetted siRNA (Stat3i) containing nanoparticles in the size of 200 nM. (b) Nanoparticles delivered systemically Stat3i combined with radiation (IR). iRGD peptides interact with integrins on the GBM cells and BBB, allowing Stat3i to transcytosis into tumor cells. (c) Antigens are released into the tumor microenvironment (TME) by dying GBM cells. (d) When GBM antigens are encountered, DCs in the TME become activated. DCs absorb and digest GBM antigens before moving towards the local lymph nodes, where they can present antigens to CD8+ T cells. (e) T cell activation is facilitated with clonal proliferation. (f) Cytotoxic T lymphocytes migrate to the TME to kill GBM cells [97].

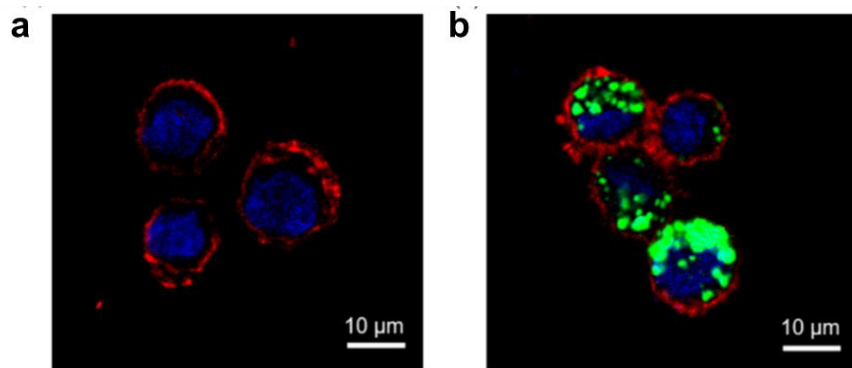
Under clinical investigations, liposomes made up of cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DTOP) loaded with RNA tested on a murine model directed to target the activated DCs, resulting in the proliferation of activated T cells with enhanced survival. Adding the PD-L1 (monoclonal anti-bodies) immune checkpoint inhibitors can increase the following effect. In the canine glioma model, nanoparticles loaded with RNA consider as

safe [43]. The surface modifications of liposomes by encapsulating them with iron oxide allow dendritic cell transport along with MRI. The findings in the murine model showed MRI signal intensity as an early predictor of anti-tumoral immune response and increased survival. The advent of the following techniques may help quick detection of non-responders so the therapy can be adjusted quickly. Furthermore, the mRNAs nanoparticles encode the transcription factor, including the activating kinase IKK β and interferon regulatory factor 5 (IRF5) [98]. These transcription factors may shift macrophages (conversion of M2 to M1 associated with malignancy) that reduce tumor size and increase tumor cell immunity. LNPs were used in one study to distribute miR-124, a microRNA that modulated the activator and Stat3 pathway, reducing tumor immunosuppression. In a mouse model, the miR-124 loaded nanoparticle resulted in enhanced survival [99].

PEGylated lipids, often known as PEG-lipids, are essential components on the surface of LNPs. The PEG domain is attached to the LNP surface in such lipids, while the alkyl chain is attached to the LNP bilayer. PEG-lipids are composed of a hydrophilic PEG molecule connected to a hydrophobic alkyl (or lipid) chain [100, 101]. Thanks to their steric barrier impact, PEGylated lipids in LNPs can lengthen the circulation time of LNPs. These characteristics inhibit the binding of the LNPs to the plasma proteins. In this context, solid LNPs (comprised of low-density lipoproteins) coupled to PEGylated c-MET siRNA reduced c-Met expression in tumors with slower growth. Both naturally derived and synthetic lipids are the most used for RNA delivery. Likewise, solid LNPs (SLNs) comprised of LDL (low-density lipoprotein) components were coupled to PEGylated c-Met siRNA, which down-regulated c-Met expression in tumors with minor progression. Cationic lipids can form electrostatic complexes with negatively charged nucleic acids, resulting in “lipoplex” nanoparticles that cells can endocytose [102]. Significant improvements in siRNA transfection can be achieved through co-formulation of ionizable or cationic lipids with other excipients to create a more stable LNP. In this connection, the cationic/ionizable lipids encapsulated with nucleic acid are frequently combined with naturally occurring phospholipids, cholesterol, or PEGylated lipids to avoid the non-specific uptake and enhance the in-vivo circulation time [103].

Lozada-Delgado et al. synthesized a DOPC-PEG cholesterol-based nanoliposome formulation and targeted miRNA-143 to inhibit glioblastoma tumor progression. Their result reported that the multiple injections of glioblastoma tumor-bearing mice with a miR-143-inh-liposomal formulation significantly reduced tumor growth compared to control mice [104]. Similarly, Lee et al. proposed targeted inhibition of oncogenic miRNA-21 to treat glioblastoma by rescuing tumor suppressors, PTEN and PDCD4. They synthesized three-way-junction (3WJ) based RNA nanoparticles, artificially derived from pRNA of bacteriophage phi29 DNA packaging motor. Their result showed that multi-valentfolate (FA)-conjugated 3WJ RNA nanoparticles constructed to dock anti-miR-21 Locked Nucleic Acid (LNA) sequences (FA-3WJ-LNA-miR21) specifically targeted and delivered anti-miR-21. Furthermore, LNA knocked down miR-21 expression in glioblastoma cells *in-vitro* and *in-vivo* with favorable biodistribution [105]. Shabana et al. developed a strategy for miRNA 603 (miR-603) therapeutic delivery that depresses glioblastoma radiation resistance and causes down-regulation of insulin-like growth factor (IGF1) by forming a complex between miR-603 and polyethyleneimine (PEI), then encapsulating into liposomes modified with polyethylene glycol (PEG) and fibronectin-mimetic peptide (PR_b). $\alpha_5\beta_1$ integrin is overexpressed in glioblastomas and PR_b specifically targets this integrin. They reported that intracellular miR-603 levels increased approximately 22-fold thanks to integrin targeting and

363 complexation of the miRNA with PEI. PR_b liposomes were taken by the patient-derived glioblastoma line (GBM-
 364 CCC-001 cells) while no detectable uptake was observed with non-targeted liposomes (Figure 5) [106].



365
 366 **Figure 5.** Non-targeted liposomes and (d) PR_b-functionalized liposomes incubated for 48 h at 37 °C with GBM-
 367 CCC-001 cells. Liposomes with encapsulated calcein are shown in green, cell membranes in red and nuclei in blue
 368 [106].

369 On the other hand, miRNAs are involved in different cellular processes, such as cell growth and migration, cell
 370 differentiation, angiogenesis, chemoresistance, and programmed cell death [107], and are classified into tumor
 371 suppressors and oncogenic miRNAs (oncomiRs) based on their roles in tumorigenesis [108]. Non-coding RNAs
 372 (ncRNA) dysregulation is seen in almost all tumor forms, including GBM. Previous reports have shown the
 373 dysregulation of miRNAs and lncRNAs in GBM [109, 110]. Such short or long RNA molecules are now considered
 374 biomarkers for GBM prognosis [111].

375 Ciafrè et al. examined the expression levels of 245 miRNAs in GBM using a microarray technique. Their result
 376 showed that miR-221 is markedly upregulated in GBM and brain-enriched miRNAs such as miR-128, miR-181a,
 377 miR-181b, and miR-181c are down-regulated in glioblastoma. Margaret et al. demonstrated that miRNA-138
 378 suppresses GBM cell proliferation through downregulation of CD44. They reveal that miR-138 inhibits CD44
 379 expression by binding directly to the 3' UTR of CD44. Inhibition of CD44 by miR-138 resulted in cell cycle arrest in
 380 GBM cells in-vitro, as demonstrated by a significant increase of p27 and its translocation into the nucleus. In mice
 381 with an intracranial xenograft tumor produced from human patient-derived primary GBM cells, ectopic expression of
 382 miR-138 improved survival rates [112].

383 Several researchers have evaluated the expression levels of miRNA-21, both in individual samples and in glioma cell
 384 lines. Most of their results reported that significant increase in miRNA-21 levels compared to healthy controls [113-
 385 116]. Therefore, these studies show that targeting the miRNA is the best method for GBM therapy. However, applying
 386 RNAi-based modalities for cancer treatment is limited due to several issues. RNAi-based compounds break down
 387 quickly in the bloodstream and are excreted via the kidneys. They induce immunological responses and cannot cross
 388 cell membranes due to their negative charge. Nonetheless, nanoparticles, which lengthen the half-life of RNAi
 389 molecules in circulation, have helped to overcome some of these challenges.

390 The nanoparticle platform is notable for being the most frequently studied for mRNA delivery [43]. The advantage of
 391 this technique is that cancer cells have a high affinity for the nanoparticles' uptake and quickly deliver their cargo,

392 which improves efficacy. However, off-target effects, poor delivery to the target site, degradation by nucleases,
393 undesirable toxic effects, poor binding affinity, endosomal entrapment, and activating the immune system are some
394 limitations of such a delivery system that needs to be tackled [117].

395 Selecting the nanoparticle for the mRNA delivery is necessary, as it influences the preparation technique and
396 nanoparticles' final properties and structures. miRNA-loaded nanoparticles have been prepared using a variety of
397 approaches, including single or double emulsions and nanoprecipitation. The constituent material and required surface
398 properties determine the most appropriate technique selection. The most prevalent method for making miRNA-loaded
399 nanoparticles is emulsion-based. High-speed homogenization or ultrasonication are used in these procedures [118].

400 Some biomaterials used for mRNA delivery are polymer nanoparticles (synthetic polymer and natural polymer),
401 LNPs, and inorganic nanoparticles. The synthetic polymers such as poly(ethylene imine)s (PEIs), poly(ϵ -caprolactone)
402 (PCL), PLGA, and polyurethanes (PUs) have been extensively studied for miRNA delivery [119-122]. However,
403 synthetic polymers effectively encapsulate and deliver miRNAs; their positive charge density and non-
404 biodegradability can impact cell growth and proliferation, limiting their application. Natural polymers such as chitosan
405 and hyaluronic acid are promising RNA carriers, which are non-toxic, non-immunogenic, and have good
406 biocompatibility. The protonated amino groups of chitosan bind quickly and efficiently with opposite-charged RNA
407 molecules at acidic pH [123].

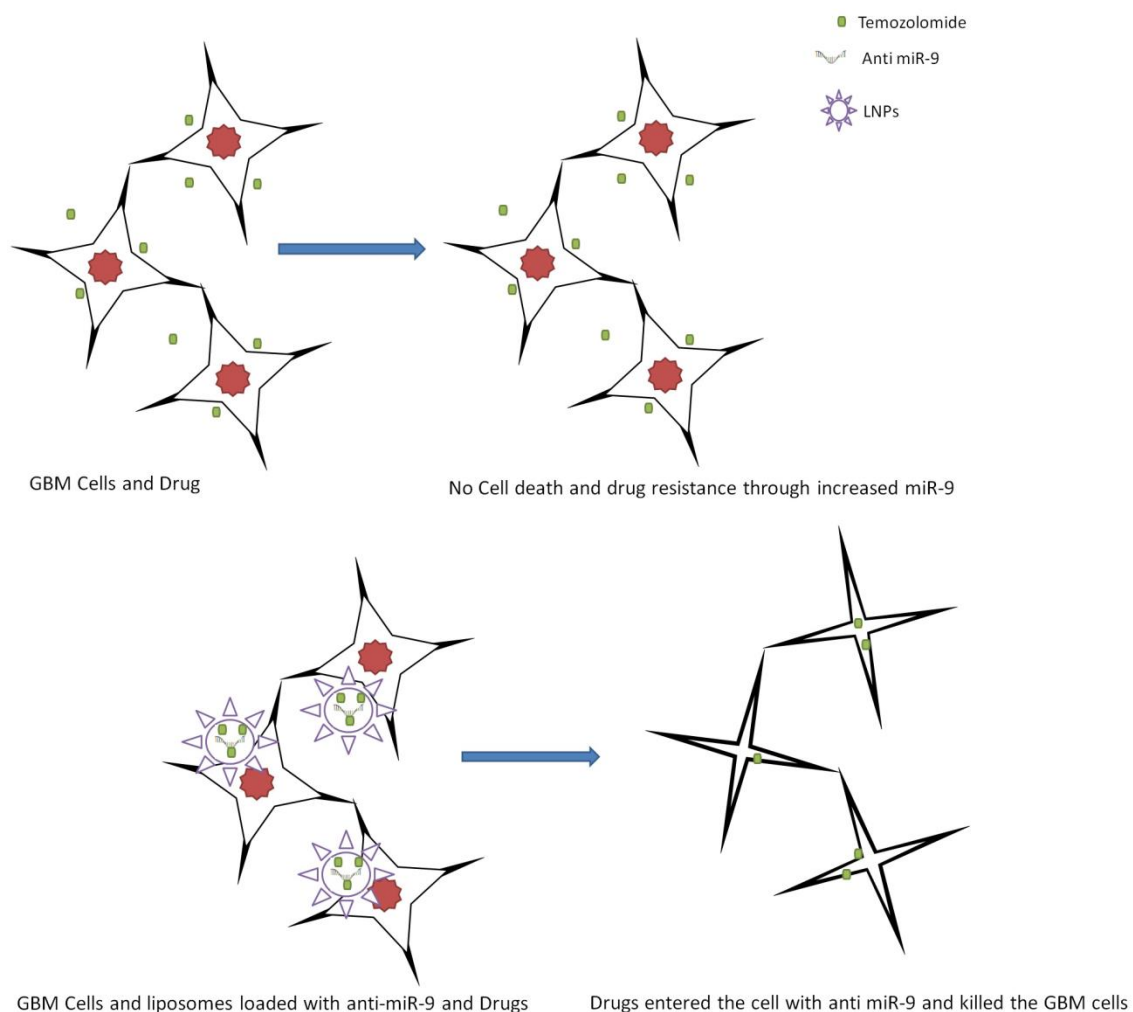
408 Unluckily, chitosan nanoparticles strongly interact with loading agents resulting in inefficient complex unpacking in
409 the cytoplasm, therefore, have limitations for RNA delivery. Hyaluronic acid is a highly hydrophilic anionic
410 natural polysaccharide; HA receptors can recognize that on cells [123]. However, surface modification with a
411 synthetic polymer is necessary for efficient RNA delivery.

412 Similarly, Seo and his colleagues created two anti-miR-21 nanoparticles that suppress miR-21. One was made up of
413 RNA and a cationic poly(amine-co-ester) (PACE), while the other was composed of peptide nucleic acid (PNA) and a
414 block copolymer of poly(lactic acid) and hyperbranched polyglycerol (PLA-HPG). Their findings revealed that both
415 nanoparticle products promote the efficient intracellular distribution and miR-21 inhibition in human glioblastoma
416 cells, resulting in PTEN overexpression and apoptosis. Furthermore, when given to animals with intracranial gliomas
417 by convection-enhanced delivery, both combinations generated considerable miR-21 reduction and provided
418 chemosensitization, resulting in better survival when combined with chemotherapy [124]. This study shows that the
419 local delivery of miR-21 inhibitory nanoparticles as adjuvant therapy for glioblastoma is feasible and promising.

420 Kozielsk and his co-researcher demonstrated that siRNA delivery with synthetic bioreducible poly(beta-amino ester)
421 (PBAE) polymeric nanoparticles is glioblastoma-selective, thereby avoiding potential side effects in healthy cells. The
422 PBAE nanoparticles were loaded with siRNAs that could potentially target anti-GBM genes such as Robo1
423 (roundabout homolog 1), YAP1 (the ortholog of *Drosophila* Yorkie), NKCC1 (Na⁺/K⁺/2Cl⁻ cotransporter), EGFR,
424 and survivin. They report that the Robo1 (roundabout homolog 1) siRNA delivered by PBAE nanoparticle triggered
425 GBM cell death, combined with the other anti-GBM genes. Most importantly, their result showed that the
426 nanoformulation encapsulating anti-GBM siRNAs triggered cell death in an *in-vitro* model of GBM, suppressed cell
427 migration, and also decreased tumor volume after intratumoral administration [125].

428 Similarly, another research group targeted tumor-associated macrophages with mRNA-loaded PBAE. TAMs typically
 429 have an M2 phenotype, allowing them to fulfill both immunosuppressive and tumor-promoting roles. Zhang and his
 430 co-researcher delivered in-vitro-transcribed mRNA encoding M1-polarizing transcription factors to reprogram TAMs
 431 without causing systemic toxicity. Loading nanocarriers with mRNAs encoding interferon regulatory factor 5
 432 combined with its activating kinase [I κ B kinase or IKK] inhibited the immunosuppressive, tumor-supporting role of
 433 TAMs and reprogrammed them to a phenotype that induces anti-cancer immunity and enhanced tumor remission in a
 434 melanoma model [126]. These nanoreagents are safe to use multiple times. This immunotherapy may allow doctors to
 435 avoid systemic medications that alter immunological homeostasis while preventing suppressive tumors for clinical
 436 purposes.

437 In temozolomide (TMZ)-resistant GBM cells, miR-9 levels were shown to be higher [127]. P-glycoprotein, a drug
 438 efflux transporter, was found to be regulated by miR-9 [128]. Munoz and his team developed mesenchymal stem cell-
 439 derived exosomes with functional anti-miR-9 for GBM treatment. They delivered anti-miR-9 to resistant GBM cells
 440 and found that it reversed the expression of the multidrug transporter and made the GBM cells more sensitive to TMZ
 441 and increased cell death and caspase activity [129], as seen in Figure 6.



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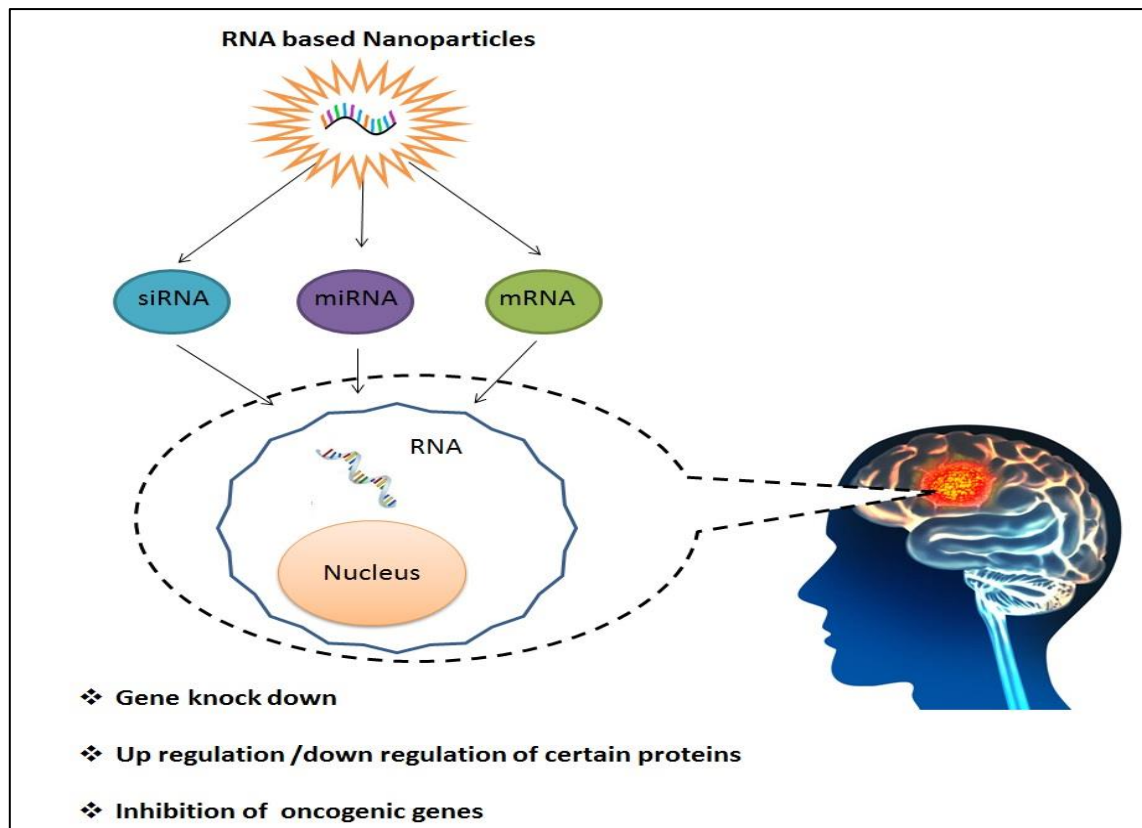
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Figure 6. The schematic illustration of exosomes loaded temozolomide and anti-miR-9 against GBM cells.

444

445 In another study, Wang and his co-researcher reported the fabrication of an iron oxide nanoparticle-based system to
446 deliver siRNAs to suppress the temozolomide (TMZ)-resistance gene. The chemotherapeutic medication TMZ is the
447 gold standard for treating GBM. However, drug resistance caused by the DNA repair protein O6-methylguanine-DNA
448 methyltransferase (MGMT), which removes the TMZ-induced DNA lesions, limits its therapeutic efficacy. Their
449 result showed that siRNA conjugated chlorotoxin-loaded iron oxide nanoparticles (NP-siRNA-CXT) overcome
450 biological barriers, binds specifically to tumor cells, and reduce MGMT expression in tumors of mice bearing
451 orthotopic glioblastoma serially-passaged patient-derived xenografts. Furthermore, this novel formulation increased
452 apoptotic cell death in GBM stem-like cells and markedly enhanced survival when compared to mice treated only with
453 TMZ [130].

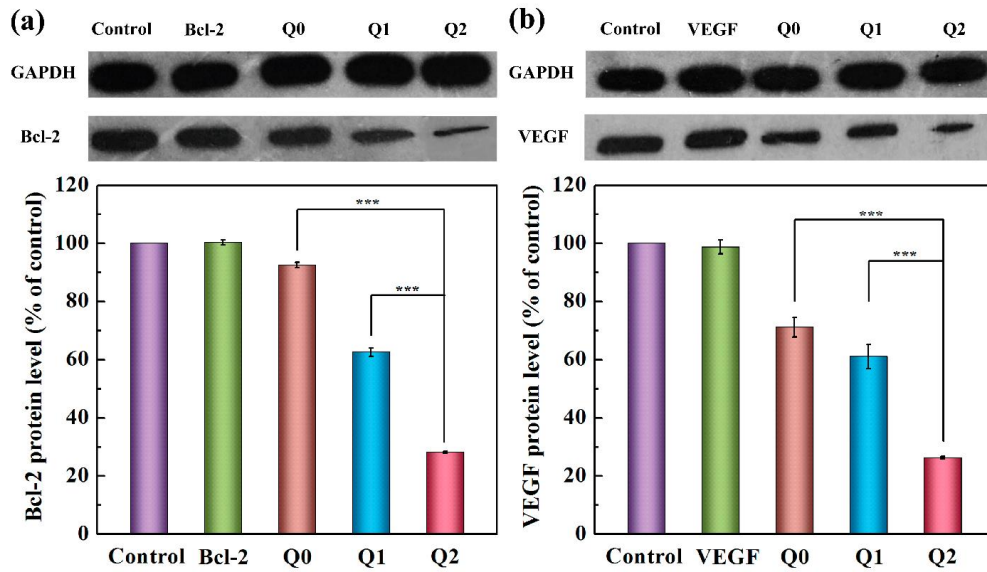
454 Moreover, the intrinsic magnetic properties of iron oxide nanoparticles make them great tools for site-specific
455 magnetic targeting and promising contrast agents for magnetic resonance imaging (MRI) [131]. Yulin Zhang and his
456 colleagues proposed innovative local chemotherapy involving the construction of cisplatin and glutathione peroxidase
457 targeting siRNA-loaded iron oxide nanoparticles as a treatment for patients with glioblastoma after surgery. Their
458 combinational nanoformulation revealed the therapeutic effects demonstrated negligible cytotoxic effects through
459 targeted ferroptosis and apoptosis [132]. Grabowska and colleagues recently reported a new strategy for RNA
460 interference treatment for GBM based on magnetic nanoparticle delivery of double-stranded RNA with homologous
461 sequences to tenascin-C (TN-C) mRNA, named ATN-RNA. The developed nanocomposite, which consisted of PEI-
462 coated magnetic nanoparticles conjugated to dsRNA, demonstrated excellent effectiveness in ATN-RNA transport,
463 resulting in considerable TN-C expression suppression as well as tumor cell movement inhibition. Furthermore,
464 produced nanomaterials have strong contrast qualities in magnetic resonance imaging and very low cytotoxicity, as
465 well as no interferon response induction [133]. Taking the recent research works together, these studies provide an
466 experimental basis for the clinical use of such therapeutic combinations. The schematic representation of the RNA
467 based nanoparticles for glioblastoma therapy (Figure 7).



468

469 **Figure 7.** The schematic representation of the RNA nanoparticles for GBM therapy.

470 Gold nanoparticles are the other nanoparticles that can be used for obtaining vector/DNA polyplexes at the nanoscale.
 471 For example, Qiu and his colleagues developed gold nanoparticles (AuNPs) based β -cyclodextrin modified
 472 polyamidoamine G5 (PAMAM G5) as a vector for enhanced delivery efficiency of two different therapeutic siRNA
 473 (B-cell lymphoma/leukemia-2 (Bcl-2)-siRNA and vascular endothelial growth factor (VEGF)-siRNA) into
 474 glioblastoma cells. They reported that modifying β -cyclodextrin-PAMAM G5 with AuNPs led to obtaining increased
 475 gene delivery efficiency. In addition, they examined the oncogene silencing efficiency of AuNPs- β -cyclodextrin-
 476 PAMAM G5-BCI-2 and AuNPs- β -cyclodextrin-PAMAM G5-VEGF polyplexes in glioblastoma cancer cells by using
 477 Western blot assays (Figure 8). They used naked siRNA as a control and GAPDH protein as a reference protein. They
 478 reported that the results showed that AuNPs- β -cyclodextrin-PAMAM G5 (Q2) exhibited excellent siRNA
 479 transfection ability and that the formed polyplexes could silence genes in cancer cells [134].



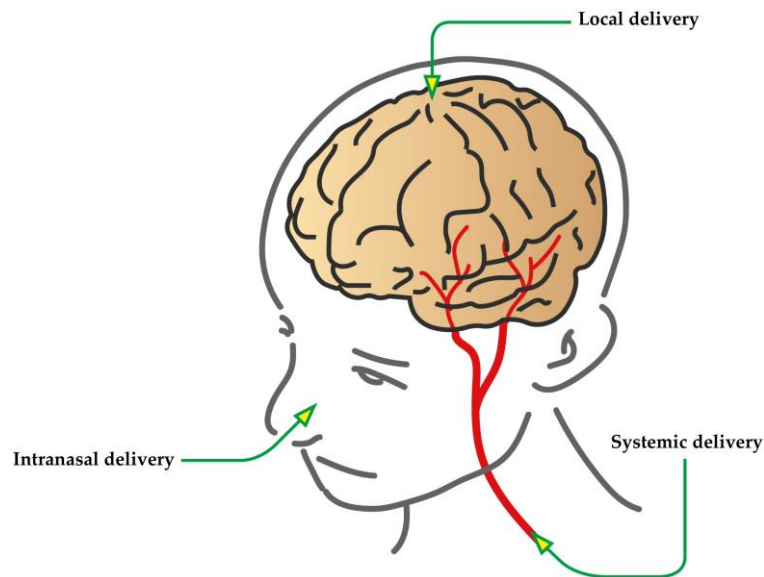
480

481 **Figure 8.** Relative Bcl-2 (a) and VEGF (b) protein expression levels in U87MG cells transfected with vector/siRNA
 482 polyplexes at an N/P ratio of 5:1. The GAPDH protein was used as a reference for the Western blot data. For (a,b),
 483 *** means that the p values of statistical analyses are <0.001 (the AuNPs- β -cyclodextrin-PAMAM G5 (Q2) vector.
 484 AuNPs-PAMAM G5 without β -CD modification (Q1) and pristine PAMAM G5 dendrimer) [134].

485

486 4. Factors affecting the *in-vivo* delivery and uptake of RNA/LNPs

487 Advancement in Molecular Medicine mRNA-based therapeutics provides pace to serious issues requiring efficient
 488 targeting delivery. The accessibility towards the target organ depends on the delivery of mRNA easily. Delivery of
 489 mRNA is still a halt to explore more means. Generally, drugs can be delivered to the brain by local brain site injection
 490 or direct drug administration by invasive techniques. Figure 9 shows various brain delivery techniques that display the
 491 major routes: local, intranasal, and systemic delivery.



492

493 **Figure 9.** Various brain delivery techniques displaying the major routes: local delivery, intranasal delivery, and systemic delivery
 494 [135].

4.1. Administration routes

4.1.1. Intravenous route

The selection of route of administration is a critical parameter for the delivery of RNA. The appropriate route provides the efficacy and shelter of RNA to reach their specific site for response [136]. LNPs deliver RNA molecules either by invasive or non-invasive route with certain limitations into the brain. CNS exhibited barriers that make drug delivery more complex to reach towards the target site. To target delivery towards the brain, encapsulate the therapeutic agent either into liposomes, dendrimers, micelles, cell-penetrating peptides, and other lipid base nanocarriers. Intravenous LNPs containing ionizable lipids have been reported to accumulate in the liver in an apolipoprotein E (ApoE)-dependent manner through the low-density lipoprotein receptor (LDLR) [137]. The brain is another tissue with a high ApoE content in terms of producing derivative particles [138]. Particles in the brain are taken up by astrocytes and neuronal cells for anti-inflammatory and antiapoptotic functions, respectively. In addition to lipid metabolism, exclusion of amyloid beta also occurs on account of ApoE [139]. Hence, it is hypothesized that ApoE-LDLR-mediated uptake of LNP occurs in the fluid of the brain as well as in the liver. ApoE mediated uptake of intravenous LNPs is one of the key features which make them useful for targeting neuronal cells [140]. Another intravenous polyplex nanomicelles of polycation poly [N9-[N-(2-aminoethyl)-2-aminoethyl] aspartamide] encapsulated mRNA targeted into CNS. This system employed high capacity, endosomal escape due to pH responsive release, easily degraded into nontoxic end product, and stable in physiological fluid.

4.1.2. Local routes

Besides the circulatory route, the local route also has certain limitations for delivering RNA to its target site. Several hurdles earn by mRNA before it can be successfully translated into protein. The local administrative route includes non-invasive nasal, pulmonary that have limitations in delivering mRNA [141]. The nasal route is considered more convenient, permeable, and highly vascularized. In the intranasal route, encapsulated mRNA/LNPs reach the brain from the nose via the trigeminal nerves by bypassing the BBB. The liposome-encapsulated " β -breaking" peptide known as H102 has been shown to exhibit low toxicity and effective drug concentration in the brain via the intranasal route [142]. In another study, it was reported that green fluorescent protein (GFP)-mRNA adsorbed to pegylated and lipid-coated cationic core nanoparticles were used for the intranasal route. The results showed 30% transfection in DC2.4 cells *in vitro* and successful expression *in-vivo* [143]. The fact that the intranasal route is a non-invasive technique and helps to reduce the amount of dose administered to the therapeutic range compared to intravenous administration with high non-specific uptake in peripheral tissues has made it a very remarkable technique [144, 145]. Another study reported by Madane et al. that curcumin encapsulated NLCs via intranasal delivery to CNS. *Ex-vivo* and *in-vivo* studies confirmed the highest permeation of curcumin into the brain through the nasal route. Temozolomide (TMZ) was used for brain tumors administered by the intranasal route and proved significant accumulation of TMZ into the brain [146]. The mucosal surface of the nasal cavity is scanty for enzymatic activity, and mucociliary clearance is a primary hurdle for any delivery system to clear rapidly [147]. Previously reported data indicated that the olfactory route had been utilized to deliver LNP-RNA into the brain. Chitosan nanoparticles reduces

the expression of certain genes in brain parts of the cortex and striatum, while siRNA was restricted to the olfactory bulb. The capillary size of CNS is small enough (100 μm), so targeted nanoparticles should be appropriate to diffuse these passages into the brain. LNP and gold nanoparticles adopted intracellular pathways to deliver their cargo into the brain efficiently [100]. However, few studies reported the administration of mRNA/LNP through transdermal route. Local delivery can also provide systemic responses. Local administration of LNP-mRNA formulations enables supplementation of therapeutic proteins in specific tissues, such as heart, eyes and brain. Previously reported studies revealed that uncharged LNP with an appropriate size and sufficient PEGylation exhibited maximum uptake by the lymphatic system [148]. The highest uptake of LNP by the lymphatic system can be achieved by the surface functionalization of LNP with sugar moiety like mannose to preferential uptake by dendritic cells found in lymph[149] to activate T cell immunity LNP-RNA can overcome barriers to reach targeted site of brain via intradermal administration [150].

4.2. Colloidal stability of nanoformulations

To ensure the therapeutic efficacy of formulation, stability is another crucial parameter to consider before administration. It is away from any suspicion that particle size, charge, and structural conformation may be significantly altered when interacting with blood proteins. Indeed, mRNA/LNP interacted with blood proteins. It becomes adsorbed on the surface of nanoparticles and forms biomolecules, which reduces the stability of LNP and forms agglomerates that cause premature release of cargo [151]. Figure 10 represents different routes of administration, formulation stability, and barriers encountered in the *in-vivo* delivery of mRNA-LNP.

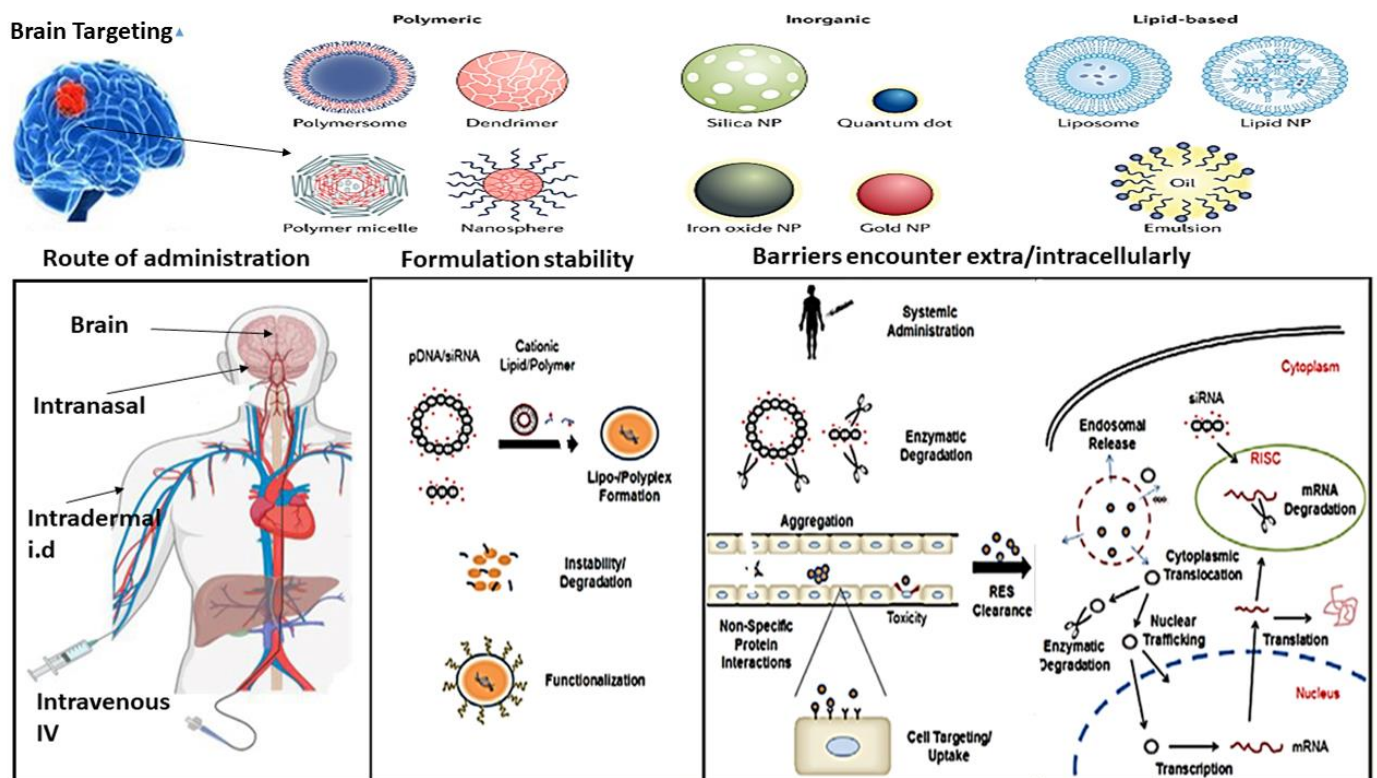


Figure 10. Representation of different nanocarriers for different routes of administration, formulation stability, and barriers encountered in the *in-vivo* delivery of mRNA-LNP to brain.

LNP stability can be achieved by alternating their lipid composition [152]. The introduction of cholesterol tends to increase the rigidity of LNP because it reduces the lipid membrane permeability and enhances the strength and stability of LNP in serum. Fabrication of stable positively charged LNP can be utilized to enhance the permeability of formulation across BBB. Cationic SLNs 3beta -[N-(N'-N'dimethylaminoethane) conjugated with TfR antibody has also been utilized to deliver baicalin into the brain efficiently [153] Another strategy to improve stability of a formulation is synthesizing PEGylated lipids that can increase the retention time of LNP into the circulatory system as it permits the escape of LNP to being engulfed by macrophages [154] present in the brain. Among lipid nanoparticles, especially solid lipid nanoparticles (SLNs) are widely used in drug delivery systems. SLNs, which have a solid hydrophobic lipid core, play an important role in crossing the reticuloendothelial system (RES) of the BBB and exhibit improved physicochemical stability, high bioavailability, sufficient drug loading capacity, and tissue distribution [155, 156].

4.3. Incorporation of targeting moieties

Receptor-based internalization and biodistribution of mRNA into targeted organs can be accomplished by incorporating targeting agents and several antibodies on the LNPs [157]. Perche et al. fabricated mannose-coated mRNA nanoparticles to promote mRNA transfection into splenic dendritic cells [158]. Moreover, targeting moieties can also facilitate an endocytic mechanism to protect nucleic acid from degradation. Previously reported data showed that capping of nanoparticles with angiopep-2 improved delivery of LNP into brain cells as it binds to lipoprotein receptor-related protein-1(LRP-1) present on BBB shown in Figure 7. Another study evidenced the efficient delivery of docetaxel via angiopep-2-coated SLN. Encapsulation of etoposide into SLN conjugated with melanotransferrin antibody permeates easily across BBB due to overexpression of melanotransferrin into endothelial cells of BBB. Although the binding of targeted antibodies to the lipid formulation to deliver mRNA with more efficacious means might escalate the complexity, cost, and monitoring issues for the LNP synthesis process [159]. Despite captivating clinical outcomes, the abovementioned factors should also pay special attention. Dan peer et al. prepared an *in-vivo* targeting stage for the specific targeting of siRNA-LNP to leukocytes. Inclusion of lipoprotein of LNP with the non-covalent binding at the Fc site of specific antibodies provides an additional layer of complexity to the LNP may increase the hurdles and cost of the process, including spare phase in production method [160, 161]. Agarwal et al. developed methotrexate encapsulated SLN conjugated with cationic bovine serum albumin (CBSA) as a ligand that enhances the permeation across BBB. The conjugated CBSA provides amino groups to become attached with the carboxylic group of SLNs to improve transport across BBB. Hence it also improves the uptake of SLN [162] [163]. Kranz et al. proposed that either change in surface charge of mRNA shifted the accumulation into another organ. Reduction of lipid content in LNP imparted negative surface charge of nanoparticles and altered the shift from lungs to the spleen. The anionic mRNA lipoplexes enhanced the accumulation of nucleic acid in the spleen of patients diagnosed with melanoma [164]. Another approach used to create LNP formulations with higher specificity for antigen-expressing cells is RNA aptamers [165]. Ray et al. (2021) were developed RNA aptamer-loaded ionizable

cationic lipid (DLin-MC3-DMA) system and showed that the developed system could traverse the BBB, and was readily taken up in multiple cell types [166].

4.4. Incorporation of adjuvants

Integration of adjuvants alongside formulation can initiate the immune response towards the antigen. Adding an adjuvant can trigger the immune system to facilitate humoral and cell-mediated responses [167]. The potential toxicity of adjuvants causes serious ailments. The physiological barrier encountered in CNS delivery allows the transport of both wanted and unwanted nanoparticles to enter into brain tissue that regulates the immune responses as well as causes localized damage. Lipid carrier gains much more excess than other nano particulate systems that affect the brain parenchyma. Some studies evidenced that lipids stimulate the immune system and cause inflammation [168]. Cationic lipids like 1,2-dioleoyl-3- trimethylammonium propane (DOTAP) in LNP might induce a pro-inflammatory response with Th1 cytokines and activate the toll-like receptor4 (TLR-4) observed in mice compared with LNP that were comprised of anionic and neutral lipids [169]. Certain adjuvants act like toll receptor agonists, unmethylated Cp-G oligonucleotides administered with protein enhanced the immune responses. Wu.et.al proposed that TLR agonist as a small immune potentiator (boost the immune responses) might be useful in formulating novel LNPs [92]. Transcriptional regulation upsurges gene coding and triggers cytokines, chemokines, and type 1 interferon by activating these receptors. Certain inorganic adjuvants like silica and TiO₂ were produced potential toxicity in the fetus [170]. Figure 11 represents the underlying mechanisms for targeting of ligands and adjuvants in CNS.

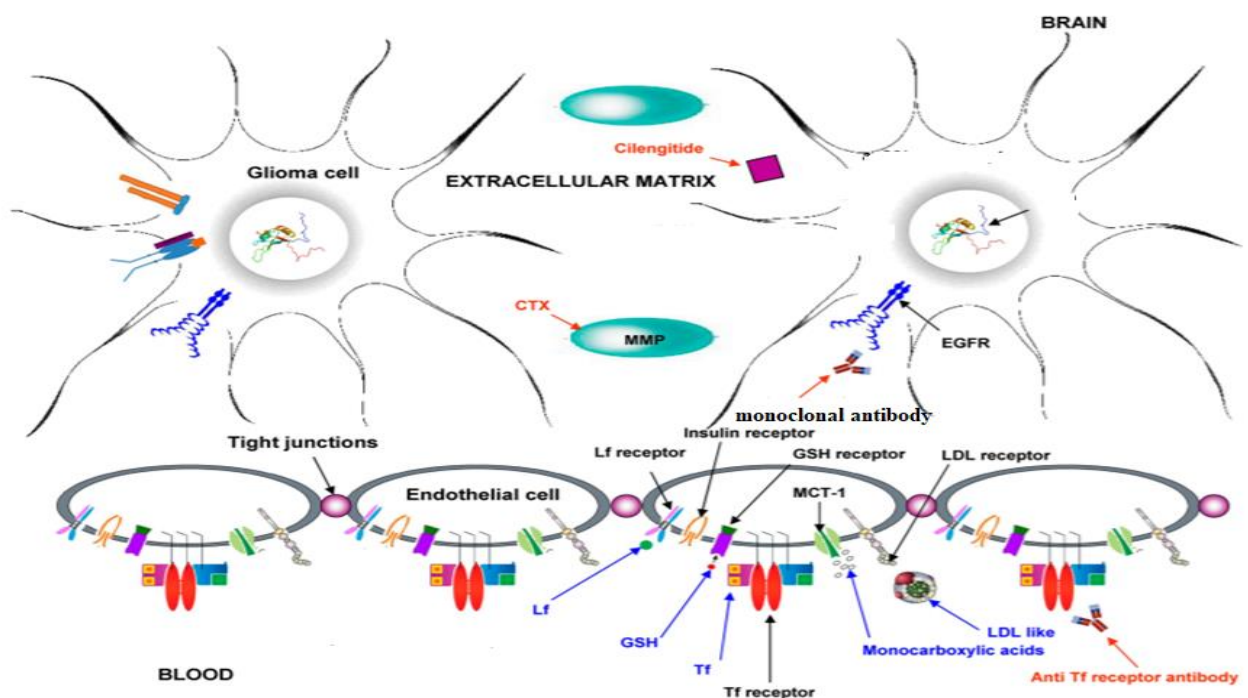


Figure 11. Schematic illustration of mechanism responsible for targeting of ligands and adjuvants in CNS, blue color indicate endogenous ligands for targeting, red exogenous ligands. Reproduced with permission from ref [171].

5. Active targeted macrophage-mediated delivery of therapeutic agents to the brain

To boost tumor-specificity and avoid unanticipated toxicity, innovation in the delivery mechanism of anti-tumor drugs is necessary to develop novel therapeutic approaches. Allavena et al. integrated a live cell-mediated drug delivery method with nanotechnology to show that blood monocytes can be used as cargo to deliver anti-cancer medicines encapsulated in polymeric PLGA-NPs (poly (D, L-lactide-co-glycolide) acid loaded nanoparticles [172]. Monocytes carrying PLGA-Cy7 nanoparticles were more efficient than free PLGA-CY7-NPs at reaching the tumor location, and it was also confirmed via bio-distribution studies [173]. It was shown that monocytes loaded with doxorubicin (DOX) encapsulated PLGA nanoparticles (PLGA-DOX NPs) had greater anti-cancer effects in-vitro. Therefore, monocytes can be considered promising tools to design live cell-mediated drug delivery systems in cancer research and therapy. Figure 12 demonstrates the *in-vivo* application of monocytes as drug delivery tools. Table 3 summarizes the macrophage/monocytes-mediated delivery of therapeutic agents to the brain that are discussed in this section.

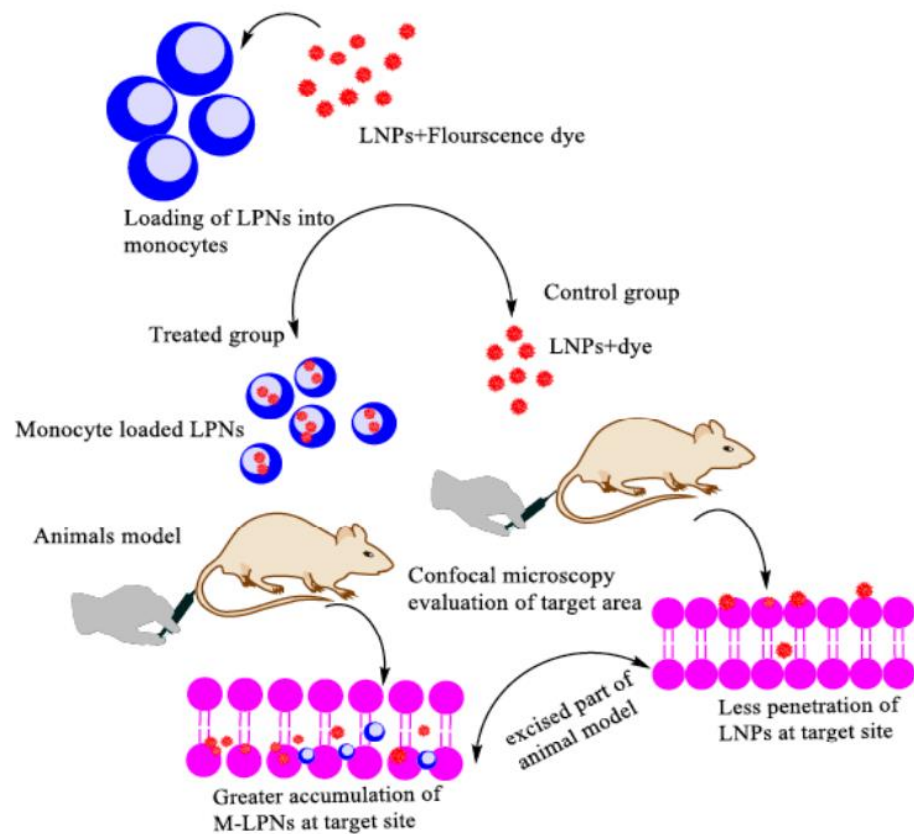


Figure 12. *In-vivo* study illustrating monocytes as drug delivery tool reproduced with permission from [174].

Table 3. Macrophage/Monocytes-mediated delivery of therapeutic agents to the brain.

Macrophage/Monocytes	Nanoparticle	Therapeutic agent	Treatment	References
Mononuclear Macrophages	Polymer patches	Catalase	Neurodegenerative disorders	[175]

Alveolar macrophages	Gold-Silica nanoshells	-	Gliomas	[176]
RAW264.7 cells	-	Catalase	Parkinson's disease	[177]
Bone marrow derived macrophages	PLGA	Doxorubicin	Gliomas	[178]
	PEI-PEG	Catalase	Parkinson's disease	[179]
	Fe ₃ O ₄	Cyanine	Gliomas	[180]
	-	Catalase	Parkinson's disease	[181]
Bone marrow derived macrophages/ THP-1 cells	poly([9,9-dioctylfluorenyl-2,7-diyl]-co-[1,4-benzo-(2,1,3)-tiadiazol]) ₂₄	-	Glioblastoma	[182]
Monocytes	Fe ₃ O ₄ SPH 30/ lentiviral vector DHIV-101	-	Brain Tumor	[183]

641

642 Tissue macrophages are an essential component of the tumor microenvironment, and they play a vital role in
643 modulating the immune response. With the ability to absorb tumors and provide cancer-specific antigens for adaptive
644 antitumor response activation, macrophages have emerged as an attractive target for cancer immunotherapy [184].
645 Monocyte drug delivery is critical in targeting several diseases, including infection, inflammation, and several types of
646 malignancies. As a result, targeting drug delivery to monocytes is a viable option. Drug delivery to the brain is a
647 challenging task. However, by using monocytes as a location for targeted delivery, disorders including Alzheimer's,
648 brain injury, encephalopathy, dementia, epilepsy, multiple sclerosis, and brain tumors will be treated effectively
649 shortly [185]. Since monocytes can easily pass the BBB, medications that are difficult to enter into the brain can be
650 loaded into monocytes, allowing for the cure of all types of brain diseases, including brain cancers. Trojan monocytes
651 are monocytes that carry drug molecules to the most challenging locations. Researchers have employed various
652 methods to confirm the penetration of monocytes into the brain, and the artificial BBB can be considered one of them.
653 It has also been suggested that recruitment of immunocytes, such as neutrophils, to the inflamed sites of the brain can
654 be a promising strategy towards GBM treatment. In this context, Zhang and colleagues effectively delivered paclitaxel
655 (PTX)-loaded liposomes through BBB to inhibit cancer cell resection [186].

656 Previous reports have highlighted macrophage-mediated localization of nanoparticles in the brain using experimental
657 glioma models. For instance, it has been observed that when QDs are delivered intravenously, macrophages could help
658 them get into the body and cocolonize the QD-bearing macro phages. We assumed that the i.v. delivery of QDs would
659 enable the macrophages that have been loaded with QDs to be deposited in the brain tissues of animals. This strategy
660 could be used to decrease surgery time while improving the diagnostic accuracy and survival of a patient diagnosed
661 with GBM [187]. In another study, macrophages originating from the bone marrow were loaded with nanoformulated
662 catalase to decrease neuroinflammation in a rodent model of Parkinson's disease (PD).

663

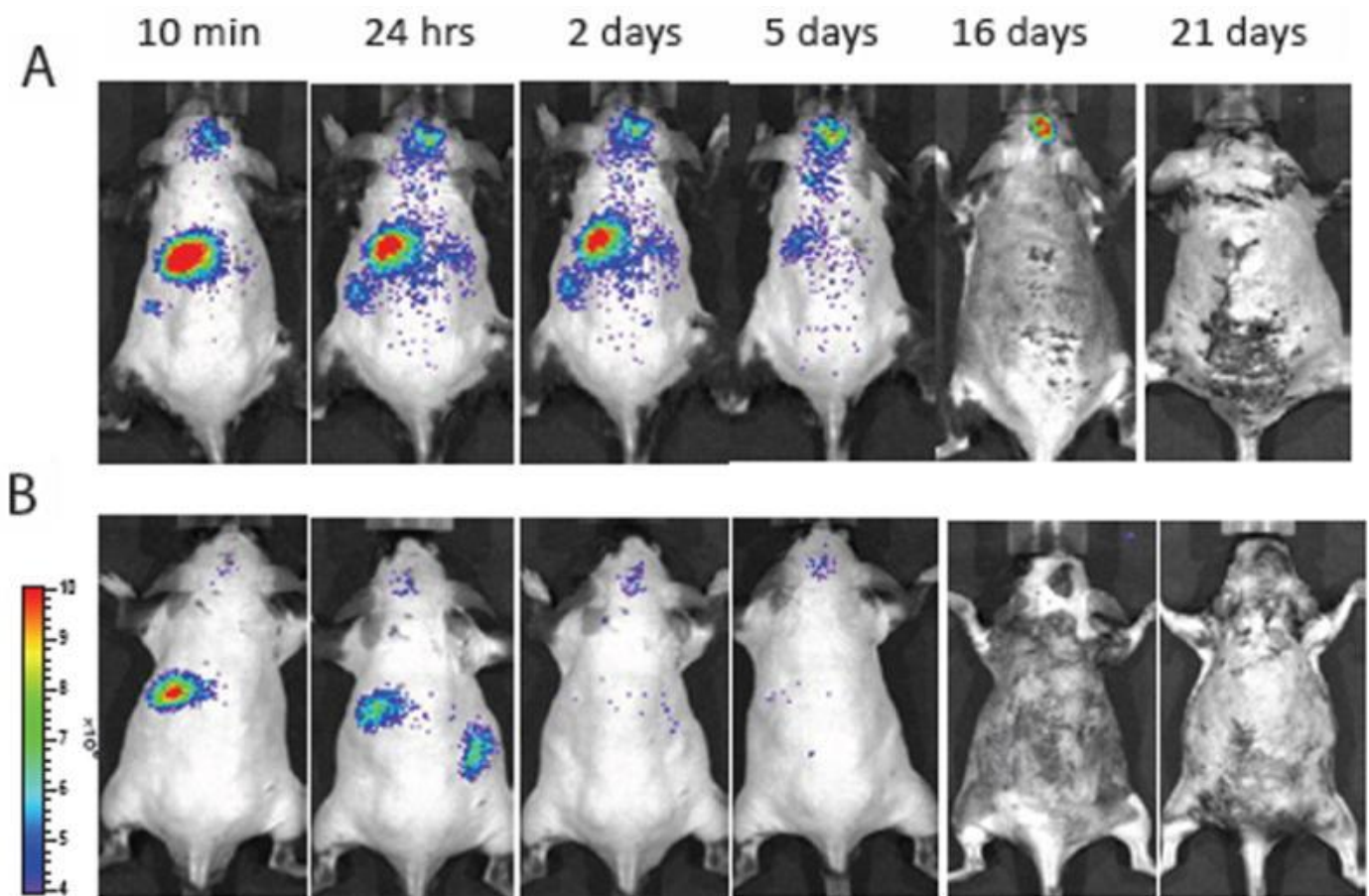


Figure 13. Illustration of the biodistribution of bone-marrow derived macrophages (BMM) carried nanozymes in mice with Parkinsons disease model by IVIS. The images show that mice with brain inflammation (A) circulate nanozyme for a longer period of time than healthy mice (B) [188].

Interestingly, the nanoformulation targeted diseased regions in the brain and increased BBB trafficking as illustrated in Figure 13 [188]. The utilization of natural immunocytes, like monocyte-macrophages, as a carrier of small molecules, opens up new possibilities for treating PD and other inflammatory and degenerative disorders, such as GBM. It is worth utilizing different routes of administration to deliver nucleic acids, such as RNAs, to the brain cells, and for this purpose, significant alterations are required when designing macrophage-associated RNA delivery nanoplatfoms. This provides a rationale for the fact that the binding of anticancer agents to a cell-surface receptor is required for receptor-mediated transport of anticancer agents to cross the BBB [189, 190].

6. RNA nanodelivery via macrophages/monocytes

RNA molecules can be "transported" to body tissue through extracellular vesicles such as microvesicles (MVs). These MVs are tiny particles with a size of 0.05-1 μm , and they are membrane-bound. They are made from the outer shedding of the plasma membrane and can be derived from macrophages, which serve as cell-to-cell communication tools. The shedding from the cell's surface occurs through stimulation, particularly in cells close to inflamed or bleeding areas [191-193]. Not much is known about the function of these bioactive MVs; however, they display

683 significance in cellular development and differentiation; and they are regarded as relatively safe when administered
 684 intravenously to patients. RNA molecules and proteins are abundantly found in injured or inflamed areas for local
 685 messaging between cells and proteins. The MVs from macrophages, as already mentioned, serve as vehicles for RNA
 686 delivery, which in turn is beneficial in RNA-based therapy [194, 195]. However, the effective delivery of RNA via
 687 macrophages is still challenging. Table 4 summarizes methods employed in the RNA nanodelivery via
 688 macrophages/monocytes that are discussed in this section. It highlights the advantages of using the methods discussed
 689 and indicates the nanoparticles and carrier in each situation.

690
 691 **Table 4.** RNA nanodelivery methods and their advantages.

Nanoparticle Type	RNA class	Delivery Vehicle	Advantages	References
Mannose-modified trimethyl chitosan-cysteine/tripolyphosphate nanoparticles (MTC/TPP)	siRNA	Caveolae-Mediated Endocytosis (CvME)	designing optimal delivery vectors to facilitate the clinical translation of siRNA drugs	[196]
Silicacoated polyion complexes (SilPICs)	mRNA	Silicacoated polyion complexes (SilPICs)	efficiently protects the mRNA payload from enzymatic degradation	[197]
Macrophages	siRNA	siRNA	potential to suppress neuro-inflammation in vivo	[198]
α -peptide based M2-like tumor-associated macrophages dual-targeting nanoparticles (M2NPs)	siRNA	M2-like tumor associated macrophages (TAMs)	specifically blocks the survival signal of M2-like TAMs and deplete them from melanoma tumors	[199]
β -1,3-D-glucan-encapsulated siRNA particles (GeRPs)	siRNA	β -1,3-D-glucan-encapsulated siRNA particles (GeRPs)	oral delivery of siRNA to reduce inflammatory responses in human disease	[200]
siRNA-lipoplexes	siRNA	Macrophages	horizontal transfer of siRNA to cancer cells via macrophages	[201]
nanohydrogel particles equipped with mannose residues on the surface (ManNP)	siRNA	ManNP	α -mannosyl-functionalized ManNP direct NP towards M2-type macrophages	[202]
glucan-based siRNA carrier (BG34-10-Re-I)	siRNA	BG34-10-Re-I	reduce gene expression of macrophage migration inhibitory factor	[203]
SPACE peptide conjugated with poly-arginine (S-R11 peptide)	Small RNA	S-R11 peptide	can overcome the challenge of gene therapies to immune cells.	[204]
Polyethyleneimine-coated Iron Oxide Nanoparticles (PEI-SPIONs)	siRNA	PEI-SPIONs	efficiently deliver siRNA to macrophages <i>in vitro</i> and <i>in vivo</i> , and silence target gene expression	[205]
fluorescent silica coated iron oxide nanoparticles (NF-SIONs)		Macrophages	intraoperative imaging of GBM by targeting tumor-associated macrophages	[206]
magnetic nanoparticles	siRNA		advancement of siRNA delivery and imaging strategies	[207]

6.1. Microvesicle RNA delivery via monocytes/macrophages

This method involves the microvesicle RNA delivery via monocytes/macrophages, reported after discovering [208] that macrophages absorb undegraded liposome-entrapped RNA molecules [209]. In 2011, Yukihiro et al. (2010) presented an innovative RNA molecule carriage technique, utilizing monocytes/macrophages in addition to their secreted MVs; Figure 10 summarizes this study [210]. The study demonstrates that transfected RNA particles from human monocytic leukemia THP-1 cells were peeled or budded from THP-1 macrophages as matter in MVs. The shedding in this experiment was performed with incubation in a serum-free medium. In addition, biochemical analyses were used to prove the shedding from the THP-1 macrophages by immunoelectron microscopy, expression of TSG101 (a membrane-associated exosomal protein), and quantitative reverse transcription (qRT)-PCR.

Firstly, the more effective miRNA intended for transfection is determined: a chemically modified miRNA (miR-143BPs) or a nonmodified one. Then, more miR-143BPs are captured by the MVs, to form MV-miR-143BPs. These MV-miR-143BPs are secreted from the THP-1 macrophages, after miR-143BP transfection, instead of transfection with nonmodified miR-143. In addition, this method involves the transfection of THP-1 macrophages through the ex-vivo miR-143BP secreted MV-miR-143BPs, within xenografted nude mice. This is evidenced by the major surge in miR-143 levels in the mice's serum, tumor, and kidney following the intravenous injection. Thus, the in-vitro and in-vivo studies concluded that some transfected miR-143BPs were secreted from THP-1 macrophages as MV-RNAs (Figure 14).

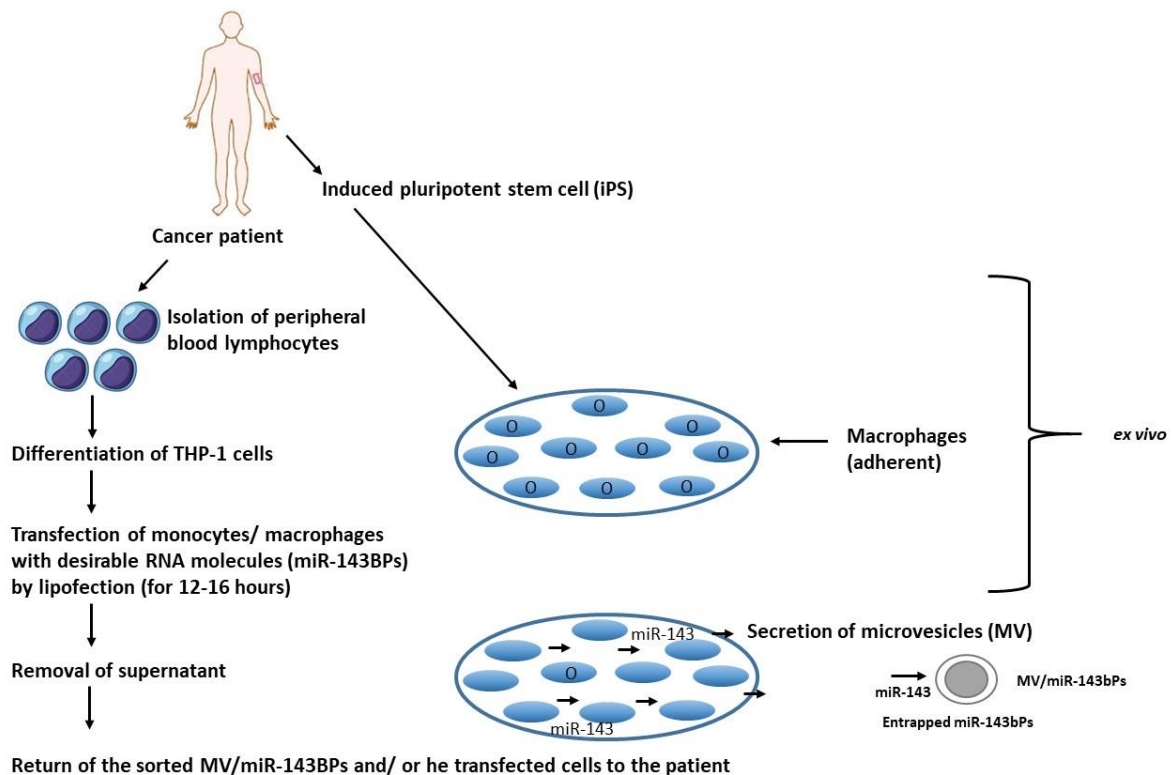


Figure 14. A schematic illustration of a method for carrying RNA molecules using microvesicles in monocytes or macrophages. (Figure modified from ref [210]).

714 Song et al. (2021) reported a method involving the small interfering RNA (siRNA) delivery of polymeric
715 nanoparticles to macrophages [196]. The study is justified because the cytosolic transport of siRNA is perplexing, and
716 it is imperative to understand the intake of siRNA delivery systems at the cellular level. Additionally, the recognition
717 of intracellular processing can considerably expand on improving siRNA-based therapeutics. This investigation shows
718 that the caveolae-mediated endocytosis (CvME) is responsible for the strong siRNA delivery involving mannose-
719 modified trimethyl chitosan-cysteine/ tripolyphosphate nanoparticles (MTC/TPP NPs) towards macrophages by
720 avoiding lysosomes. In addition, the Golgi complex and endoplasmic reticulum (ER) are essential organelles in the
721 effective carriage of siRNA to macrophages.

722 Furthermore, in some people dealing with lysosomal storing issues and Parkinson's disease, the NPC1 and Syntaxin6
723 roles get impaired. This study illustrated that Syntaxin6 and NPC1 knockout remarkably reduce the uptake at the
724 cellular level and the gene silencing of the siRNA conveyed in MTC/TPP NPs in macrophages, resulting in reduced
725 healing effects for mice with acute hepatic damage. Consequently, the study indicates that CvME is responsible for an
726 effective siRNA delivery, aiding in planning prime delivery vectors for facilitating the clinical translation of siRNA
727 drugs. Therefore, if the polymer-centered nanoparticles depend on the CvME pathway, it will lessen the carriage
728 abilities of siRNA drugs in Parkinson's disease lysosomal storage suffering patients [211, 212].

729 As already mentioned, macrophage transport of messenger RNA (mRNA) is an encouraging therapeutic tool for
730 inflammatory illnesses since it can modify macrophages' immunological activities. However, the effective distribution
731 of macrophage-specific mRNA remains an issue [213]. The latest study by Kamegawa et al. (2021) reports on polyion
732 complexes (PICs) that are covered with silica (SiPICs), using a biologically-motivated approach to envelop mRNA
733 and scavenger receptor (SR)-mediated macrophage for target therapy, as illustrated in Figure 15 [197]. In this
734 approach, the PICs are loaded with mRNA followed by coating, subsequently mixing tetramethyl orthosilicate in a
735 water-based media at approximately 25°C, with tetramethyl orthosilicate. To confirm the development of the silica
736 shell, an increase in the particle size was noted.

737 This was followed by characterization using Fourier transform infrared spectroscopy and elemental analyses (scanning
738 transmission electron microscopy energy-dispersive X-ray spectrometry). A fetal bovine serum-containing medium
739 delivered decent fortification from the enzymatic ruin of the silica shell. Additionally, a reversible shell in silica
740 allowed for mRNA release from SiPICs after silica was dissolved into silicic acids. In addition, SiPICs resulted in a
741 twenty-fold greater mRNA transfection effectiveness in macrophage cell line RAW264.7. When this was compared to
742 the efficacy of noncoated PICs, the SiPICs displayed better mRNA transfection, and the reason is prescribed to the
743 silica shell's enablement of cellular internalization. This study concluded that using SiPIC in the distribution of
744 mRNA, considering the protection of the mRNA and the targeting of macrophages, is a promising approach. Tumor
745 necrosis factor- α (TNF- α)-induced inflammation and neuronal apoptosis are standard components of several illnesses
746 related to the central nervous system (CNS) [214]. Additionally, according to reported literature, macrophages and
747 microglia are the roots of TNF- α in the CNS [215]. Therefore, a study by Kim et al. (2009) demonstrated that
748 targeting macrophage or microglial cells with siRNAs reduced the detrimental effects of lipopolysaccharide (LPS)-
749 induced neuroinflammation and neuronal apoptosis on the CNS function [216].

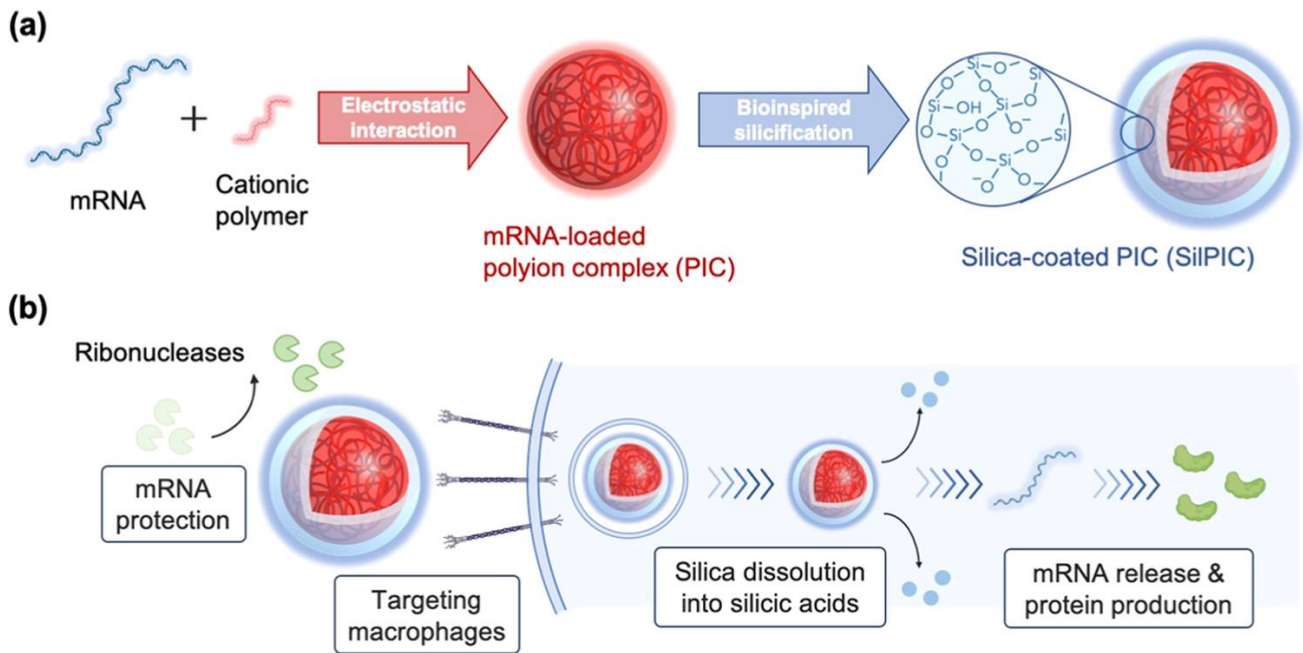


Figure 15. (a) Schematic illustration of the synthesis of silica-coated polyion complexes (PICs) (SilPICs). (b) Method of transfer of mRNA for macrophage targeting using SilPICs. Reproduced with permission from ref [197].

Furthermore, the nicotinic acetylcholine receptor (AChR) is exhibited by macrophages or microglia; therefore, a short AChR-binding peptide (bonded to nona-D-arginine deposits - RVG-9dR) as a targeting chelate ligand, based on Rabies virus glycoprotein, was used for siRNA complexation. By targeting specific genes with RVG-9dR, gene silencing was induced in macrophages and microglia cells from wild-type mice, not AChR-lacking mice. In-vitro, LPS-prompted TNF- α fabrication was effectively suppressed by anti- TNF- α siRNA bound to RVG-9dR. Also, by injecting the RVG-9dR-complexed siRNA intravenously, mice had considerably less TNF- α in their blood and brains, causing significantly lower neuronal apoptosis. Using RVG-9dR in conjunction with siRNA, Kim et al. (2009) proved that macrophages and microglia can be targeted for siRNA transfer and that TNF- α can be blocked to suppress neuro-inflammation *in-vivo*.

6.2. TAMs dual-targeting nanoparticles for siRNA delivery

Recently, researchers have been investigating TAMs for alternatives as therapeutic targets of cancer immunotherapy (Figure 16). However, Qian et al. (2017) reported difficulties perfecting direct delivery involving treatments to the tumor-promoting M2-like TAMs [199]. Therefore, they prepared M2-like TAM dual-targeting nanoparticles (M2NPs) to deliver siRNA to M2-like TAMs. The structure and function of these M2NPs are contained by α -peptide, which is a searcher receptor B type 1 (SR-B1) directing peptide, connected to M2pep, as an M2 macrophage binding peptide. Thus, the most critical components in this approach involve the following: i) biocompatibility of the combined lipid nanoparticle with functionalized peptides as dual-targeting units for specified M2-like TAM binding ii) sub-30 nm particle dimension for effective diffusion in the tumor [217], and iii) robust loaded siRNA for methodical transference

[218]. In addition, the method involves blocking the survival sign by packing the anti-colony stimulating factor-1 receptor (anti-CSF-1R) small interfering RNA (siRNA) on the M2NPs. This step in the approach aims to block the M2-like TAMs and diminish their survival on melanoma tumors. M2NP-based siRNA transfer significantly removed M2-like TAMs by a 52% margin, decreased the tumor size by 87%, and extended the endurance in mice.

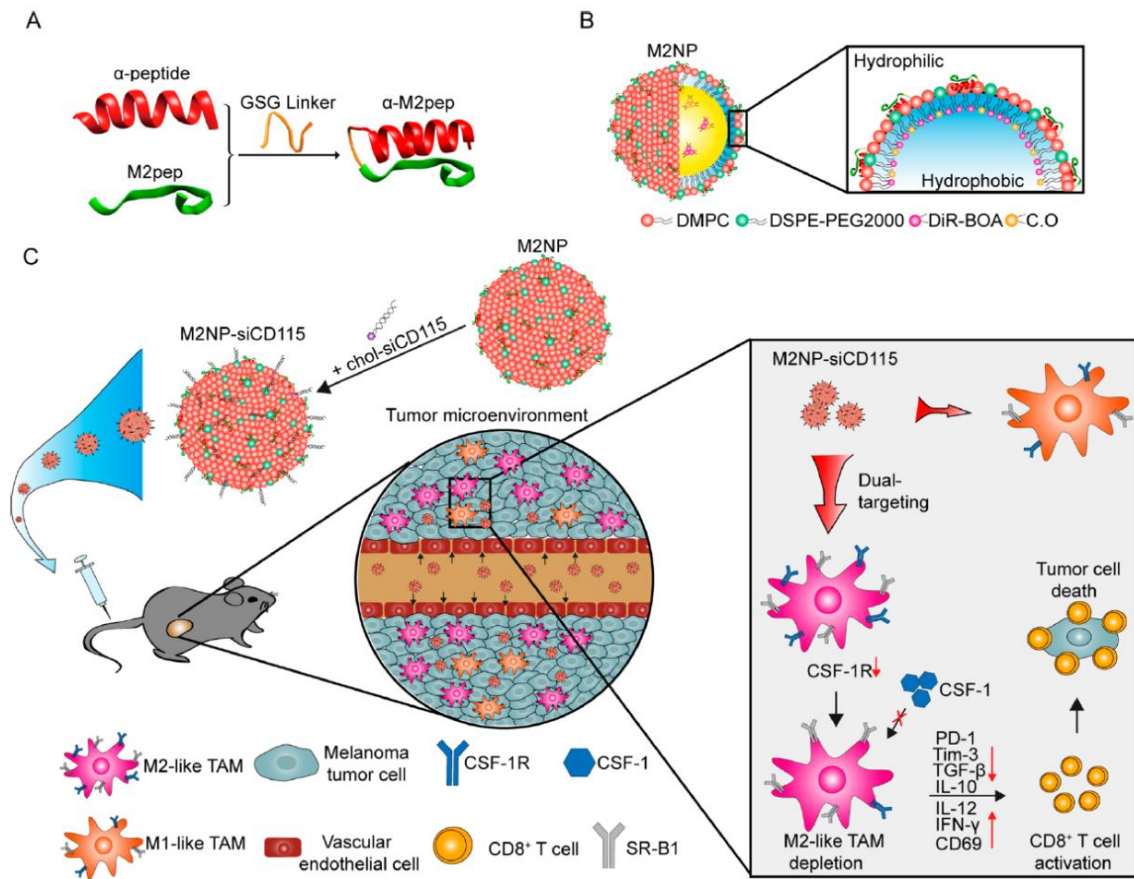


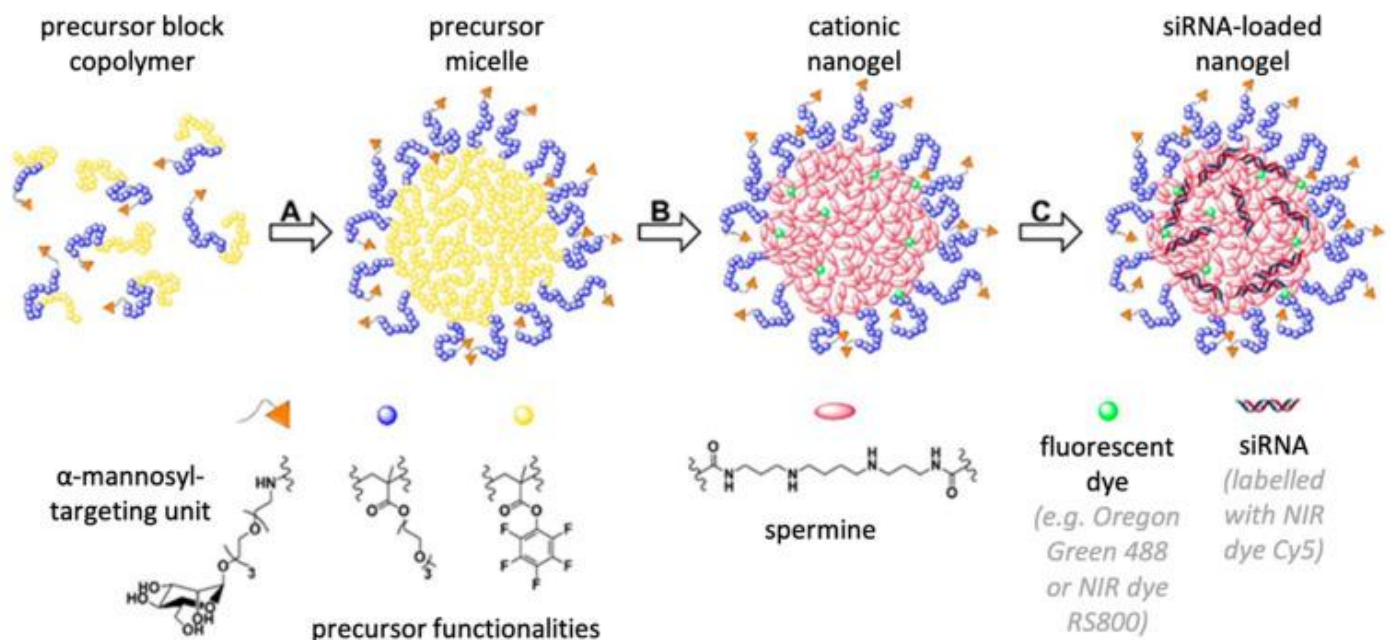
Figure 16. Schematic representation of the M2NP for the purpose of M2-like TAM-specific nano-immunotherapy. (A) Fusion of peptide α -M2pep using a mixed blend approach. (B) Assembly and constituents of M2NP. (C) M2NP-based delivery of siRNA to silence CSF-1R gene and in-vivo regulation of the immune system by synergistic dual targeting of M2-like TAMs. Reproduced with permission from ref [199].

In an *in-vivo* experiment, Aouadi et al. (2009) created siRNA particles packaged in 1,3-D-glucan (GeRPs) to deliver siRNA orally. When they retrieved the GeRPs containing siRNA against tumor necrosis factor α (Tnf- α), they found less messenger RNA in the macrophages of the mice models. They also found that Mitogen-activated Protein Kinase Kinase 4 (Map4k4) was a regulator of cytokine expression, which was previously unknown. Finally, the team found that blocking Tnf- α and interleukin-1 β synthesis in macrophages protected mice against lipopolysaccharide-induced invasiveness [200]. Conde et al. (2015) developed peptide-functionalized gold nanoparticles (AuNPs) for siRNA delivery in a lung cancer orthotopic murine model that can stop mRNA of mouse vascular endothelial growth factor (VEGF) in the inflammatory tumor M2 macrophages. The developed nano delivery system showed the highest effect (ED_{50} 0.0025–0.01 mg kg⁻¹) in the literature for both immune cells and cancer cells in an *in-vivo* cancer model. They indicated that the developed nano delivery system did not cause an innate immune response [219].

796 Recently, Wayne et al. (2019) illustrated that macrophages presented parallel transference of siRNA during coculture
 797 with tumorous cells. Moreover, the amount of siRNA transferred can be adjusted depending on the siRNA
 798 concentration and the total amount of macrophages transferred. Study results showed that loading calcium integrin-
 799 binding protein-1 siRNA onto macrophages suppressed the growth of tumorspheres and expression of CIB1 and KI67
 800 mRNAs in breast cancer cells. Furthermore, CIB1-siRNA transfected macrophages presented localization in
 801 orthotopic tumors after they were transferred, suggesting that stimulated macrophages or intracellular pathways, such
 802 as Rab27a, play a role in controlling their translocation [220].

803 The nanohydrogel particles (ManNP) engineered by Kaps et al. (2020) carried siRNA *in vitro* more capably through
 804 polarized macrophages (M2) than their non-targeted counterparts. The *in-vivo* targeting of ManNP was then evaluated
 805 in mice with tentative liver fibrosis, which was characterized by more significant infiltration of profibrotic M2-type
 806 macrophages. Optical imaging of ManNPs loaded with siRNA in fibrotic livers demonstrated robust uptake and
 807 acceptable biocompatibility. Additionally, ManNP colocalized with CD206+ M2-type macrophages but did not
 808 colocalize with untargeted NP (NonNP) and did not appear to be specific to liver cells. Further, according to
 809 serological analyses, ManNP did not result in liver and kidney inflammation. The study results suggest that M2-type
 810 macrophages can be targeted by α -mannosyl-functionalized ManNPs and avoid nonspecific uptake, suggesting that
 811 ManNPs may be used as siRNA delivery vehicles in the treatment of liver tumours (Figure 17) [221].

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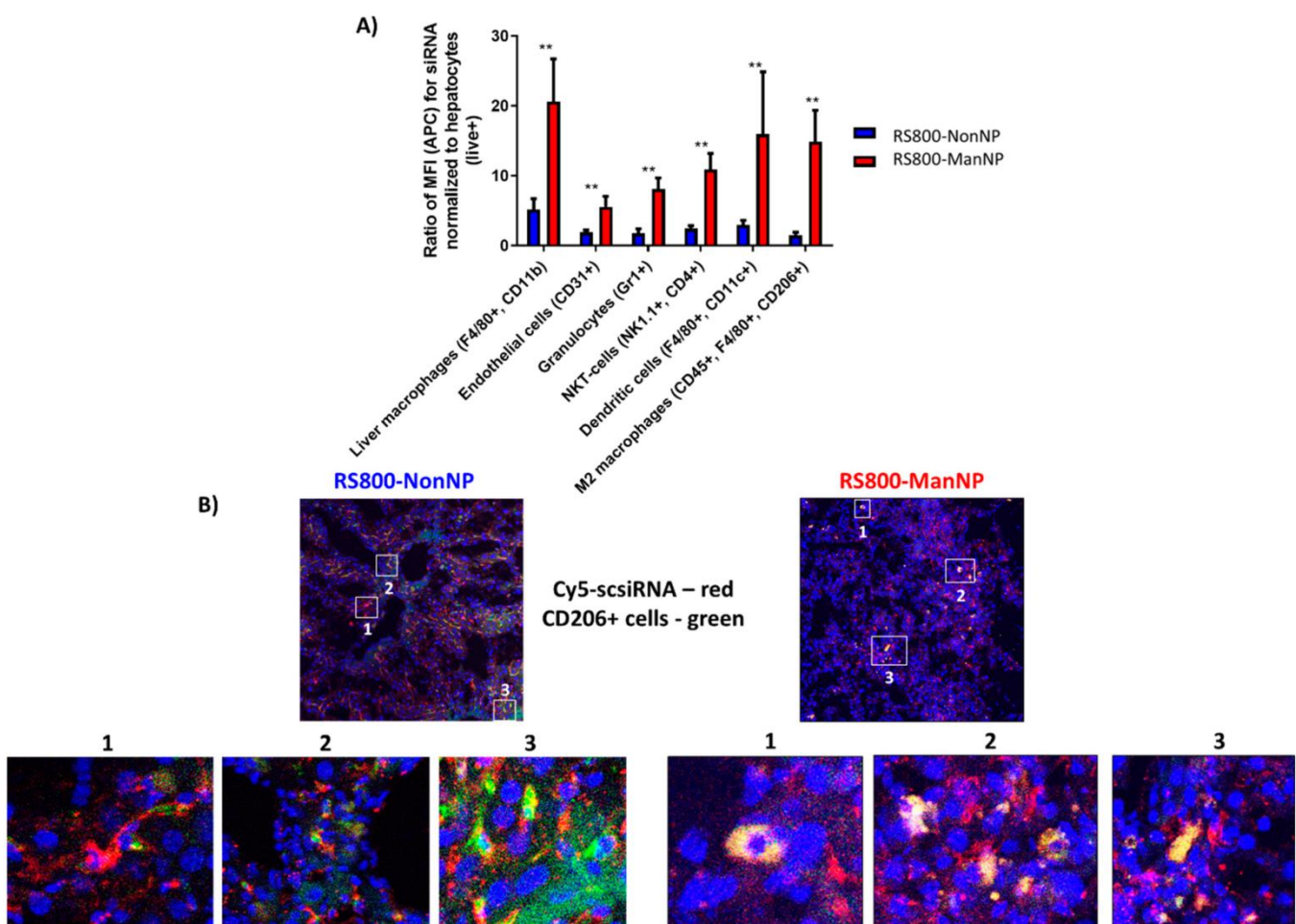
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815 **Figure 17.** A method for preparing mannose functionalized nanohydrogels (ManNP) [221].

816 Illustrated in Figure 18, Kaps et al. (2020) further assessed the *in vivo* uptake of RS800-NonNP and RS800-ManNP in
 817 liver-derived single-cell suspensions from fibrotic mice using flow cytometry. They compared cellular uptake in the
 818 presence of Cy5-siRNA/RS800-NonNP particles to that of hepatocytes that represent abundant liver cells (Figure
 819 18A). A significant number of immune cells expressed other mannose-recognizing scavenger receptors, which are
 820 mostly found on antigen-presenting immune cells. RS800-ManNPs were taken up by CD206 expressing M2-type

821 macrophages in addition to a range of other relevant immune cells. According to the results seen in vitro, the addition
 822 of α -mannosyl functionalization results in reduced unspecific uptake in non-targeted cell populations and increased
 823 uptake for targeted cell types. When superimposed with Cy5-scsiRNA (red), the Cy5- scsiRNA/RS800-ManNP
 824 exclusively localized with CD206+ macrophages (Figure 4B). RS800-NonNP carrying Cy5-scsiRNA was found to be
 825 less colocalized with CD206+ M2-type macrophages in liver cryosections, thus exhibiting occasional yellowish
 826 costaining (Figure 18B). In fibrotic liver, ManNP promotes efficient uptake into immune cells expressing the mannose
 827 receptor, such as macrophages (CD206+). Furthermore, the carbohydrate-functionalization impairs uptake by non-
 828 targeted (non-)parenchymal liver cells. This study illustrated that it can be considered that ManNP helps to deliver
 829 siRNA selectively to macrophages (and possibly other immune cells expressing the mannose receptor) in vivo.

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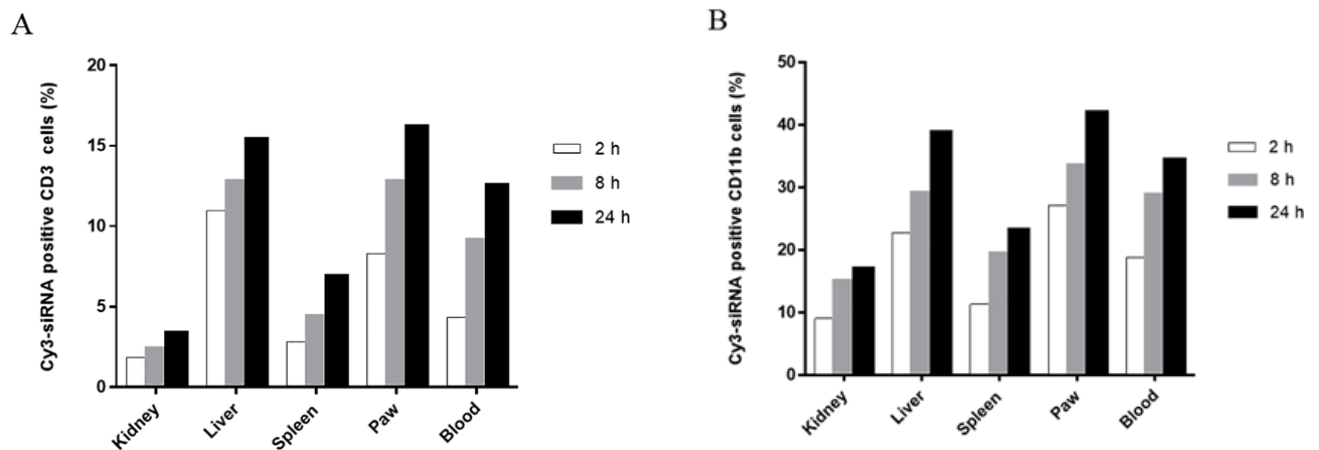
839 Using an in vitro and in vivo macrophage migration inhibitory factor MIF nanoparticle system, Zang et al. (2015)
840 studied the advancement of non-viral, direct-targeted MIF nanoparticles. In this study, the researchers developed an
841 RNA carrier structure (BG34-10-Re-I) based on glucan that provides a core shell for siRNA nanoparticles. BG34-10-
842 Re-I/siRNA nanoparticles significantly decreased macrophage migration inhibitory factor's (MIF) protein and mRNA
843 levels. Furthermore, the nanoparticles reduced MIF within macrophages, and the systemic injection of the
844 nanoparticles reduced MIF in macrophages associated with 4T1 mammary cancer cells. The siRNA-core structure and
845 glucanshell have been shown in vitro and in vivo studies to facilitate siRNA delivery to macrophages [203].

846 Ryu et al. (2022) developed a method to deliver small RNAs into immune cells that are difficult to transfect, based on
847 the skin permeating and cell entering (SPACE) peptide conjugated with polyarginine. S-R11 peptides and small RNAs
848 formed hydrogen bonds, which resulted in a self-assembling nanocomplex. The nanocomplex permeated RAW 264.7
849 macrophage cells about 5.3-fold better than Lipofectamine™ 2000. Further, polyinosinic polycytidylic acid (poly
850 (I:C)) suppressed 66.2% of the target gene in activated cells, with the fusion peptide ensuring cell viability at high
851 nano complex concentrations [222].

852

853 **6.3. Magnetic nanoparticles as carriers of siRNA to macrophages**

854 Within the biomedical field, superparamagnetic iron oxide nanoparticles (SPIONs) have displayed favorable physical
855 properties such as biocompatibility, equivalence in size to biological systems, increased surface-area-to-volume ratio,
856 and simply adjustable surface for bioactive agent addition [223]. The preceding facts inspired Jia et al. (2019) to
857 introduce a method utilizing polyethyleneimine-coated superparamagnetic iron oxide nanoparticles (PEI-SPIONs) as a
858 carrier for the targeted transfer of siRNA to macrophages [205]. The PEI-SPIONs bind and condense the siRNA when
859 the iron:siRNA weight ratio is four times higher. As a result, the nanoparticles (PEI-SPIONs) capably deliver siRNA
860 into macrophages and macrophage-like RAW 264.7 cell lines in-vitro and in-vivo. Furthermore, these multimodal
861 PEI-SPION/siRNA particles can regulate macrophages' function and image and trace them. This study introduced an
862 easy, non-toxic, and promising method for delivering siRNA to macrophages in-vitro and in-vivo to induce gene
863 silencing functionally. In Figure 19, is an illustration of good cellular uptake of the PEI-SPION/siRNA NPs, showing
864 the particles within the different areas of the body i.e. in the blood, spleen, liver, kidneys and inflamed paw, after
865 monitored hours (2, 8 and 24 h). Additionally the authors indicated that the PEI-SPION platform could be useful in
866 delivering siRNA therapeutics systemically for the treatment of rheumatoid arthritis, a disease whose pathogenesis is
867 linked to macrophage dysfunction and for which local siRNA delivery is not preferred due to the involvement of
868 multiple organs.



869

870 **Figure 19:** The uptake of PEI-SPION/siRNA NPs by the cells in arthritic rats, *in vivo*. Using (A) anti-CD3 and (B) anti-CD11b
 871 monoclonal antibodies, the cellular uptake of PEI-SPION/Cy3-siRNA nanoparticles was measured with flow cytometry. % of
 872 Cy3-siRNA uptake within gated CD3+/CD11b+ cells [205, 224].

873 While researchers have not actively investigated the development of targeted therapies of RNA delivery via
 874 macrophages towards GBM, Lee et al. (2018) [206] developed an imaging device for GBM. By targeting TAMs, these
 875 fluorescence-coated iron oxide nanoparticles are near-infrared (NF-SION), highly fluorescent, and possess an
 876 enhanced water dispersion capacity for internal imaging of GBMs [206, 225-227]. The study was justified by
 877 indicating that a prominent characteristic of GBM margins is macrophage infiltration, which is well correlated with a
 878 deprived diagnosis. Therefore, the team designed NF-SIONs, as seen in Figure 20, with an approximate particle size
 879 of 37 nm to exploit endocytosis movement. These NF-SIONs envision tumor-associated macrophages by imaging in
 880 *in vitro* live cells and *in vivo* fluorescence in GBM models. The study also successfully reported that the synthesized NF-
 881 SIONs infiltrated the BBB and explicitly delineated the cancerous mass. The accomplishment of this study opens
 882 possibilities that researchers can now develop nanoparticles with therapeutic characteristics towards GBM using RNA
 883 delivery via macrophages.

884 Jia et al. (2019) used polyethyleneimine-coated superparamagnetic iron oxide nanoparticles (PEI-SPIONs) to perform
 885 targeted siRNA delivery to macrophages with *in vitro* and *in vivo* studies. They found that developed PEI-
 886 SPIONs/siRNA particles displayed successful modulation on macrophage function and provided imaging of
 887 macrophages [228]. Medarova et al. (2007) developed a probe siRNA conjugated Cy5.5 dye-labeled magnetic
 888 nanoparticles for siRNA delivery and imaging of its accumulation in tumors by using magnetic resonance imaging
 889 (MRI) and near-infrared *in vivo* optical imaging (NIRF). They indicated that they could manage to image delivery of
 890 the probe by MRI and NIRF. Furthermore, they could follow the silencing process by the optical imaging technique
 891 [229].

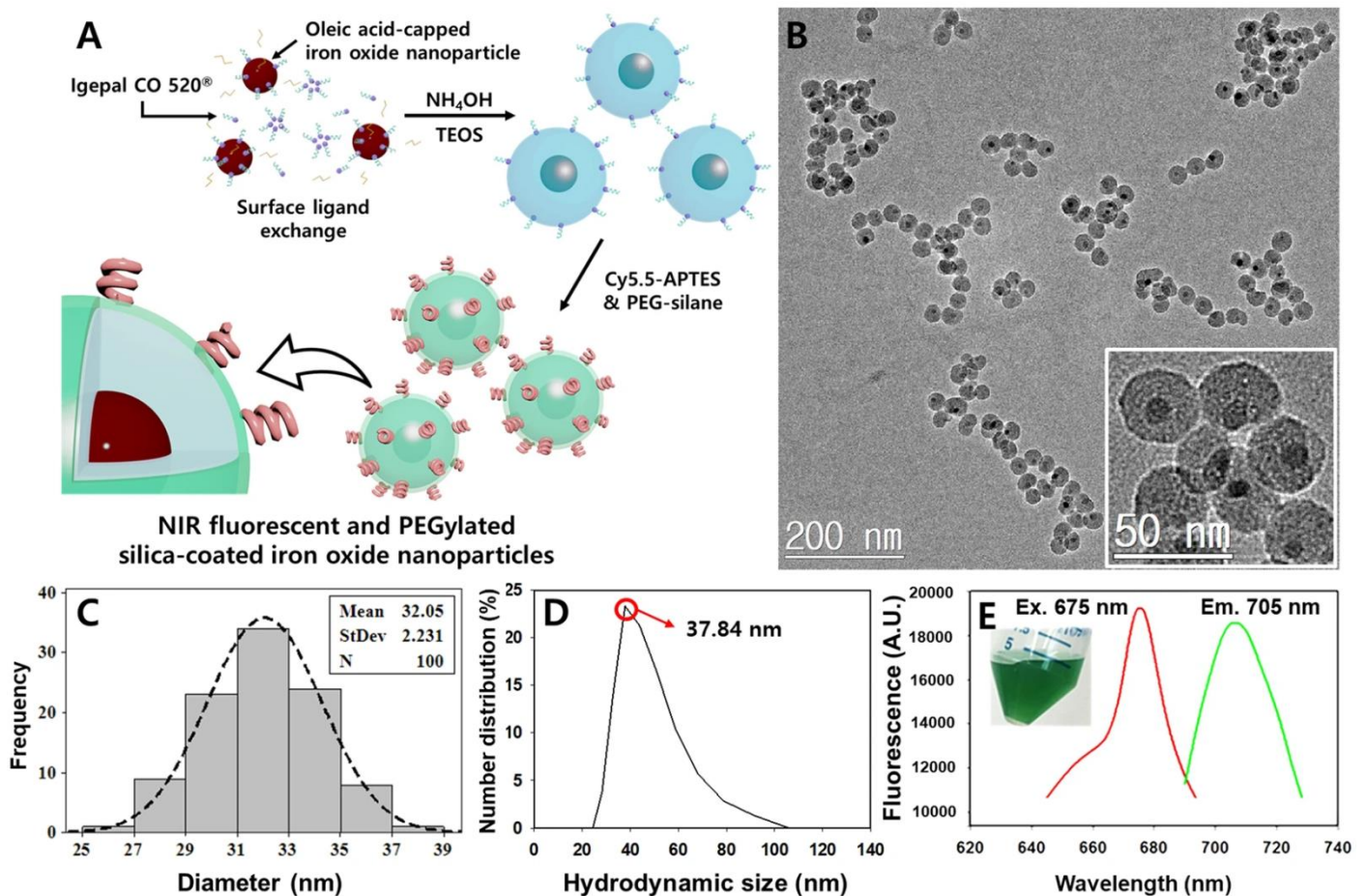


Figure 20. (A) Illustration of the synthesis of the NIR-fluorescent silica-coated iron oxide nanoparticles (NF-SIONs). (B) Synthesized NF-SIONs imaged under TEM. (C) A histogram of the size distribution of nanoparticles. (D) An image of the hydrodynamic size distribution of the NF-SIONs in 0.01M PBS. (E) The NF-SIONs' emission and excitation profiles. Reproduced with permission from ref [206].

7. Conclusion and outlook

In the clinical setting, various tools for GBM diagnosis are currently available. Even though several therapeutic modalities against GBM are in the clinic or clinical trials, this progressive type of brain cancer remains an incurable and fatal disease. Several miRNAs and lncRNAs are deregulated in glioblastoma cell lines and tumor tissues, making them attractive targets for GBM treatment. However, delivering RNA molecules to the brain is difficult since the BBB prevents most chemicals from entering the brain. Nanoparticles have been shown desirable properties to deliver RNA molecules to glioma locations. On the other hand, the use of immunocytes as a drug delivery tool to treat various brain diseases, including glioma, is a growing subject of research. Monocytes or macrophages could be potential carriers for delivering mRNA to the glioma location. The present review described six successful methods to prepare nanovehicles for RNA-based therapeutic transfection in macrophages. Based on these studies, it is clear that different compositional elements of the formulations can trigger responsiveness and determine cellular uptake, which is extremely important to developing an efficient RNA transfection vector for transfer via macrophages. The two-way exploitation of immunocytes has been introduced as a promising approach for the targeted delivery of RNA molecules at GBM sites. Another method we discussed is to stimulate the innate immune response, allowing immune cells to

transport drug molecules more efficiently from the injection site to the desired location. Loading mRNAs into LNPs protects them from extracellular degradation while allowing for precise transport of the targeting moiety. RNA encapsulated LNPs have offered several intracellular and extracellular barriers that could be overcome by conjugation with several targeting moieties or using adjuvants. These conjugations result in the induction of humoral and adaptive immunity. Further studies are needed to develop sensible LNPs that can effectively traverse the BBB, making RNAi-based therapeutics a clinical reality to treat GBM.

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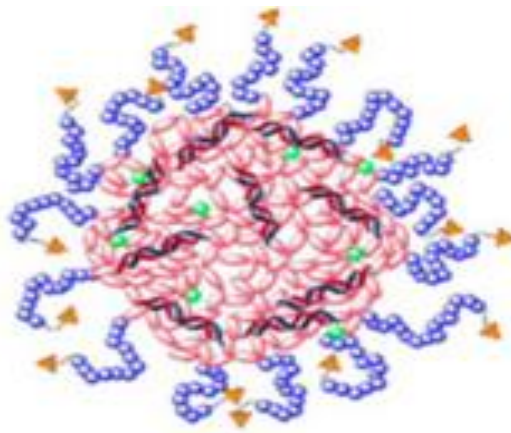
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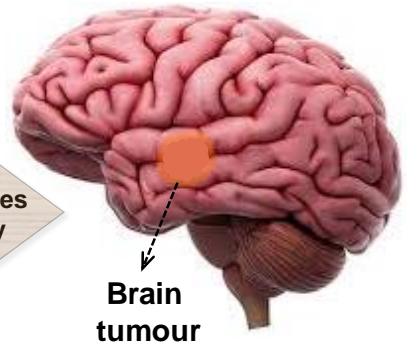
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RNA-loaded nanoparticles
for macrophage delivery



Brain
tumour