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Review Article

Aptamer-functionalized liposomes for drug delivery

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ABSTRACT

Among the various targeting ligands for drug delivery, aptamers have attracted much interest in recent years because of their smaller size compared to antibodies, ease of modification, and better batch-to-batch consistency. In addition, aptamers can be selected to target both known and even unknown cell surface biomarkers. For drug loading, liposomes are the most successful vehicle and many FDA-approved formulations are based on liposomes. In this paper, aptamer-functionalized liposomes for targeted drug delivery are reviewed. We begin with the description of related aptamers selection, followed by methods to conjugate aptamers to liposomes and the fate of such conjugates *in vivo*. Then a few examples of applications are reviewed. In addition to intravenous injection for systemic delivery and hoping to achieve accumulation at target sites, for certain applications, it is also possible to have aptamer/liposome conjugates applied directly at the target tissue such as intratumor injection and dropping on the surface of the eye by adhering to the cornea. While previous reviews have focused on cancer therapy, the current review mainly covers other applications in the last four years. Finally, this article discusses potential issues of aptamer targeting and some future research opportunities.

1. Introduction

Targeted drug delivery, compared to the systemic administration of drugs, offers potential advantages of increased efficacy and reduced toxicity [1]. This approach has led to the development of various nanomaterial-based drug formulations, with liposomes being a commonly used example with great clinical and commercial success [2]. Well-known liposome-based drug formulations include Doxil and Arikayce. Doxil, approved in the US in 1995, encapsulates doxorubicin hydrochloride within PEGylated liposomes and relies on passive targeting of tumor tissues. Arikayce, approved in 2018, employs liposomes containing amikacin sulfate for the treatment of lung infections. Another notable example of lipid-based drug delivery is the mRNA vaccine developed during the Covid pandemic [3,4].

Liposomes possess several advantages as drug carriers. They exhibit high biocompatibility, rendering them biologically inert with minimal toxicity. Liposomes, which are bilayered vesicles, were initially discovered by Bangham et al. in the mid-1960s. They can encapsulate both hydrophobic and hydrophilic drugs [2,5,6]. They demonstrate excellent properties in terms of circulation, penetration, and diffusion. Additionally, liposomes provide benefits such as extended half-life, improved safety, and enhanced efficacy [5,7]. Liposomes can be classified into different types based on their size and the number of layers they possess, including unilamellar, oligolamellar, multilamellar, and multivesicular liposomes [5,7]. Over the years, liposomes have been extensively investigated as potential vehicles for cancer chemotherapy, ocular drug delivery, colon-specific drug delivery, and various other applications [8–10].

Recent efforts have been devoted to developing active targeting delivery formulations with various targeting ligands and stimuli-responsive systems to improve their specificity and efficacy. For example, liposomes have been functionalized with small molecules such as folate [11] and glucose [12]. These molecules act as targeting ligands, facilitating the selective binding and uptake of liposomes by cells that overexpress the corresponding receptors [7]. Moreover, liposomes have been functionalized with macromolecules such as antibodies, peptides and aptamers as targeting ligands [7,13].

In addition to ligand-based targeting, functionalized liposomes can be engineered to respond to external stimuli. By incorporating light-sensitive, pH-sensitive, temperature-sensitive, or enzyme-responsive

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components, liposomes can exhibit controlled drug release at the intended site [2]. This feature enables precise timing and dosage of drug delivery, maximizing therapeutic efficacy, while minimizing potential side effects [13,14]. To further enhance the performance of liposomes, they can be conjugated with poly (ethylene glycol) (PEG) and polysaccharides on their external surfaces. PEGylation decreases surface fouling of liposomes, reducing their clearance by the immune system and prolonging their circulation time in blood [15]. Additionally, polysaccharides can provide both passive and active targeting functions, aiding in the accumulation and interaction of liposomes with target cells [Fig. 1] [7,13,16].

Aptamers are single-stranded nucleic acids that selectively bind to target molecules upon DNA/RNA folding to secondary and tertiary structures, which include features like loops, bulges, hairpins, and pseudoknots [18]. The high-affinity binding of aptamer to target involves intermolecular hydrogen bonding, electrostatic interactions, aromatic π–π stacking and van der Waals forces [18,19]. Also, salt concentration, metal ions can impact the tertiary structure of aptamers and their ability to bind to targets through their charge distribution and forming metal bridges [20]. A stable structure is formed in guanine-rich sequences, called a G-quadruplex, which has been found in many aptamers [21].

Given their favourable characteristics, aptamers have attracted significant interest in targeted drug delivery. Although numerous aptamers have been developed for various targets, only a limited number have progressed to clinical trials. For instance, the *anti*-nucleolin aptamer (AS1411) and the *anti*-CXCL12 aptamer (NOX-A12) have been undergoing clinical trials for cancer therapy, demonstrating the potential of aptamers as therapeutic agents [22]. Furthermore, a recent phase 1 clinical trial evaluated the safety and pharmacokinetic profile of the Toll-like receptor 4 antagonist aptamer, indicating the expanding scope of aptamers in therapeutic development [23].

To obtain nucleic acid aptamers, systematic evolution of ligands by exponential enrichment (SELEX) is a gold-standard strategy that involves iterative rounds of selection and amplification of nucleic acid libraries. Aptamers targeting cell surface biomarkers have been reported by selecting aptamers against purified proteins [24], cell-SELEX [25], and tissue-SELEX [26]. A few review papers were published to summarize aptamer-liposome conjugates for cancer therapy [27,28]. Aptamer-functionalized nanomaterials have shown effectiveness in delivering drugs in cancer therapy [29]. Over the last four years, many new developments have been made and the range of applications has gone beyond cancer therapy to other applications such as ocular drug delivery, drug delivery to skeletal muscle and oral macromolecules delivery, which will be the focus of this review.

2. Aptamers for cell surface targets

High-quality aptamers form the basis for targeted drug delivery. Thus, we first describe the screening of aptamers targeting the cell surface through multifaceted approaches: protein-based SELEX and cell-based SELEX [Table 1]. They have been used to identify aptamers with high affinity and specificity for cell-surface targets.

2.1. *SELEX methods for cell surface targets*

The SELEX process commences with a large pool of random oligonucleotides which are incubated with the target molecule, those oligonucleotides demonstrating an affinity for the target are discerned and isolated from the non-binding oligonucleotides. The identified binding oligonucleotides are then subjected to amplification through polymerase chain reaction (PCR). This amplified cohort of oligonucleotides undergoes successive cycles of selection and amplification. This iterative procedure is reiterated multiple times until oligonucleotides evincing the highest affinity for the target molecule are ultimately isolated [19,30].

Several SELEX methods have been used to select aptamers for cell surface proteins in the past few years [Table 1]. Cell-SELEX offers various advantages, including unbiased biomarker discovery, maintenance of the native folding, and post-translational modifications of target proteins. Using cell-SELEX, aptamers can be generated without prior knowledge of the molecular composition of cell surface targets, allowing the identification of novel biomarkers and potential therapeutic and diagnostic targets. In addition, modified cell-SELEX

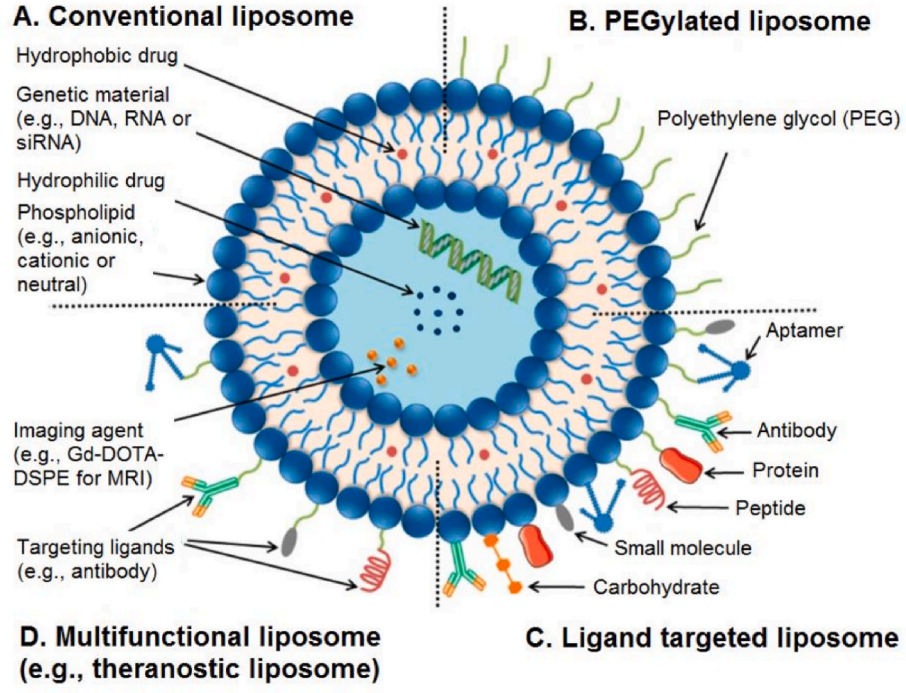

Fig. 1. Representation of conventional, PEGylated, targeted, and multifunctional liposomes. Image adapted from Ref. [17] with permission. Copyright ® 2018 *Multidisciplinary Digital Publishing Institute.*

Table 1
SELEX for cell surface.

Cell SELEX						
Aptamer name	Target	SELEX methods	Number of nucleotides	K_d (method)	Usage	Ref.
Aptamer VRF-CSRB-01	Unknown protein targets on the surface of retinoblastoma Weri-RB1 cell line	Cell-SELEX for Weri-RB1	87	49.41 ± 7.87 nM (Flow cytometry)	Diagnosis and targeting	[47]
PAp7T8	Unknown target for pancreatic ductal adenocarcinoma (PDAC)	Cell-SELEX for CMLu-1 cells isolated from PDAC lung metastases	32 Truncated and consist only the internal stem and a long hairpin loop region	110.07 nM to CFPAC-1 cells for the unmodified PAp7T aptamer (Flow cytometry)	Treatment	[45]
Apt-T-M3	Unknown target	Cell-SELEX for intestinal epithelial cells Microfold cells (M cells)	45Truncated the fixed primer region from the sequence of aptamer M3	176 ± 108 nM to M cells (Flow cytometry)	Targeting delivery of macromolecules	[44]
ZAJ4a	Receptor-Type Tyrosine-Protein Phosphatase F	Cell-SELEX for highly metastatic salivary adenoid cystic carcinoma cell line SACC-LM cells	49 Truncated the fixed primer region	8.1 ± 0.7 nM to DU145 cells (Flow cytometry)	PTPRF detection	[49]
HF3–58 and HA5–68	Primary identified as to glycoprotein on paclitaxel-resistant ovarian cancer cell line (A2780T)	Cell-SELEX for A2780T	HF3–58: Truncating 27 nucleotides from its 3′ end and mutating 2 nucleotides HA5–68: Truncating 27 nucleotides from its 3′ end and mutating 2 nucleotides	HF3–58: 0.30 ± 0.24 nM (Flow cytometry) HA5–68: 4.5 ± 1.6 nM (Flow cytometry)	Recognising drug resistance cells	[46]
A40s	Ephrin Receptor Tyrosine Kinase A2	GBM tissue-derived stem cells (GSCs)	30	41.92 nM to GSCs (TaqMan small RNA assays) 0.76 ± 0.26 nM to recombinant human EphA2 (Aptamer-based ELONA)	Delivery miRNA	[51]
Aptamer S1	/	Cell-SELEX for human breast cancer MCF-7 cell line	84	29.9 ± 6.0 nM (Flow cytometry)	Identification	[43]
Apt2	/	Cell-SELEX for human mammary epithelial MDA-MB-231 cell line	90	17.7 ± 2.7 nM (Flow cytometry)	Targeted therapies	[42]
CH1	Membrane proteins	Cell-SELEX for BRAF V600E-mutated human melanoma cells A375	49Truncated 15 nucleotides from its 5′ end and 8 nucleotides from its 3′ end, and mutating 1 nucleotide	19.9 ± 4.2 nM (Flow cytometry)	Detection	[48]
HSM01	Unknown target. Potential targets include fibronectin, collagen-3 and the filament protein actin	Cell-SELEX for HSKMCs	42	109.5 ± 9.8 nM (Flow cytometry)	Drug delivery to skeletal muscle	[71]
Apt-5	Neuron-specific enolase (NSE) serum tumor biomarker in clinical practice for small cell lung cancer	Cell-SELEX for SELEX immobilized onto the MBs	88	12.26 nM (Surface plasmon resonance)	Aptasensor	[72]
Modified cell SELEX						
B85.T2	CD19 transmembrane glycoprotein cluster of differentiation 19	Cell-SELEX for COS-7 transiently transfected with the human CD19 glycoprotein	25	49.9 ± 13 nM to purified human recombinant CD19 glycoprotein (Bio-Layer Interferometry)	Delivery miRNA	[31]
EA-3	CD98hc and EphA2 in Nasopharyngeal carcinoma	Cell-SELEX for epithelial tumor HONE1- Epstein-Barr virus cells	50 Truncated reverse primer	13.86 ± 5.45 nM to HONE1-EBV (Flow cytometry)	Identification	[50]
C24S	CD 44	Cell-SELEX for circulating tumor CHO–K1 cells overexpressing HBsAg CD44	40 Truncated primer binding region	14.54 nM (Flow cytometry)	Capturing	[33]
Cm-4	/	Cell-SELEX for HEK293T overexpressing HBsAg	51	148.4 nM (Flow cytometry)	/	[32]
Aptamer-A5	GLUT1 glucose transporter	CRISPR-Mediated Isogenic cell-SELEX for Caco2 with SLC2A1 gene knockdown	40	160 ± 49 nM (Flow cytometry)	Binding	[34]
Protein SELEX						
RNV-L7	Low-density lipoprotein receptor	Protein SELEX for human recombinant LDL-R protein	81	19.6 ± 3.2 nM to human recombinant LDL-R protein (ELONA) 295 ± 18 nM to positive cells at (Flow cytometry)	Delivering miR-21	[56]
uPAapt-02-FR	Human Urokinase-Type Plasminogen Activator (uPA)	Protein SELEX for human uPA	>40 Truncated by both primer binding sites	0.7 nM (SPR)	Detection and interfering with the pathological functions	[55]
Aptamer H2	Human lipocalin 6 (hLCN6)	Protein SELEX for recombinant hLCN6-His Tag protein on magnetic beads	60	3.21 ± 0.75 nM (ELONA)	Sperm Capture	[57]
Other SELEX						
8–60	Recombinant programmed cell death-ligand 1 (PD-L1)	REase-mediated SELEX for recombinant PD-L1	60	1.4 nM to PD-L1 (SPR)	Diagnostics	[37]

(*continued on next page*)

Table 1 (*continued*)

Cell SELEX						
Aptamer name	Target	SELEX methods	Number of nucleotides	K_d (method)	Usage	Ref.
				82.5 ± 25.5 nM to PD-L1 expressing BCPAP cells (Flow cytometry)		
SU-3	Fibroblast growth factor receptor binding protein3, K650E	SPR-based aptamer selection on gold-chip	94	28.2 ± 19.6 nM (SPR)	/	[38]
CS1 and CS2	RNA-binding protein (RBP) LS4 and LS12	Phage Display SELEX (PD-SELEX) for RBP	CS1:59 CS2:56	CS1: 6.82 ± 0.98pM CS2 : 7.24 ± 2.29 pM (SPR)	Engineering and analysis of RNA-protein interactions	[54]
D-10-3 and BD-10-1	Intercellular adhesion molecule-1 (ICAM-1)	Cell-SELEX for ICAM-1$^{(+)}$ CHO-ICAM- 1 cells and Protein SELEX for ICAM-1 protein	Both 81	D-10–3: 13.8 ± 0.7 nM to CHO-ICAM-1 cells BD-10–1: 18.2 ± 1.6 nM to CHO-ICAM-1 cells (Flow cytometry)	Inhibition of cell migration and invasion	[36]

incorporating transient transfection [31], overexpression [32,33], or CRISPR-based genetic manipulation [34] to the target could increase the expression of desired target molecules in cells and improve the selection of aptamers with higher specificity and affinity. Protein-SELEX was used to select aptamers that specifically bind to recombinant surface proteins. While the selected aptamers can target the intended protein, they have a few potential disadvantages. These include the risk of isolating aptamers that bind to non-functional regions of the target protein, limited binding due to buried sites and the possibility of selecting ineffective aptamers for the natural form of the protein [35]. Despite these disadvantages, a study carried out both cell-SELEX and protein-SELEX for intercellular adhesion molecule-1 (ICAM-1)-positive cells and ICAM-1 protein, respectively [36], where the most replicated sequences of the cell-SELEX and protein SELEX were the same.

In addition to these SELEX methods, REase-mediated SELEX (REase-SELEX) was utilized for *in vitro* evolution of a highly specific PD-L1aptmaer [37]. In this REase-SELEX work, the DNA library contained a recognition site for a type II restriction endonuclease, *Alu*I, in the middle of the 40-nt random sequences, against recombinant PD-L1. *Alu*I cleaved the non-binding sequences at the recognition site. This enzymatic digestion enriched the pool of aptamers that specifically bind to PD-L1. An additional advantage of REase-SELEX is that the digestion by *Alu*I induced mutations within the library, increasing its diversity and potentially leading to the generation of aptamers with improved binding properties [37]. In 2019, an aptamer SU-3 targeting fibroblast growth factor receptor binding protein 3 $K^{65}0E$ was selected using surface plasmon resonance (SPR)-based aptamer selection on library immobilized gold chips [38]. SPR-based aptamer selection provided a real-time examination of the binding kinetics of each SELEX cycle throughout the selection process without further experimental effort [38].

Many aptamers generated through various SELEX methods have shown high binding affinities in the nanomolar range, which is indicative of strong and specific interactions with their target molecules. These results demonstrated that the achievement of high binding affinity does not solely depend on the SELEX method used, but also on the proper and optimized conditions of the SELEX process. The commonly used aptamers for targeted drug delivery are the tyrosine kinase 7 protein aptamer, sgc8 [39], mucin 1 aptamer, S2.2 [40], and prostate-specific membrane antigen aptamer, A10–3.2 [41]. The secondary structures of a few most commonly used aptamers for targeted drug delivery are shown in [Fig. 2].

2.2. Target identification of aptamer

Identifying the target molecules of aptamers can be a significant challenge, particularly in cell-SELEX. Several studies have not identified

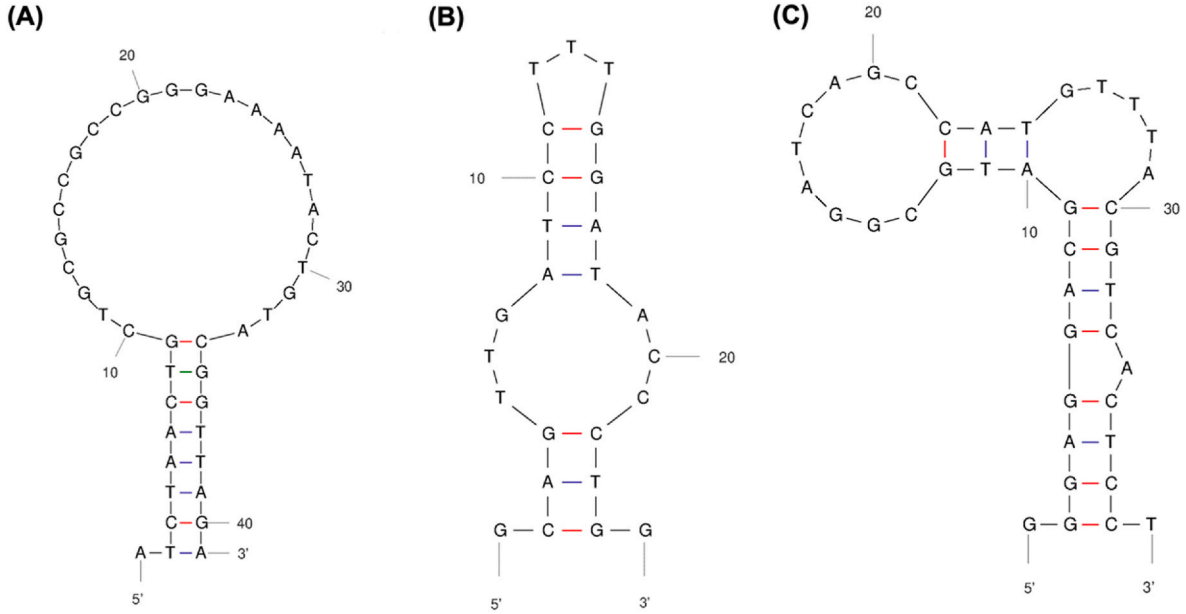

Fig. 2. Secondary structures of three common aptamers predicted using Mfold software: (A) sgc8, (B) S2.2 and (C) A10–3.2 aptamers.

the specific targets of their aptamers [32,42–45]. However, in some studies, various approaches have been employed to determine aptamer targets. One common and straightforward approach involved treatments with high temperature and agents such as tunicamycin and trypsin to ensure that the aptamer targets were proteins [46–48]. A paper employed stable isotope labeling using amino acids in cell culture (SILAC)-based quantitative proteomic methods to identify the receptor-type tyrosine-protein phosphatase F (PTPRF) as the target of ZAJ4a [49]. The findings were further validated by measuring the binding of ZAJ4a to PTPRF-knockdown cells using flow cytometry [49]. Moreover, protein pull-down experiments and immunoblotting are additional techniques used for target identification and validation. In particular, protein pull-down experiments involving incubating protein extracts with biotinylated aptamers, followed by the analysis of eluted proteins using mass spectrometry [50] or a receptor tyrosine kinase proteome profile array [51].

2.3. Specificity and quality of aptamers

While many aptamer sequences have been reported, it is important to realize that not all reported aptamers can specifically bind to their claimed targets. Our group and many other groups have found that many small-molecule-binding aptamers cannot bind [52]. An article published in 2021 pointed out that several aptamers cannot bind to their reported targets. For instance, aptamers, such as A10-3 (selected by protein SELEX for PSMA targeting), GL21. T (selected by cell-SELEX for AXL targeting), and EpDT3 (selected by cell-SELEX for EpCAM targeting) were found to exhibit no specific binding to their reported protein targets. This lack of specificity was confirmed through validation experiments, including the use of siRNA knockdown of the specific target in cells [24]. The Ellington group performed a systematic flow cytometry study on seven different aptamers and 14 cell lines, and nonspecific adhesion was observed in a few aptamers/cells [53]. Five research papers [32,54–57] discussed in this review, did not include counter-selection or negative selection steps in their SELEX processes. This omission raises concerns about the specificity and potential off-target binding of the resulting aptamers. The Levy group also performed a systematic comparative study, raising concerns about specific aptamer binding [24].

These findings raised concerns about the aptamers' ability to effectively target cells and emphasized the importance of thorough validation and verification of aptamer binding to ensure their specificity and functionality in practical applications. In our opinion, it is important to have control sequences that are not aptamers and perform experiments side-by-side to ensure that the observed targeting behaviour is due to aptamer binding. A paper was published to recommend minimal publication standards of new aptamers [58]. Unfortunately, several papers reviewed in the current study lacked side-by-side control sequences. Additional validation studies are required before considering these aptamers for targeted delivery applications to ensure their suitability and specificity.

To optimize the SELEX process and generate more specific aptamers, one approach is to implement counter selections. The libraries were then incubated with structurally similar target molecules or cells. This helps to discriminate and remove non-specific oligonucleotides that may bind to the structurally-similar targets [59]. Another non-specific DNA or aptamer-mediated interaction is observed in spherical nucleic acids, which can be internalized by various cells irrespective of DNA sequence, as long as a high DNA density is achieved [60].

2.4. Advantages and challenges of aptamers

Aptamers are often referred to as "chemical antibodies". Antibodies are Y-shaped proteins belonging to the immunoglobulins, a family of globular proteins. For example, the common IgG antibody is composed of four polypeptide chains: two heavy chains and two light chains. These chains are held together by disulphide bonds. The heavy and light chains form pairs in each arm of the Y, generating two identical antigen-binding sites. These sites, located at the tips of the arms, contribute to antigen binding in the nanomolar to picomolar range, enabling the recognition of specific foreign entities [61]. Similar to antibodies, aptamers bind to their targets. However, most functional aptamers exhibit binding affinities to small molecules in the micromolar to nanomolar range. Indeed, the specificity of aptamers can be a concern when compared to antibodies. Molecules with similar structures may bind to the same aptamer. For instance, the tetracycline DNA aptamer was reported to cross-react with oxytetracycline (129.7 %), chlortetracycline (32.4 %), and doxycycline (17.6 %), antibacterial agents with structures related to tetracycline, when compared to the tetracycline antibody (10 %, 13.7 % and 0 %, respectively) [62]. This potential for cross-reactivity underscores the need for rigorous validation and careful consideration of aptamer specificity in practical applications. Nevertheless, the advantages of aptamers over antibodies are becoming increasingly evident [29,63]. Although antibodies offer high specificity and affinity for their targets, they can be limited by their susceptibility to high temperatures and pH changes. In contrast, aptamers, particularly DNA aptamers, exhibit enhanced stability, making them more suitable for various experimental conditions, diagnostic and therapeutic applications. Aptamers also offer several practical benefits such as lower production costs, much less batch-to-batch variation, easier chemical modification, and smaller size. These features allow for higher immobilization densities and facilitate the development of more efficient targeted delivery vehicles [25,64]. Additionally, aptamers are relatively non-immunogenic and have comparatively quick tissue penetration with negligible toxicity, making them suitable for *in vivo* applications. Finally, the targeting effect of aptamers can be removed by adding its complementary DNA [65]. Therefore, the interest in using aptamers for targeted and controlled drug delivery has been enormous [66–68].

2.5. Challenges of unmodified aptamers for therapeutic applications

When it comes to clinical use, the inherent physicochemical characteristics of nucleic acids can result in undesirable pharmacokinetic patterns due to vulnerability to nuclease breakdown, rapid removal through the kidneys, swift dispersion from the bloodstream to specific tissues (such as the liver or spleen), polyanion effects, and a non-specific immune response [19]. In serum, the reported RNA lifespans are in the range of seconds. This duration is slightly prolonged, to minutes, when they are in a double-stranded state [69]. Furthermore, DNA typically exhibits longer reported lifespans, around 1 h. The inclusion of a 3′ inverted dT residue can further extend this duration to several hours [69,70]. All of these factors hinder their effectiveness as therapeutic agents in living organisms. To advance the clinical adoption of aptamer-based therapeutics, substantial modifications to aptamer structures and their combination with functional molecules are crucial. Thus far, aptamers have been linked with PEG or cholesterol to enhance their availability and extend their presence within the organism. In a pharmacokinetic analysis of Macugen, a vascular endothelial growth factor aptamer PEGylated with a 40 kDa PEG, it exhibited a half-life of 9.3 h in plasma following intravenous administration and 12 h after subcutaneous injection. Notably, its half-life was markedly extended to 94 h in the vitreous humor [19]. A review paper has outlined various methods that have been developed to enhance the stability and half-lives of aptamers on their own within the body [19].

3. Aptamer-functionalized liposomes

3.1. Conjugation of aptamers to liposomes

Nucleic acids are highly-charged and unmodified DNA or RNA cannot be conjugated to phospholipid bilayers [73]. Conjugating aptamers to liposomes can be achieved in a few methods: covalent

binding, non-covalent strategies, or post-insertion techniques, allowing the construction of aptamer liposomal spherical nucleic acids [Fig. 3] [73,74].

3.2. Covalent conjugation

The direct attachment of aptamers to liposomes could be achieved by carbodiimide conjugation [Fig. 3A] or thiol-maleimide crosslinking [Fig. 3B] [75]. Carbodiimides (RN = C=NR) find frequent applications in organic synthesis, bioconjugation, and drug delivery. A notable example is 1-ethyl-3-(3-(dimethylaminopropyl)-carbodiimide, a water-soluble carbodiimide commonly utilized to activate carboxylic acid (*R*–COOH) residues. This activation allows them to react with ligands containing amino groups (-NH_2), resulting in the formation of amide bond linkages (*R*–$CONH_2$) [74]. In a study, the AS1411 aptamer was conjugated onto liposome-COOH through carbodiimide chemistry. The liposome-COOH was dispersed in a buffer at pH 6.0 with continuous stirring. In the presence of carbodiimides, the 5′–NH_2–AS1411 aptamer was coupled to the liposomes, forming an amide bond linkage [76].

Thiol-maleimide crosslinking is a simple and swift reaction involving a thiol-modified DNA and a maleimide-modified lipid, yielding a thiosuccinimide product. This reaction represents a specific form of Michael addition, where a thiol or thiol derivative, characterized by the structure R–SH, is capable of interacting with polar and charged residues in highly polar solvents. Meanwhile, the maleimide group selectively reacts with –SH, establishing a thioether bond at pH between 6.5 and 7.5 [75]. In [Fig. 3B], C_6-thiol-modified Apt1 was deprotected in water by Tris (2-carboxyethyl)phosphine hydrochloride to produce Apt1-SH and then purified by precipitation. Apt1-SH was resuspended in binding buffer (pH 7.4) and conjugated maleimide-functionalized PEG-liposomes [75].

3.3. Non-covalent strategies

Avidin is a tetrameric biotin-binding protein, composed of four identical subunits (forming a homotetramer). Each subunit exhibits a high degree of affinity and specificity for binding to biotin. The avidin–biotin complex has an extraordinarily low dissociation constant, measured at approximately femtomolar, ranking it among the strongest known non-covalent bonds [77]. In [Fig. 3C], the authors utilized avidin–biotin coupling to conjugate biotinylated aptamers onto avidin-treated liposomes [Fig. 3C] [78].

3.4. Post-insertion methods

Post-insertion methods involve the incorporation of aptamers into preformed liposomes, either by using lipid moieties linked to the aptamer or by hybridizing complementary DNA strands to the liposomes [79]. Cholesterol-tagged aptamers can spontaneously insert into

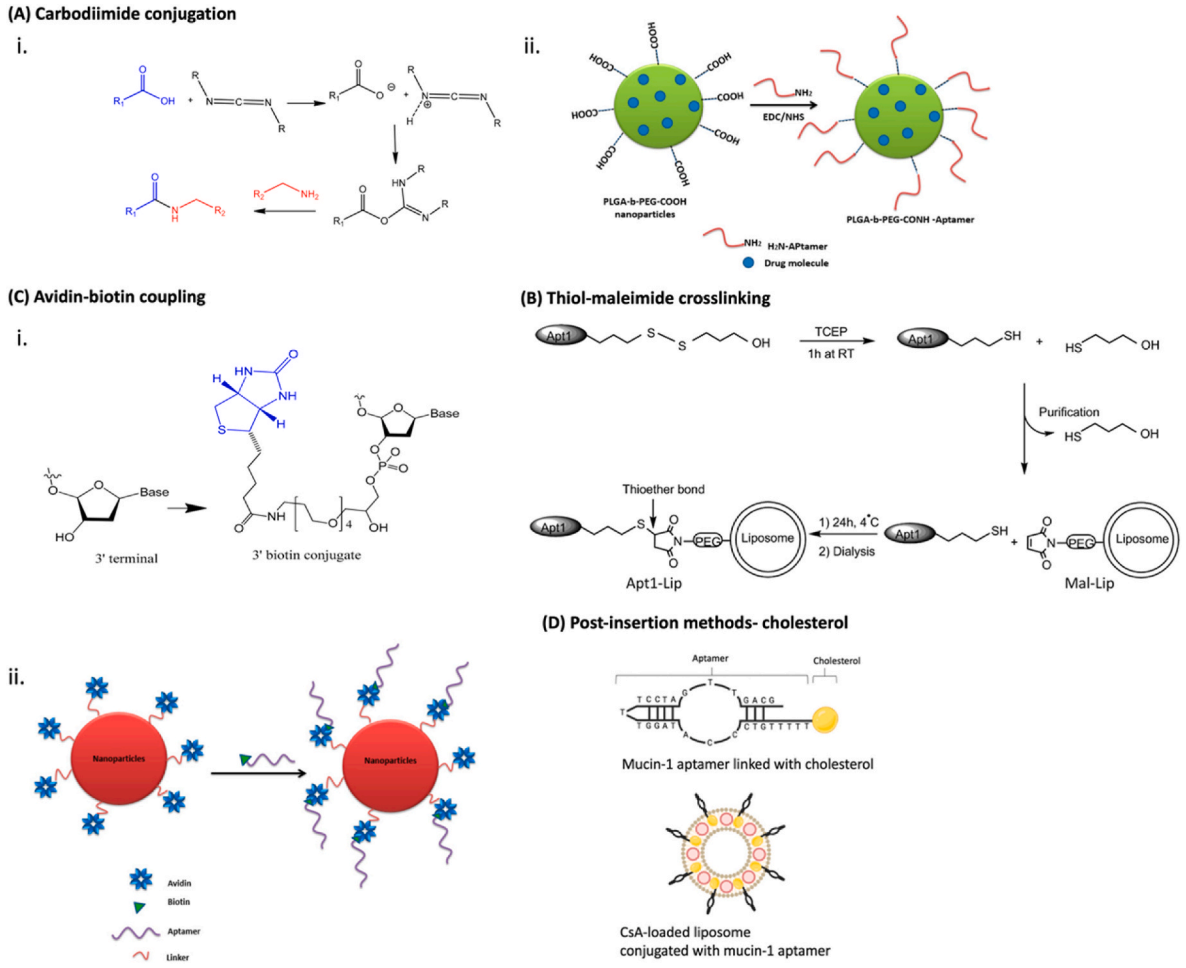

Fig. 3. Chemistry of conjugation aptamers to liposomes. (Ai) Formation of a covalent amide bond between carboxylic acid and carbodiimide; (Aii) carbodiimide bioconjugation approaches of aptamer-functionalized on PLGA-b-PEG-COOH nanoparticles (Image adapted from Ref. [74] with permission. Copyright ® 2019 *Multidisciplinary Digital Publishing Institute.*). (Bi) Apt1-Liposome Functionalization of liposomes with aptamers using thiol-maleimide reaction. Image adapted from Ref. [75] with permission. Copyright ® 2015 American Chemical Society (C) Formation of biotin aptamer; (Bii) Attachment of biotin aptamer to nanoparticles with avidin linker (Image adapted from Ref. [74] with permission. Copyright ® 2019 *Multidisciplinary Digital Publishing Institute.*). (D) Post insertion of cholesterol-aptamer into the lipid bilayer of liposomes. Image adapted from Ref. [79] with permission. Copyright ® 2023 Royal Society of Chemistry.

liposomes based on the tag [Fig. 3D] [79]. Aptamers can be added to liposomes during formation or conjugated to preformed liposomes. Conjugating during liposome formation allows aptamers to be distributed on both the outside and inside surfaces, but the interior aptamer may limit the space for drugs, reducing the drug-loading efficiency. Post-insertion methods offer customization advantages for preformed liposomes, providing better control over aptamer distribution and drug-loading capacity [74,80]. Conjugation efficiency can be determined using various methods, such as gel electrophoresis and spectrophotometry after ultracentrifugation [79].

In comparison with aptamers, common approaches to conjugate an antibody to liposomes include chemical modification and protein-liposome conjugation [13]. Chemical modification involves modifying antibodies and phospholipids with reactive groups like amino, thiol, imine, or aldehyde groups, allowing for covalent bond formation between the antibody and the liposome surface [13]. Even though the conjugation methods are similar, the size difference between the aptamer and antibodies can impact the conjugation strategies and the overall properties of the liposome-antibody or liposome-aptamer conjugates. In addition, it is difficult for antibody conjugation to achieve

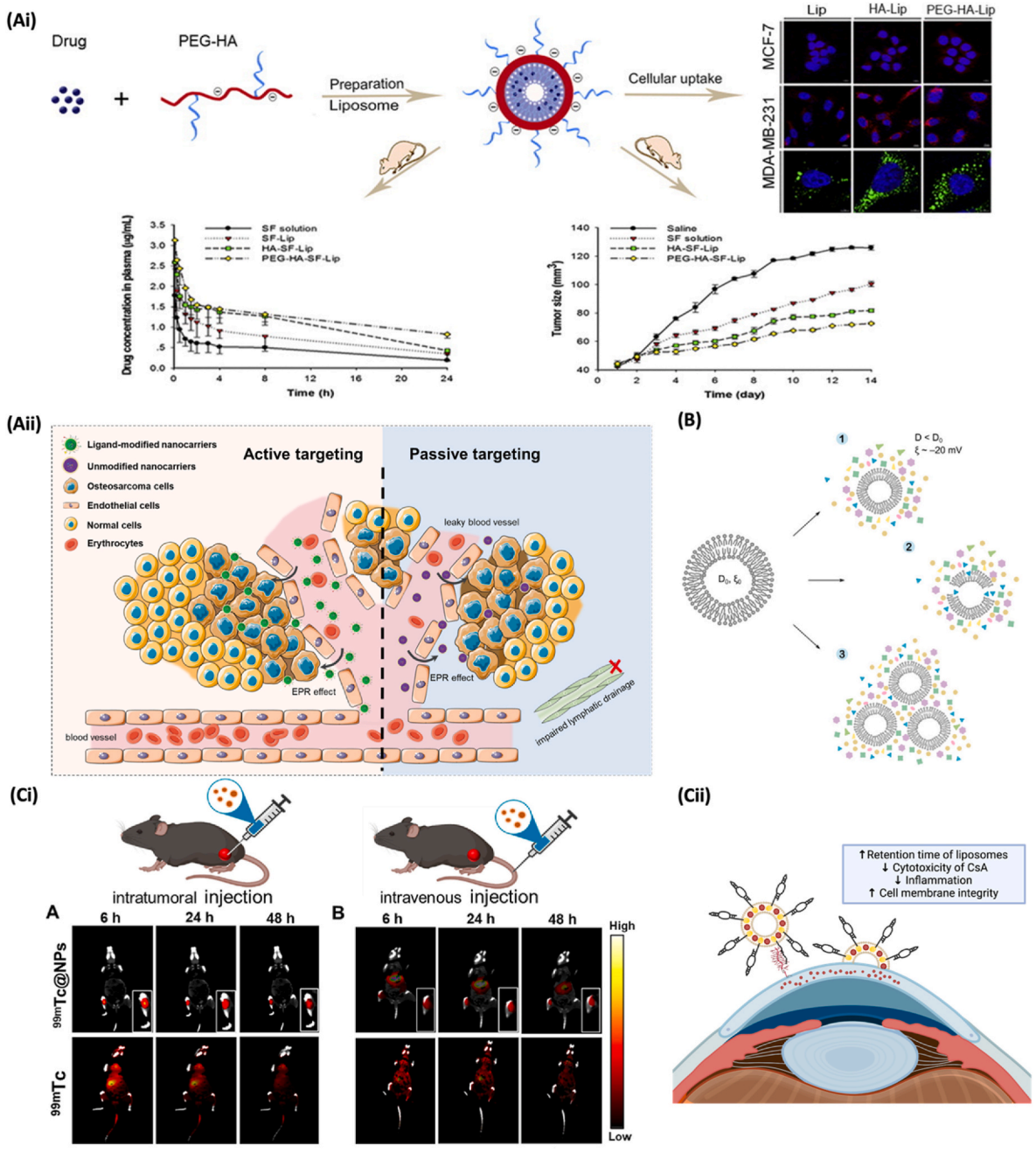

Fig. 4. Delivery and distribution of liposomes (Ai) PEGylated liposomes for higher systemic exposure and enhanced efficacy after intravenous administration (Image adapted from Ref. [83] with permission. Copyright ® 2018 Elsevier). and (Aii) passive and active tumor targeting via the interstitial space. Image adapted from Ref. [92] with permission. Copyright ® 2023 BioMed Central.) (B) Possible effects of the protein corona on liposome properties: (1) shrinkage of liposomes and evening out of the zeta-potential, (2) lipid bilayer disruption, and (3) protein-mediated aggregation. Image adapted from Ref. [93] with permission. Copyright ® 2021 Elsevier. (Ci) Targeted delivery of nanoparticles through intravenous and intratumoral injection (Image adapted from Ref. [91] with permission. Copyright ® 2023 Elsevier) and (Cii) local and topical administration of liposomes to cornea. Image adapted from Ref. [79] with permission. Copyright ® 2023 Royal Society of Chemistry.

site-specificity [81].

3.5. Fates of liposomes in body

Even though liposomes are a successful platform for drug delivery, only a small fraction of the formulations have advanced to clinical use. This low success rate can be attributed to the suboptimal biodistribution and safety profile of liposomes after administration [82]. PEG has been conjugated with aptamers or incorporated into liposomes to increase the size and molecular weight to decrease renal clearance and increase the circulation time *in vivo* [15,83]. Long-circulating liposomes capitalize on the distinctive characteristics of blood vessels found in tumor tissues [15]. Tumor blood vessels have larger gaps (100–700 nm) than non-tumor tissues (5–10 nm), allowing liposomes to easily enter the tumor interstitial space [15]. Moreover, solid tumors have disorganized vascular structures, leading to the absence of a functional lymphatic system. This combination of leaky tumor vasculature and limited lymphatic drainage by disorganized vascular structures are known as the enhanced permeability and retention (EPR) effect [15,83,84]. An example of this is when PEGylated hyaluronic acid-coated liposomes were employed to enhance the delivery of sorafenib, specifically targeting CD44 in the MDA-MB-231 tumor xenograft mouse model. This resulted in a prolonged presence of sorafenib in the bloodstream after intravenous administration, ultimately leading to an increased effectiveness in combating cancer in mice [Fig. 4i] [83]. [Fig. 4] Aii illustrates two strategies employed in nano-delivery systems for anti-tumor therapy. Passive targeting (right panel), utilizing the EPR effect, involves nanocarriers circulating in the bloodstream, followed by their extravasation and accumulation within tumor tissue, exploiting the permeable nature of tumor vasculature. On the other hand, active targeting (left panel) incorporates the customization of nanocarriers with targeting ligands. These ligands can selectively bind to receptors overexpressed on tumor cells, enabling precise drug delivery locally or facilitating internalization through receptor-mediated endocytosis.

Aptamer-functionalized liposomes are liposomal spherical nucleic acids that often have a high density of DNA [85], enhance uptake by cells [86] and penetrate various tissues including the blood-brain barrier [87]. Numerous aptamer-functionalized liposomes have been designed for medical applications, typically administered intravenously [84,88]. This delivery method facilitates the widespread distribution of liposomes throughout the body, allowing them to reach organs non-invasively. However, in the bloodstream, these liposomes interact with circulating proteins, forming a protein corona on their surface [82,84]. The protein corona makes liposomes easier to be recognized by the innate immune system and eliminated by phagocytic cells in organs, such as the lungs, liver, and spleen. The opsonization of plasma proteins could also affect the size, charge and lipid bilayer formation of liposomes and lead to protein-mediated aggregation, potentially prevent liposomes from binding to their targets [Fig. 4B] [82]. Therefore, simply coating liposomes with aptamers may not achieve the desired targeting function *in vivo*.

PEGylation of liposomes [89] or pre-coating liposomes with dysopsonic proteins [90] has been suggested to reduce protein corona formation during in circulation. Albumin, for instance, decreases the opsonization of PEGylated liposomes by blocking binding sites for serum proteins, enhancing hydrophilicity, and changing the PEG molecule structure into a “brush” conformation [82,90]. Local administrations of aptamer-functionalized liposomes, like intratumor injection [91] or topical administration to the eye surface [79], might enhance targeted delivery regionally rather than systemically [Fig. 4C].

4. Targeted therapy using aptamer-functionalized liposomes

Aptamer-functionalized liposomes exhibited effectiveness in targeted drug delivery for cancer therapy, showing enhanced uptake in tumor cells in both *in vitro* and *in vivo* studies. These liposomes have been used for delivering aptamers, siRNA, or drugs via intravenous injection for cancer treatment [Table 2]. Beyond that, recent studies showed other applications in the eyes, skeletal muscles, and intestines [Table 2].

4.1. Delivery to tumors

Targeted delivery of chemotherapeutic agents to tumors has been a major application of aptamer-functionalized liposomes. Recently, five research groups achieved targeted delivery using this system for liver [94], breast [94,95], colorectal and colon cancers [96,97]. These liposomes were administered to animals through intravenous injection. Caelyx/Doxil, PEGylated liposomal doxorubicin, was the first approved nanoparticles with significantly improved pharmacokinetics and prolonged circulation time compared to free doxorubicin, making it a promising candidate for cancer treatment [97]. In one study, Jaafari et al. utilized an EpCAM RNA aptamer to functionalize Caelyx through an EDC/NHS coupling reaction. This modification allowed targeted delivery of doxorubicin to C26 tumor cells inoculated in mice [97]. The results showed that the aptamer-functionalized Caelyx was preferentially distributed to the tumor site and exhibited reduced accumulation in the heart, liver, and spleen. Consequently, it significantly reduced tumor growth compared to regular Caelyx [97]. In another study focused on colon cancer, PEGylated lipid nanoparticles containing docetaxel were prepared and modified with AS1411 *anti*-nucleolin aptamers. *In vivo* experiments conducted on C26 tumor-bearing mice showed that these aptamer-targeted lipid nanoparticles improved the antitumor activity and inhibited tumor growth compared to non-targeted counterparts [96].

Chemotherapeutic agent resistance is a significant challenge in cancer therapy. Overexpression of the forkhead box M1 (FOXM1) transcription factor in breast cancer is known to contribute to such resistance [95]. Moosaviana et al. developed PEGylated liposomes functionalized with FOXM1 aptamers (FOXM1-apts). These aptamers were electrostatically attached to cationic liposomes just before *in vitro* experiments. The combination therapy of these liposomes/FOXM1 aptamers with chemotherapeutic agents demonstrated a significant enhancement in cytotoxicity on cancer cells and increased apoptosis compared to treatment with chemotherapeutic agents alone. This study suggested that liposomes/FOXM1 aptamers could overcome chemotherapeutic agent resistance, possibly due to passive delivery to the tumor site and inhibition of FOXM1 by aptamer. Overall, the use of aptamer-functionalized liposomes for targeted delivery of chemotherapeutic agents showed potential in improving the efficacy of cancer treatment [95]. However, further research is needed to fully understand the pharmacokinetics and biodistribution of these liposomes to optimize their therapeutic potential.

4.2. Delivery to eyes

Ocular drug delivery has been challenging due to barriers in the eyes and tear flow, which reduce the retention and bioavailability of ocular drugs in the eyes [79]. Our group developed mucin-targeting liposomes for ocular drug delivery, using a post-insertion method with cholesterol-linked S2.2 aptamer. Encapsulating cyclosporine A (an FDA-approved drug for treating dry eye diseases) into these liposomes reduced cytotoxicity and increased retention in human corneal epithelial cells. In a rat model, local and topical administration of these liposomes effectively relieved dry eye disease symptoms, performing similarly to commercial eye drops. Aptamer-functionalized liposomes outperformed non-aptamer liposomes in reducing inflammation and maintaining corneal integrity [Fig. 5A]. This highlights their potential as a promising strategy for improved ocular drug delivery with reduced side effects [79]. Further studies are needed to investigate the pharmacokinetics and biodistribution of liposomes in eye. In addition, Cruz et al. conducted a study where they developed nucleolin-targeting PEGylated liposomes to enhance the retinal antiangiogenic effects of

Table 2
Recent studies on aptamer-functionalized liposomes for targeted delivery.

Target	Aptamer	PEGylated liposome	Drug	Method of administration	Ref
Cancer					
Breast cancer	CD44 aptamer and PD-L1 aptamer	DSPE-PEG2000	Doxorubicin and IDO1 siRNA	Intravenous injection	[94]
Breast cancer	FOXM1-apt	/	Deliver aptamer	Intravenous injection	[95]
Colorectal cancer	AS1411	PEG2000-DSPE	Docetaxel	Intravenous injection	[96]
Colon carcinoma	EpCAM aptamer	Yes	Doxorubicin	Intravenous injection	[97]
Liver cancer	TLS11a	MAL-PEG2000-DSPE, PEG2000- DSPE	Doxorubicin	Intravenous injection	[98]
Eye					
Cornea	Mucin-1 S2.2	/	Cyclosporin A	Topical eye drop (locally)	[79]
Human Umbilical Vein Endothelial Cells	AT11-L0	DSPE-PEG2000	Dexamethasone and acridine orange derivative termed C_8	No *in vivo* study	[99]
Muscle					
Skeletal muscle	HSM01	DSPE-PEG	/	Intravenous injection	[71]
Intestine					
Intestinal epithelial cells Microfold cells (M cells)	Chol-Apt-T-M3	/	Exenatide	Incubation of liposomes with Peyer's patches and intestinal epithelium tissues *ex vivo*	[44]

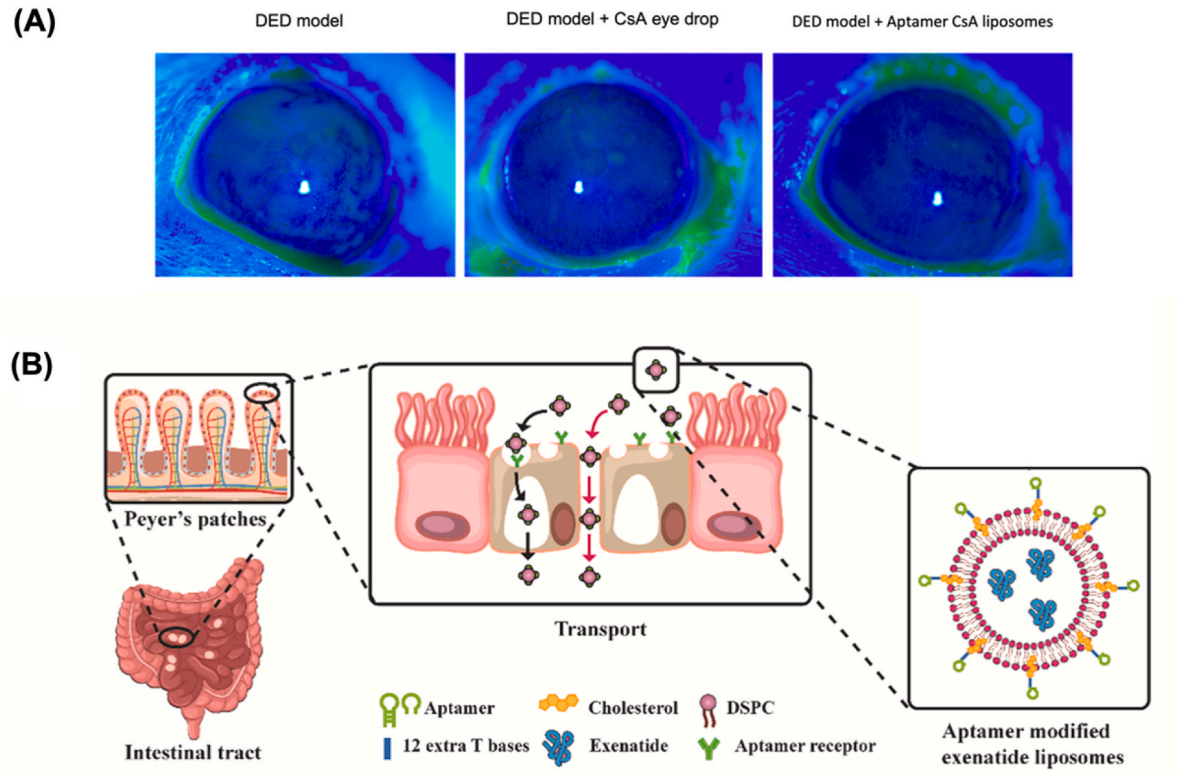

Fig. 5. Aptamer-functionalized liposomes for targeted delivery to the eye, skeletal muscles, and intestine. (A) The efficacy of mucin-targeting cyclosporin A liposomes promoted corneal integrity, similar to the commercial drugs, in dry eye diseases rat models. Image was adapted from Ref. [79] with permission. Copyright ® 2023 Royal Society of Chemistry. (B) The delivery of macromolecules encapsulated in Apt-T-M3-functionalized liposomes to intestinal tract. Image reproduced and adapted from Ref. [44] with permission. Copyright ® 2023 Elsevier.

the anti-inflammatory drugs dexamethasone and C8 (acridine orange derivative) in the human umbilical vein endothelial cell (HUVECs) model [99]. In their approach, amide-AT11-L0, an aptamer derivative of AS1411, was conjugated to NHS-terminated PEGylated liposomes. Notably, the liposomes alone exhibited no cytotoxicity in HUVECs. However, when loaded with C8 and dexamethasone and functionalized with the aptamer, the angiogenic process were not notably successful, possibly due to the influence of the modified AT11-L0 G4 on nucleolin recognition, suggested by the authors [99]. It is important to consider further research using an unmodified AS1411 aptamer [99]. To gain a comprehensive understanding of the targeted and localized anti-angiogenic effects in the retina, future investigations should include an assessment of retinal pigment epithelial cells or other retinal cells. This is crucial since the binding affinity of aptamer-liposomes may differ depending on the cell type and cell surface protein profile [99]. By conducting such studies, a better understanding of the applicability and efficacy of this approach can be attained.

4.3. *Delivery to skeletal muscles*

In recent years, significant progress has been made in the development of drugs for the treatment of skeletal muscle diseases. However, a major challenge is the lack of muscle cell specificity, leading to unpredictable systemic adverse effects [71]. To tackle this issue, Su et al.

employed cell-SELEX to select HSM01 aptamers that specifically target human skeletal muscle. These aptamers were then incorporated into PEGylated liposomes through a sulfhydryl group linkage. For comparison, control library-conjugated liposomes were also used in the study. The binding of skeletal muscle-targeted liposomes to the tree shrews' skeletal muscle peaked at 120 min post-injection, gradually decreasing by the 180-min mark. Crucially, it was observed that these liposomes had no noticeable effects on the liver and kidney metabolism. These findings highlight the potential of the developed skeletal muscle-targeted liposomes as a promising approach to enhance drug delivery and specificity for treating skeletal muscle diseases [71].

4.4. Delivery to intestine

Liposomal supplements or drugs are widely used to deliver drug to GI tract. However, delivery of macromolecules has been difficult due to its instability in the GI tract and the low permeability of macromolecules in the intestine [44]. To address this issue, Guan et al. employed a novel approach, using aptamer (Apt-T-M3) specifically targeting M cells, which are epithelial cells found in Peyer's patches in the intestine [44]. They selected Apt-T-M3 through cell-SELEX and then developed Apt-T-M3-modified liposomes, incorporating cholesterol-linked Apt-T-M3 using a microfluidics system, to facilitate the delivery of exenatide to M cells [Fig. 5B]. The study demonstrated that the aptamer-functionalized liposomes significantly enhanced the transport efficiency of exenatide in M cells, effectively reducing the transepithelial electrical resistance of M cell monolayers [44]. In *ex vivo* intestinal absorption studies, these aptamer-functionalized liposomes exhibited high absorption rates in Peyer's patches and demonstrated their specific targeting ability for M cells. Altogether, the Apt-T-M3-liposomes proved to be a promising and efficient M cell targeted delivery system for the oral administration of macromolecules, as they promoted both M cell-mediated transport and improved paracellular permeability [44]. To further validate the potential of this delivery system, additional research on biodistributions *in vivo* might be necessary.

5. Conclusions and future perspectives

The broader scope of applications of aptamer-functionalized liposomes highlights their potential to revolutionize drug delivery in various medical fields, offering a more precise and efficient approach to treating a range of diseases and conditions. In this review, we briefly introduce methods to isolate aptamers for specific cells and tissues, and the potential problems of using these aptamers as targeting ligands. We further reviewed the conjugation of aptamers to liposomes, and the fate of these conjugates in animals. Finally, a few applications of such conjugates have been discussed, including targeted drug delivery to tumors, corneas, muscles, and intestines. Although significant progress has been made, the field is still far from being clinically applicable. We identified the following problems that may become future research opportunities. One of these challenges is the degradation of DNA in the bloodstream. To address this, research have been focusing on modifying aptamers to enhance their stability while preserving their binding properties [63]. For example, Macugen, the first approved aptamer drug for treating age-related macular degeneration, was discontinued. Macugen is an extensively modified RNA aptamer, and the high cost associated with its synthesis diminishes the cost advantage compared to antibodies [100]. In addition, using modified nucleic acids complicates the aptamer selection process. Second, it is crucial to improve the SELEX process, obtain high-quality aptamers, and identify the aptamer targets. High-quality and specific aptamers are the basis of targeted drug delivery and can be used to separate targeting effects from nonspecific effects, especially *in vivo*. The *in vivo* binding properties of aptamers must be thoroughly evaluated using appropriate negative controls to ensure effective targeting and drug delivery. Third, because aptamer-functionalized liposomes interact with various molecules in the body, comprehensive pharmacokinetic studies are essential. These studies provide insights into the safety, circulation, biodistribution, and clearance of aptamer-functionalized liposomes, particularly when administered via intravenous injection. By addressing these aspects, aptamer-functionalized liposomes demonstrate their potential for clinical applications beyond cancer therapy. They can potentially be integrated into soft materials such as hydrogels, wound healing patches, and microneedles, presenting a hopeful approach for precise and effective drug delivery. Further exploration of the synergies between aptamer-functionalized liposomes and emerging technologies such as CRISPR or nanotechnology, which combines the specificity of aptamers with the precision of CRISPR and the advanced capabilities of nanotechnology, holds the potential to provide valuable insights into addressing genetic disorders and complex medical conditions.

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