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Adenosine and lipids: A forced marriage or a love match?

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ABSTRACT

Adenosine is a fascinating compound, crucial in many biochemical processes: this ubiquitous nucleoside serves as an essential building block of RNA, is also a component of ATP and regulates numerous pathophysiological mechanisms *via* binding to four extracellular receptors. Due to its hydrophilic nature, it belongs to a different world than lipids, and has no affinity for them. Since the 1970's, however, new discoveries have emerged and prompted the scientific community to associate adenosine with the lipid family, especially *via* liposomal preparations and bioconjugation. This seems to be an arranged marriage, but could it turn into a true love match? This review considered all types of unions established between adenosine and lipids. Even though exciting supramolecular structures were observed with adenosine-lipid conjugates, as well as with liposomal preparations which resulted in promising pre-clinical results, the translation of these technologies to the clinic is still limited.

Contents

1. Introduction

Adenosine is a nucleoside involved in multiple essential biochemical processes both intra- and extracellularly (Fig. 1). In the cell, adenosine can be phosphorylated into adenosine monophosphate (AMP), which is used by the cell as an RNA building block. This phosphorylation process can be repeated in order to generate adenosine triphosphate (ATP), the "molecular currency" furnishing energy to the cell. ATP can also be converted by adenylyl cyclase (AC) into cyclic AMP (cAMP), a very common second messenger which triggers intracellular signal transduction cascade. Outside the cell, adenosine regulates essential processes by binding to four G-protein-coupled receptors, A_1, A_{2A}, A_{2B} and A_3 [1], which govern major pathophysiological functions. Equilibrative nucleoside transporters (ENTs), which are widely expressed by the cells [2], maintain the balance between intra- and extracellular concentrations of adenosine. Under pathological conditions, extracellular

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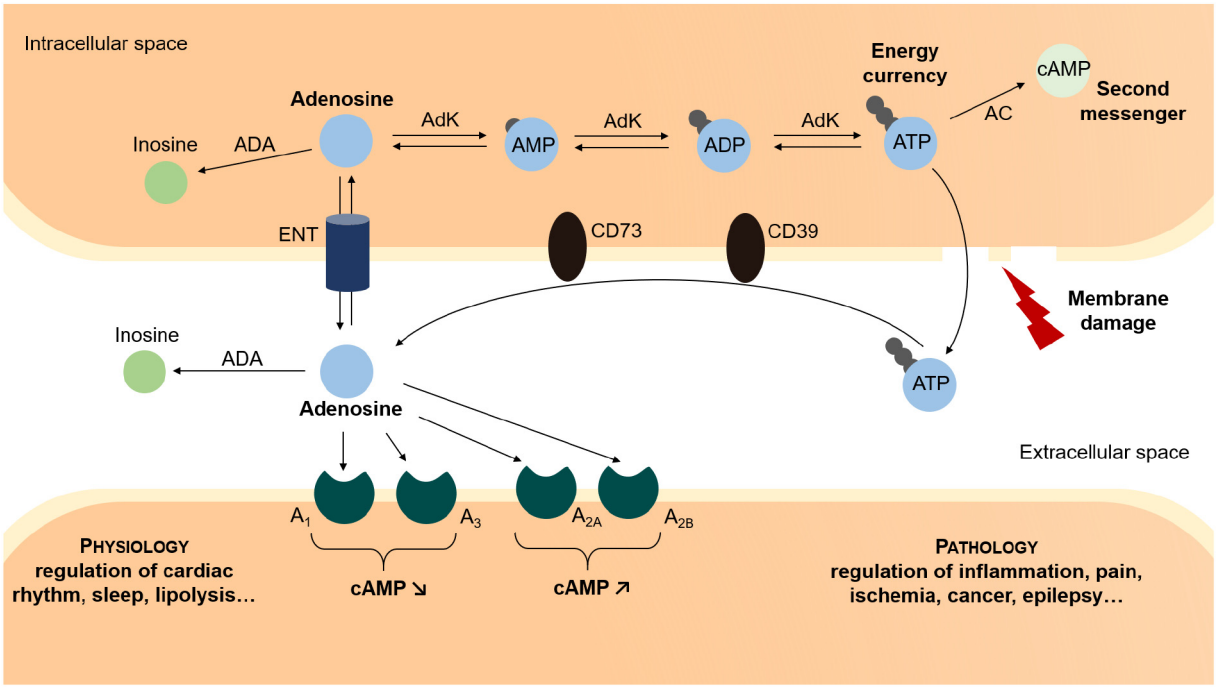

Fig. 1. Intracellular and extracellular adenosine. In the cell, adenosine is degraded by adenosine deaminase (ADA) into inosine or phosphorylated by adenosine kinase (AdK) into adenosine mono-phosphate (AMP), di-phosphate (ADP) or tri-phosphate (ATP), which is used as substrate by adenylyl cyclase (AC) for cyclic AMP (cAMP) formation. Outside the cell, adenosine binds to four receptors involved in various pathophysiological processes.

adenosine concentrations dramatically rise due to the release of abundant intracellular ATP by damaged cells [3] and its prompt transformation into adenosine by ectonucleotidases CD39 and CD73.

Adenosine is a hydrophilic molecule that has no affinity with lipids. Like Romeo and Juliet, they come from two separate worlds that rarely mingle. But, if forced to join, would they repel each other, accommodate or start a fusional relationship? Scientific discoveries in the 70's and 80's encouraged research on that topic.

The development of liposomes for drug delivery in the early 1970's [4] was seen by some specialists as a very suitable match for adenosine and its derivatives. Indeed, adenosine and ATP have very short plasmatic half-life [5,6] due to their degradation by plasmatic enzymes and rapid uptake by erythrocytes and endothelial cells, which could be circumvented by liposomal encapsulation. First proposed applications were the delivery of liposomal ATP to treat rat ischemic brains [7] and liposomal adenosine to examine its effect on the contraction of rat aortic smooth muscle cells [8]. As will be presented in the first part of this review, the first matchmakers' intuition was accurate and liposomal forms of adenosine, ATP and even ADP have been developed and studied since this consensual marriage with expected successful outcomes.

Completely independently, a few naturally occurring nucleolipids were discovered in the 1970's, such as tunicamycins, which were isolated from *Streptomyces lysosuperficus* [9]. This drove chemists to create multiple artificial nucleolipids a few years later, including adenosine nucleolipids like adenosine-5′-alkylphosphates [10]. There were two main rationales for studying such adenosine nucleolipids: (i) authors saw them as possible intermediates in enzymatic reactions involving ATP and lipids and wanted to study their physiological significance [10,11], or (ii) they wanted to explore the existence of hydrophobic regions in the active sites of ATP-dependent enzymes [12].

With a totally different mindset, a third rationale for developing adenosine nucleolipids was established in the late 1980's. Adenosine congeners were functionalized by covalently attaching lipids at a permissive site (*e.g.* N6 or C2 positions of adenosine) with the idea to alter the drug distribution and metabolism *in vivo* while conserving good binding properties without any prior cleavage [13,14].

Even though these approaches have opened the field of adenosine nucleolipids and given some interesting results, no author really anticipated that this forced marriage would become a perfect love match between adenosine and lipids. Nucleolipids, and adenosine nucleolipids in particular, present remarkable properties that will be exposed in the second part of this review.

2. Adenosine and adenosine derivates loaded liposomes

Liposomes are lipid-bilayer vesicles made of combinations of natural or/and synthetic lipids. These lipids are perfectly biocompatible and biodegradable and have already been used as excipients in numerous vaccines. As a result, liposomes can be used as safe delivery systems for both hydrophilic and hydrophobic molecules. Up until now, > 15 liposomal formulations already reached the market, Doxil® being the most well-known among them [15].

When adenosine, ADP or ATP are loaded into the liposomes' hydrophilic core, they are protected from the blood stream and their pharmacokinetics and biodistributions are completely modified, offering new therapeutic possibilities. Table 1 describes the composition and size of the liposomes that will be described in this part, as well as their drug loading and/or encapsulation efficiency when available. Regrettably, not all reports provide a full and comprehensive characterization of their liposomal system, which leads to uncertainties in terms of dosage, especially because liposomal formulation often present very poor drug loadings.

2.1. Liposomal adenosine

The main objective of adenosine encapsulation into liposomes is to prolong its half-life in the blood stream. Liposomal formulations can also enhance the quantity of active drug at the site of action by passive or active targeting.

Table 1
Adenosine, ADP and ATP-loaded liposomes.

Drug loaded	Lipid composition	Liposome size (nm)	Drug loading (μmol drug / μmol lipid)	Encapsulation efficiency (%)	Therapeutic target	References
Adenosine	DSPC:DSPG:chol	168 ± 18	n.d.	12.9	Inflammation	[16]
	DSPC:chol	206 ± 40	n.d.	8.6		
	DSPC:DODAB:Chol	202 ± 27	n.d.	10.5		
	HSPC:chol:DSPE-PEG	134 ± 21	n.d.	n.d.	Myocardial ischemia	[17]
	Soybean oil:PC:chol:glycerin	n.d.	n.d.	n.d.	Osteoarthritis	[18]
ADP	DMPC:chol:DHSG:DSPE-PEG: H12-PEG-Glu2C18	240 ± 68	n.d.	n.d.	Hemostasis	[19–22]
	DPPC:chol:DHSG:DSPE-PEG: H12-PEG-Glu2C18	285 ± 78	n.d.	n.d.		
	DSPC:chol:DHSG:DSPE-PEG: H12-PEG-Glu2C18	298 ± 107	n.d.	n.d.		
ATP	PC:chol:SM4	~ 140	≤ 0.39	n.d.	Cerebral ischemia	[7,23]
	PC:chol	n.d.	n.d.	n.d.		[24]
	PS	~ 100	n.d.	n.d.	Retinal ischemia	[25,26]
	PC		n.d.	n.d.		
	PS:PC		n.d.	n.d.		
	PC:chol:stearylamine	> 2000	0.32 ± 0.02	n.d.	Myocardial	[27]
	PC:chol:DSPE-PEG:DOTAP	~ 200	c.a. 0.40	n.d.	ischemia	[28,29]
	PC:chol:DSPE-PEG:2G4-PEG–PE	~ 200	n.d.	n.d.		[30]
	PC:chol:DSPE-PEG:DOTAP:2G4-PEG–PE	190 ± 45	c.a. 0.40	n.d.		[31]
	PC:chol	n.d.	n.d.	8	Liver ischemia	[32]
	PC:chol:PG	~ 200	0.35 ± 0.06	n.d.		[33,34]
	PC:chol	~ 100	n.d.	n.d.		[35,36]
	PC:chol:PG		n.d.	n.d.		
	PC:chol:ODA		≤ 0.07 ± 0.01	n.d.		
	PC:chol:DOTAP		n.d.	n.d.		
	PC:chol:DOTAP:DOPE		n.d.	n.d.		
	PC:chol:DOTAP:Lac-10-chol		0.04 ± 0.01	n.d.		
	PC:chol:DOTAP:DOPE:Lac-10-chol		0.03 ± 0.01	n.d.		
	DPPC:chol:PEG	~ 120	0.33 ± 0.11	n.d.	Islet transplantation	[37]
	DSPC:DSPE-PEG:chol	n.d.	n.d.	n.d.	Intestinal ischemia	[38]
	VitaSol	n.d.	n.d.	n.d.	Wound healing	[39]
	ATPv	n.d.	n.d.	n.d.		[40]
	DOPC:DOPC-E	n.d.	n.d.	n.d.		[41]
	PC:DOTAP	120–160	n.d.	n.d.		[42–45]
ATP + pentobarbital + suramin	DOPE:CHEMS:PEG-PE	~ 150	n.d.	n.d.	Cerebral ischemia	[46]

CHEMS: cholesteryl hemisuccinate; Chol: cholesterol; DHSG: 1,5-dihexadecyl-N-succinyl-l-glutamate; DODAB: dimethyldioctadecyl ammonium bromide; DOPE: 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPC: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPC-E: 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine; DOTAP: 1,2-dioleoyl-3-trimethyl-ammonium-propane; DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DSPC: 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPE-PEG: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)]; DSPG: 1,2-distearoyl-*sn*-glycero-3-phosphorylglycerol; H12-PEG-Glu2C18: dodecapeptide HHLGGAKQAGDV-polyethylene glycol-Glutamate 2C18; HSPC: hydrogenated soy phosphatidyl choline; n.d.: not determined; ODA: octadecylamine; PC: phosphatidylcholine; PEG-PE: polyethylene glycol-phosphatidylethanolamine; PG: phosphatidylglycerol; PS: phosphatidylserine; SM4: sulfatide.

Based on this background knowledge, Gutman et al. developed adenosine loaded liposomes to target inflammation [16]. They showed that negatively charged liposomes composed of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), distearoyl-phosphatidylglycerol (DSPG) and cholesterol could be prepared by reverse phase evaporation method and loaded with adenosine (reaching a drug loading charge of 12.9%), and that these monodisperse liposomes of around 170 nm of diameter were internalized by human monocytes and reduced inflammation *in vitro*. Regrettably, the only parameter assessed *in vivo* was the absence of adenosine loaded liposomes toxicity on monocytes, whereas their influence on inflammation was not monitored.

Another approach for passive targeting *via* liposomal formulation was developed by Takahama et al. for myocardial ischemia and reperfusion [17]. It is known that cellular permeability is enhanced in the zone of infarct due to the disruption of the vascular endothelial integrity. As a result, the authors decided to prepare PEGylated adenosine liposomes with prolonged circulation time for reaching higher concentration of adenosine in the target zone. For this purpose, neutral monodisperse liposomes were prepared by the hydration method, with hydrogenated soy phosphatidylcholine, cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000). The use of fluorescent liposomes showed that they indeed accumulated in the necrotic area, which correlated with a prolonged residence time of liposomal radiolabeled adenosine in the same zone. This led to a reduction of the infarct size which was not associated with usual adenosine side effects (hypotension and bradycardia), confirming the advantage of using liposomal delivery.

Very recently, Corciulo et al. developed the idea of using liposomal adenosine for treating osteoarthritis [18], based on the fact that extracellular adenosine downregulation was known to be implicated in the development of the disease, more specifically *via* its interaction with A_{2A} receptor. Unexpectedly, they observed that injection of A_{2A}R agonists did not protect rats against a model of post-traumatic osteoarthritis. Hence, they decided to load adenosine in liposomes made of soybean oil, phosphatidylcholine, cholesterol and glycerin and injected them locally in the joint. Using this formulation of adenosine, they witnessed important prevention and protection effects against the disease, with reduced joint swelling, cartilage and joint protection, as well as lowered OARSI scores, and demonstrated that these effects depended on A_{2A}R activation. Even though these results constitute a promising pre-clinical proof-of-concept, it seems unfortunate that liposomal formulations were so poorly defined (basic information on their composition can only be found in the corresponding patent [47]) and

scarcely characterized in this work, with no mention of physico-chemical parameters.

2.2. Liposomal ADP

An original liposomal ADP system has been developed for 10 years in Takeoka's lab for platelet substitution. Pegylated liposomes were loaded with ADP in the intraliposomal aqueous core and functionalized with a fibrinogen γ-chain peptide. This peptide corresponded to the carboxyl terminal 12 amino acids of the fibrinogen γ-chain and is a ligand for glycoprotein IIb/IIIa receptors which are expressed on activated platelets at the site of injury. As a result, these "H12-(ADP)-liposomes" specifically accumulated within the sites of injuries after intravenous injection [22] and reduced the bleeding time in thrombocytopenic rats [21] and rabbits [20,48] by playing two different roles: (i) they enhanced platelet aggregation *via* cross-linking between activated platelets thanks to their functionalization with the fibrinogen γ-chain peptide and (ii) they enhanced platelet activation by releasing ADP, which acts through platelet surface receptors $P2Y_1$, $P2Y_{12}$ or $P2X_1$. Interestingly, authors could modulate this ADP release by tuning the vesicles lamellarity and flexibility [21].

Recently, this team made a brilliant conceptual and experimental move: instead of continuing to consider ADP only as a drug for platelet activation, they started looking at it as an adenosine prodrug. This led them to evaluate the pharmacological efficacy of H12-(ADP)-liposomes on blast lung injury, where ADP would be useful in treating the hemorrhage, while adenosine would exert anti-inflammatory protective effects [48] (Fig. 2). They obtained an important increase in mice survival after lung injury in the group treated with H12-(ADP)-liposomes, going with reduced tissue damage/hemorrhage and inflammation. Moreover, they proved the implication of adenosine in this phenomenon, as treatment with A_{2A} and A_{2B} antagonists abrogated the beneficial effects of the H12-(ADP)-liposomes.

2.3. Liposomal ATP

Like adenosine, ATP cannot be efficiently delivered intracellularly by itself. It is very rapidly metabolized in the bloodstream and the hydrophilic nature of this nucleoside prevents it from crossing cell membranes. As a result, ATP-loaded liposomes have been used for several decades to improve ATP delivery (Fig. 3A).

As ATP is the main energy currency in the cell, intracellular ATP delivery has mainly been used for pathologies involving a phenomenon of energy failure. The main application has been ischemia, *i.e.* the lack of blood supply in a tissue, leading to a shortage in oxygen, decreased cell metabolism and ATP depletion. Ischemic insults can virtually affect any organ or tissue, the most common and severe cases being heart, brain and liver ischemia.

Early proof of concept studies on ATP-liposomes pharmacological efficacy against cerebral ischemia were conducted on rats in the 1990's. Liposomes efficiently protected ATP from plasma degradation, could cross the blood-brain-barrier under hypoxic conditions [24] and protected the rats against cerebral ischemic insults [7,23]. A closely related model of retinal ischemia [49] was later used to better understand the mechanism of action of these ATP-liposomes. ATP delivery led to increased cell survival and decreased cell necrosis in primary retinal ganglion submitted to glucose-oxygen deprivation *in vitro*, and to decreased neurons death in the ganglion cell layer *in vivo* after ischemia-reperfusion. Pro-inflammatory genes were also down-regulated in ischemic retinas following ATP-liposomes treatment [25,26]. These simple ATP-liposomes were recently redesigned into pH-sensitive "energy formula"-loaded liposomes [46] (Fig. 3B). The lipid composition was chosen in such a way that liposomes responded to pH and released

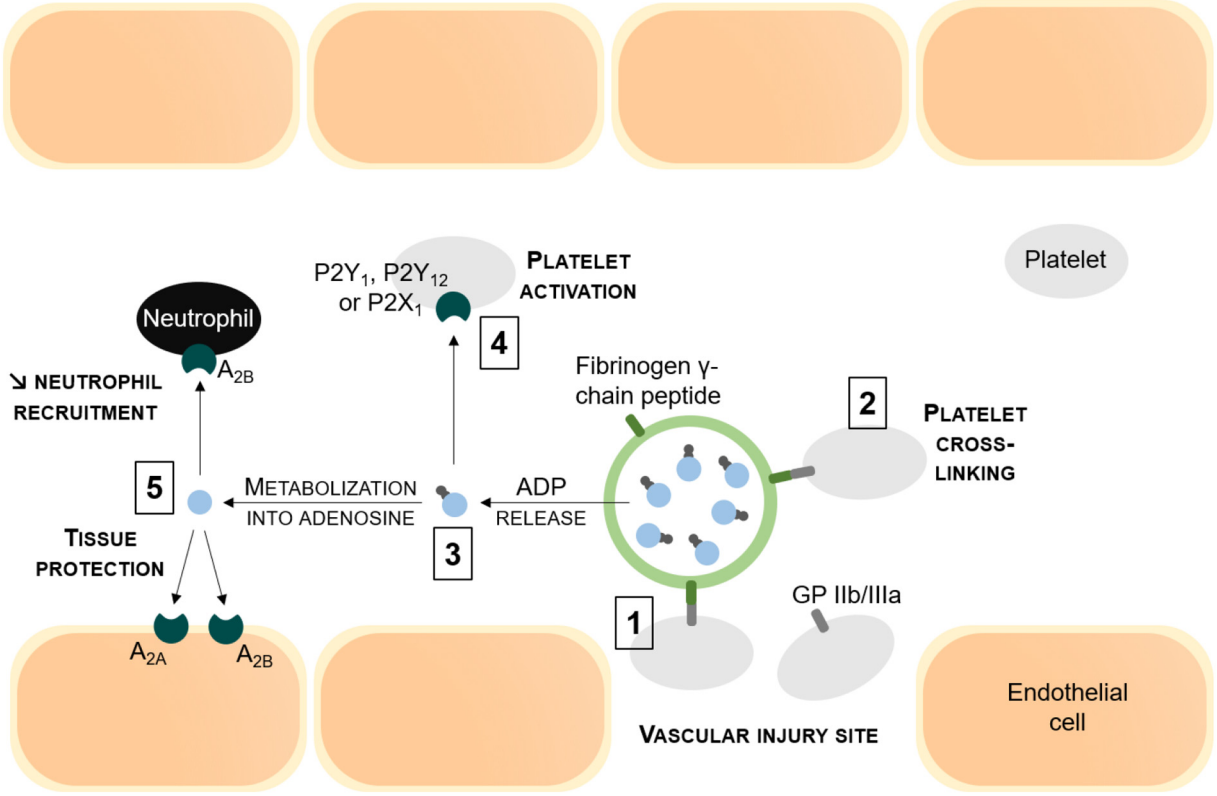

Fig. 2. Hypothetic mechanism of action of H12-(ADP)-liposomes. (1) H12-(ADP)-liposomes accumulate at the site of injury by binding to glycoprotein (GP) IIb/IIIa on activated platelets. (2) This also enhances platelet cross-linking, as the same liposome presents several fibrinogen γ-chain peptide moieties on its surface and can therefore bind several activated platelets. (3) ADP released from the liposomes (4) further activates platelets *via* $P2Y_1$, $P2Y_{12}$ or $P2X_1$ receptors, and (5) is also metabolized into adenosine, which has anti-inflammatory protective effects.

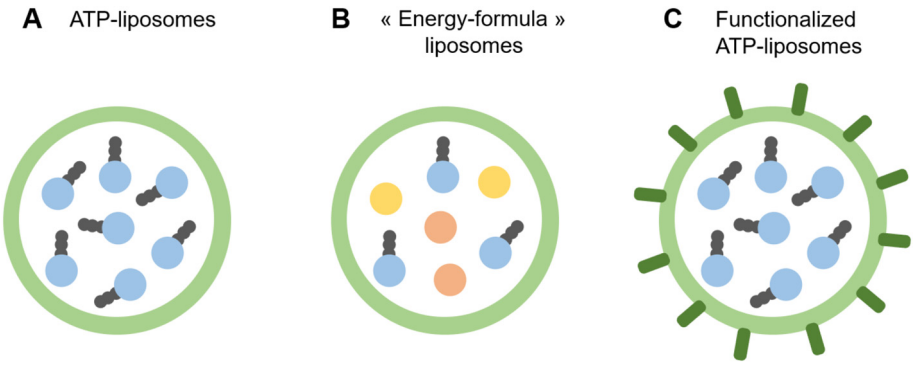

Fig. 3. Different types of ATP-liposomes. (A) Basic ATP-loaded liposomes can be complexified by (B) co-loading with complementary energy molecules like pentobarbital and suramin or (C) by functionalization with targeting moieties like antibodies or receptor ligands. The lipid bilayer composition can also be tuned to provide pH-sensitivity, fusogenicity or stealthiness.

their content at the penumbral intracellular pH. In addition, ATP was co-loaded with pentobarbital, which inhibits energy consumption, and with suramin, for blocking ATP toxic extracellular actions. Even though these liposomes protected a neuronal cell line against ATP depletion *in vitro*, they failed to protect astrocytes or endothelial cells under the same conditions and no *in vivo* data were reported. Hence, the superiority of these "energy-formula"-liposomes, compared to simple ATP-liposomes, remains to be proven.

ATP-liposomes were also shown to accumulate in myocardial infarct tissues in dogs [27]. Torchilin's team confirmed this result in isolated rat hearts perfused with a Langendorff instrument and showed that ATP-liposomes could protect the mechanical functions of the myocardium during ischemia/reperfusion [29]. Similarly, ATP-liposome treatment reduced the fraction of the irreversibly damaged heart area within the total area at risk in a model of myocardium infarction in rabbits [28]. Further improvement of the liposomes was realized by functionalizing them with the monoclonal 2D4 antibody which specifically binds cardiac myosin [30] (Fig. 3C). Intracellular cardiac myosins become, indeed, exposed to the blood-stream after sarcolemmal disruption happening during ischemia. Hence, targeting these proteins should lead to higher accumulation of antibody-coated ATP-liposomes compared to bare ATP-liposomes. Even though this mechanism was not demonstrated *in vivo*, it was shown that the pharmacological efficacy of antibody-coated ATP-liposomes was enhanced compared to bare ATP-liposomes on isolated rat hearts perfused with a Langendorff instrument [31].

Regarding liver ischemia, ATP liposomes have shown protective activity on rat models of both warm ischemia *in vivo* after a hypovolemic shock [32] and cold ischemia *ex vivo* during liver preservation for transplantation [33,34]. In both cases, authors witnessed an enhanced preservation of the ATP cellular content. Functionalizing ATP-liposomes with an asialoglycoprotein receptor ligand did not show any improved hepatocytes targeting or delivery efficacy so far [35], and their freeze-drying led to ATP leakage [36].

Regarding organ preservation, protective effects of ATP-liposomes were also tested on a model of ischemic pancreatic β cells for possible application on islet transplantation [37]. ATP-liposomes were functionalized with a fibronectin-mimetic peptide, which favored their cell internalization. Surprisingly, metabolic protection against the ischemic insult was shown to arise from both ATP and the lipids constituting the liposome (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, PEG2000). However, ATP-liposomes could not restore insulin production from ischemic islet cells.

Finally, in a model of mesenteric ischemia/reperfusion, authors showed that liposomal ATP could restore crypt proliferation but did not prevent cell death in the ischemic gut, arguing towards important complementary cytoprotective roles of other glucose metabolites like pyruvate [38].

In terms of commercial applications, highly fusogenic ATP-liposomes have been developed by the company Energy Delivery Solutions, a spin-off from Chien's lab at the University of Louisville, under the product name of ATPv (formerly VitaSol). These liposomes are composed of a stable vesicle former, for example 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or soy phosphatidylcholine (soy PC), and an unstable vesicle former, like 1,2-dioleoly-*sn*-glycero-3-ethylphosphocholine (DOPC-e) or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [50]. Dressings were saturated with these ATPv and applied to wounds to study the liposomes effect on skin healing. Very promising results were obtained on wounds performed on mice heads [41] and on ischemic ears of normal [42,45] or diabetic [44] rabbits. A very special finding was the discovery of a completely new healing process, with extremely fast granulation attributed to massive macrophage migration, their *in situ* proliferation and direct collagen production [42–45]. On a different therapeutic area, using a model of hemorrhagic shock on rats, these ATP-liposomes also improved survival when injected during resuscitation [39,40]. ATPv liposomes are currently sold as holding solutions for hair transplants. Clinical trials on other applications are eagerly awaited.

In a completely different mindset, ATP-liposomes have also been used as a helper system for ATP-responsive nanocarriers. In this strategy, the delivery of an anticancer drug by fusogenic liposomes containing ATP-responsive DNA scaffold was enhanced by the co-administration of ATP-liposomes [51].

All these studies show that there are still interesting perspectives in ATP-liposomes and ATP-vesicles development, particularly by tuning the cargo composition with other energetic molecules, by decorating the carrier surface with specific peptides or antibodies for enhanced targeting, and by finding new areas of applications. A better understanding of the ATP-liposomes mechanism of action may help move this technology to the clinic. In particular, it would be interesting to confirm whether ATP-liposomes act only by delivering intracellular ATP or if this ATP delivery leads to adenosine extracellular delivery [52,53] as in the case of ADP-liposomes [48].

3. Adenosine-lipid conjugates or nucleolipids

Nucleolipids are composite molecules formed by the assembly of a lipidic component and a nucleobase, nucleoside or nucleotide.

3.1. General synthetic strategies for adenosine nucleolipids

Different chemical approaches can be employed to obtain artificial adenosine nucleolipids. A commonly employed strategy was first proposed by Shuto and colleagues in 1987 [54] for obtaining 5′-phosphatidyl-nucleosides (Fig. 4A). This method's popularity arises from its simplicity: a single-step enzymatic reaction in a two-phase system (chloroform and buffer) is necessary, in which phospholipase D catalyzes the transfer reaction of the phosphatidyl residue to the primary hydroxyl group of nucleosides. One of the first artificial

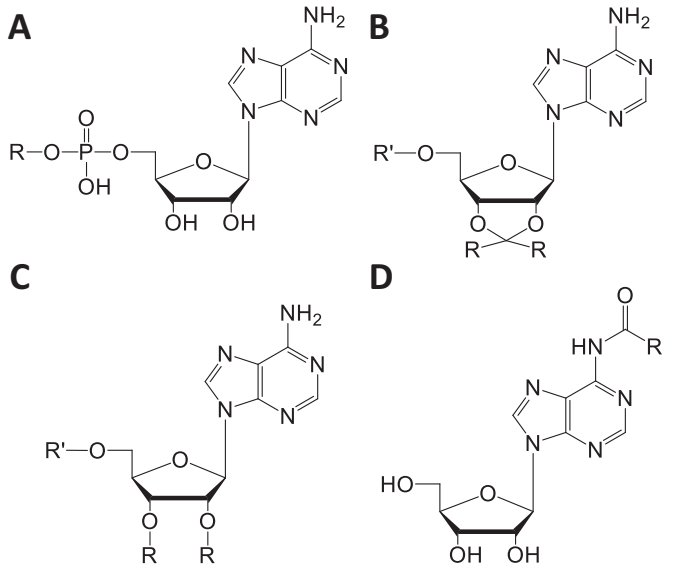

Fig. 4. Adenosine nucleolipids. R = lipids, R' = H or phosphocholine. Lipids can be conjugated to adenosine by a phosphate diester link on the primary hydroxyl group of ribose to form 5′-phosphatidyl-adenosines or fatty acyl phosphate adenosines (A), or directly attached to 2′,3′-hydroxyl groups of ribose through a ketal linkage (B) or ether bond (C), or coupled *via* an amide bond on the nucleobase (D).

adenosine nucleolipid, 5'-(l,2-dipalmitoyl-*sn*-glycero-3-phospho)-adenosine (DPPA), was synthetized by this method with a yield of 52% [54]. A few years later, Itojima et al. made use of this technique for creating a library of four 5′-phosphatidyl-adenosines with different alkyl chain lengths [55].

Other organic strategies consist of activating either the adenosine monophosphate or the lipid moiety before coupling them. For example, 5′-phosphatidyl-adenosines can also be obtained by condensation of fatty alcohols with AMP activated by N,N′-dicyclohexylcarbodiimide [10,56] or by reaction of AMP tetrabutylammonium salt with alkyl bromides [57]. Ahlers et al. created a new type of adenosine nucleolipids (Fig. 4B, R' = H) by coupling N-hydroxysuccinimide actived esters of 2′,3′-O-(3-carboxy-1-methylpropyliden)adenosine [58] with mono or dialkylamines. More recently, Moreau et al. synthesized other types of adenosine nucleolipids (Fig. 4B, R' = H) by reacting adenosine with dimethoxy ketal of palmitone [59]. These adenosine nucleolipids, such as 2′,3′-O-16-hentriacontanyliden-adenosine, were also reacted with 2-chloro-1,3,2-dioxaphospholane-2-oxide and trimethylamine in order to obtain 2′,3′-O-16-hentriacontanyliden-adenosine-5′-phosphocholine (PAPC) (Fig. 4B, R' = phosphocholine) [59]. The same team also used imidazole activated fatty acids with ′′5′-(phosphocholine)-adenosine to form the corresponding bis-(2′,3′-oleoyl)-5′-(phosphocholine)-adenosine (Fig. 4C, R' = phosphocholine) [60]. Over the same period of time, Couvreur's team synthesized squalene-based adenosine conjugates [61,62] (Fig. 4D).

A completely different strategy was recently introduced by Ceglie's team using micellar catalysis. Practically, the 1,2-epoxydodecane was coupled to 5′-phosphoribonucleotide in an aqueous cationic microemulsion. The cationic interface favored the coupling between the water insoluble epoxide and the anionic nucleotides [63].

3.2. Adenosine nucleolipids self-assembly

The attraction for nucleolipids in general (and adenosine nucleolipids in particular) has increased with the discovery of their capacity to form macro- or nanostructures. This unique property arises from both members of the couple: lipids have self-association ability and are known to organize in membrane layers and other structures such as liposomes, while nucleosides have base-pairing properties *via* hydrogen bonding or π-stacking.

Ahlers et al. were the first to explore this intriguing aspect. They compared single-chain or double-chain adenosine lipid monolayers and found that, to form stable structures, a balance had to be found between the lipophilicity procured by the alkyl chain and the size of the headgroup. Indeed, single-chain adenosine lipid did not form stable monolayers, while single-chain adenine and double-chain adenosine did [58]. Some of these adenosine nucleolipids were even found to form various helical structures, with an impact of the alkyl chain length on the efficacy of the formation of these supramolecular assemblies [55]. It was also reported that the pH of the solution greatly affected the structure: as an example, 5'-(1,2-dimyristoyl-*sn*-glycero-3-phospho)-adenosine (DMPA) formed multihelical strands in alkaline conditions, while it formed tubular cigar-like scrolls in acidic conditions (Fig. 5).

Since these early explorations, adenosine nucleolipids have been described to form many different structures including micelles and liposomes (Table 2), depending on the conjugated lipid and the buffer conditions.

Compared to other nucleolipids, adenosine lipids possess some special properties. In addition to forming hydrogen bonds, adenosine, like other purines, has a high stacking tendency (Table 3), which leads to a negative Gibbs free energy for adenosine-adenosine association in aqueous solution [73]. These specificities affect several structural parameters of adenosine lipid self-assemblies, thus contributing to the final supramolecular structure.

Indeed, when comparing 5'-(l,2-dilauroyl-*sn*-glycero-3-phospho)-adenosine (DLPA) and 5'-(l,2-dilauroyl-*sn*-glycero-3-phospho)-uridine (DLPU) wormlike aggregates, Bombelli et al. reported very different behaviors upon aging: while DLPU structures remained identical, DLPA aggregates self-assembled into giant twisted helicoidal aggregates [69]. The authors attributed this dissimilarity to higher association stacking constants of adenosine compared to uridine. This property also induced tighter nucleolipid arrangement in micelles in the case of 1,2-dioctanoyl-*sn*-glycero-3-phospho-adenosine (diC_8PA) compared to 1,2-dioctanoyl-*sn*-glycero-3-phospho-uridine (diC_8PU) [67]. Yet, this is not the case with all adenosine nucleolipids, as PAPC, for example, displayed a larger surface area per lipid compared to its equivalent 2′,3′-O-16-hentriacontanyliden-uridine-5′-phosphocholine (PUPC) [80].

Other phenomena attributed to adenosine high base-stacking potential were observed on several occasions by Baglioni's team with 5'-(1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho)-adenosine (POPA) structures. First, anhydrous POPA bilayers displayed a powder-like nature, which could be explained by a specific orientation of the stacked adenosine moieties, compared to 5'-(1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho)-uridine (POPU) [73] (Fig. 6). Secondly, POPA/POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) bilayers exhibited the presence of POPA-enriched microdomains, due to partial demixing of POPA and POPC [81]. The adenosine stacking interactions responsible for this behavior could be detected by hypochromism [72]. Finally, POPA/POPC vesicles were found to be more stable than the equivalent POPG (1-palmitoyl-2-oleoyl-phosphatidyl-glycerol)/POPC, where the adenosine polar head was replaced by a glycerol head group bearing the same charge. According to the authors, only the base stacking interactions could explain this behavior [74].

Mixing adenosine nucleolipids with uridine nucleolipids can also lead to original supramolecular architectures. Indeed, besides π-stacking, the presence of a nucleoside headgroup on a nucleolipid can also drive molecular recognition through hydrogen bonding with the complementary nucleoside on another nucleolipid. A very striking example of this phenomenon was presented by Moreau et al. by mixing PAPC and PUPC. While individual nucleolipids formed large amellar structures, mixed nucleolipids self-assembled into small vesicles, which could be visualized in real-time at the micrometer scale [59]. A similar phenomenon

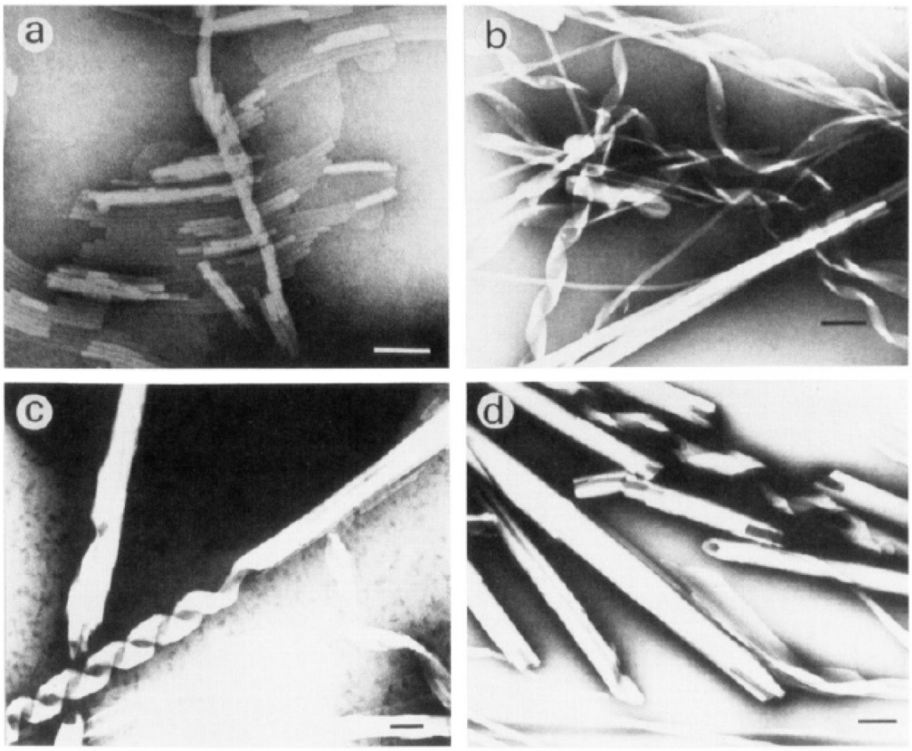

Fig. 5. Electron micrograph of the formation process of cigar-like scrolls from DMPA in acidic solution (pH 5.0): (A) linear double-helical strand; (B) ribbon structure; (C) incomplete cigar-like scrolls from ribbon structure; (D) complete cigar-like scrolls. All scale bars represent 0.1 μm. Reprinted with permission from [55]. Copyright 1992 American Chemical Society.

could be observed at the macroscopic scale between 2′,3′-O-18-pentatriacontanyliden-adenosine-5′-phosphocholine (SAPC) and 2′,3′-O-18-pentatriacontanyliden-uridine-5′-phosphocholine (SUPC): when separate, these two nucleolipids form hydrogels and fluid lamellar phases above a certain temperature. When mixed, however, they assemble into a new stable combined supramolecular system, which forms a precipitate upon heating [77].

3.3. Adenosine nucleolipids as delivery systems

3.3.1. Adenosine nucleolipids lipoplexes for gene delivery

As presented above, adenosine headgroups on nucleolipids can lead to molecular recognition with complimentary nucleolipids bearing uridine headgroups. Interestingly, this phenomenon of molecular

Table 2
Adenosine nucleolipids and observed macrostructures.

Chemical modification site	Conjugate name	Lipidic chains	C:D	Conjugated lipid	Type of link	Observed structures	References
5′-hydroxyl group of ribose	AMPC16	1	16:0	*n*-hexadecyl-phosphorylcholine	Phosphate diester	Micelles	[64,65]
	diC_8PA	2	8:0	diC_8PC		Vesicles aging into double helical strands Quasi-spherical micelles	[55,66–68]
	DDPA	2	10:0	DDPC		Vesicles aging into double helical strands	[55]
	DLPA	2	12:0	DLPC		Long helicoidal micelles and wormlike structures	[55,67,69,70]
	DMPA	2	14:0	DMPC		Multihelical strands	[55]
	POPA	2	16:0 18:1	POPC		Liposomes	[71–74]
	DOP-Ade	2	18:1	DOPC		Liposomes	[71,75,76]
2′,3′-hydroxyl groups of ribose		1		Octadecylamine	Ketal		[58]
		2		Dioctadecylamine		Stable monolayers	[58]
		2		N,N-dioctadecyl-l,3-propanediamine		Stable monolayers	[58]
	PAPC	2	16:0	Palmitone		Large lamellar structures Hydrogels	[59,77]
	SAPC	2	18:0	Stearone		Hydrogels Lamellar structures	[77]
	DOAPC	2	18:1	Oleyl fatty acids	Ether	Small unilamellar vesicles, worm-like structures and giant vesicles	[60]
6-amino group of adenosine	SQAd	1	25:5	1,1′,2-trisnorsqualenic acid	Amide	Nanoparticles	[61]
		1	30:6	squalenylacetic-acid			[78]

"C:D" represents the total amount of Carbon atoms *versus* the number of Double unsaturated bonds in the conjugated lipid chain. AMPC16: 5′-(hexadecylphosphate)-adenosine; DDPA: 1,2-didecanoyl-*sn*-glycero-3-phospho-adenosine; DDPC: 1,2-didecanoyl-sn-glycero-3-phosphocholine; diC_8PA: 1,2-dioctanoyl-*sn*-glycero-3-phospho-adenosine; diC_8PC: 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine; DLPA: l,2-dilauroyl-*sn*-glycero-3-phospho-adenosine; DLPC: l,2-dilauroyl-*sn*-glycero-3-phosphocholine; DMPA: 1,2-dimyristoyl-*sn*-glycero-3-phospho-adenosine; DMPC: l,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOAPC: di-oleyl-adenosine-phosphocholine; DOP-Ade: 1,2-dioleoyl-*sn*-glycero-3-phospho-adenosine; DOPC: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; PAPC: 2′,3′-O-16-hentriacontanyliden-adenosine-5′-phosphocholine; POPA: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-adenosine; POPC: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SAPC: 2′,3′-O-18-pentatriacontanyliden-adenosine-5′-phosphocholine; SQAd: squalene-adenosine.

Table 3
Computed RNA nucleoside pairs stacking free energies [79].

RNA nucleoside pair	AA	GG	CC	UU
ΔG_{stack} (kcal/mol)	−2.41	−2.77	−1.76	−1.51

recognition was also described between adenosine headgroups on nucleolipids and nucleic acids such as RNA or DNA. As a result, self-assembled adenosine nucleolipids may be considered as promising platforms for gene delivery [82]. A great advantage of this system is that it relies on specific Watson-Crick pairings (hydrogen bonding and π-stacking) between nucleolipids and polynucleotides, offering an interesting alternative to other non-viral technologies for gene delivery which are based on aspecific electrostatic interactions [83].

It was discovered by Baglioni's team that when adenosine nucleolipids and polyuridilic acids (polyU) were brought together, the formation of nucleolipoplexes occurred without the presence of divalent cations (Fig. 7). The proof-of-concept was obtained with diC_8PA globular micelles decorated with single-stranded complimentary RNA: this association resulted in a polynucleotide-micelle adduct that could be characterized by SAXS as a hexagonal phase (Fig. 7A) [84]. Other supramolecular structures were obtained with adenosine derivatives bearing a longer lipidic chain. Indeed, POPA bilayers swollen with a polyU-containing buffer exhibited an increase in their smectic period due to the appearance of a 1D lattice of polynucleotides between the POPA lamellar stacks (Fig. 7B) [85]. The formation of these nucleolipoplexes was specific to the presence of adenosine as a functional headgroup leading to selective molecular recognition of nucleic acids. For example, POPG [72,74] and POPU were shown to be less able than POPA to associate with polynucleotides [86].

Despite these interesting observations, the *in vitro* and *in vivo* proofs of concept for using these nucleolipoplexes as gene delivery systems remain extremely limited. In particular, enhanced transfection efficacies at high lipid concentrations were obtained for two uridine nucleolipids compared to conventional lipid agents (DOTAP, TransFast™ and Lipofectamine® 2000) [87,88] but, so far, no study has been published regarding adenosine nucleolipids for gene delivery on pre-clinical models and no data are available in the literature regarding their transfection efficacy.

The most advanced study regarding the capacity of adenosine nucleolipid to deliver DNA inside cells was performed by Montis et al.. These authors investigated the adhesion and fusion processes of DNA-loaded POPA liposomes (formulated with POPC or 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine – DOPE – helper lipids) with giant unilamellar vesicles (GUVs – composed of POPC or 1:1 = POPC:POPG) used as a model of biological cell membranes. Both nucleolipoplexes and GUV membranes could be labeled with fluorescent probes and the interaction followed by laser scanning confocal microscopy and fluorescence correlation spectroscopy. It was observed that DNA-loaded POPA liposomes entered in contact with GUVs and fused, simultaneously delivering their DNA payload. Authors claimed that they were able to reproduce *in vitro* a recently highlighted possible non-endocytic internalization pathway happening *in vivo* for nucleic acid delivery, based on the fusion of lipoplex with the cell plasma membrane [89].

For gene delivery purpose, a different approach was imagined by Arteta et al.: instead of making use of the nucleolipid self-assembling properties, these authors non-covalently decorated poly(amidoamine) (PAMAM) dendrimers with l,2-dilauroyl-*sn*-glycero-3-phospho-adenosine (DLPA) which has the ability to associate with nucleic acids. It is known that PAMAM dendrimers have an excellent ability to condense and translocate nucleic acids for gene delivery. However, this association is only driven by electrostatic interaction, which is not very specific and could happen with any other negatively charged surface or molecule. In addition, due to their positive charge, PAMAM dendrimers exhibited some toxicity. In that respect, as an alternative, non-covalent association of anionic DLPA is a simple way of giving PAMAM dendrimers molecular recognition ability for specific association with nucleic acids [90].

As seen so far, this field of research tends to exclusively see adenosine nucleolipids as a new material for gene delivery. However, it seems to omit that adenosine itself is more than a polar head with base recognition ability and could be worth delivering for itself.

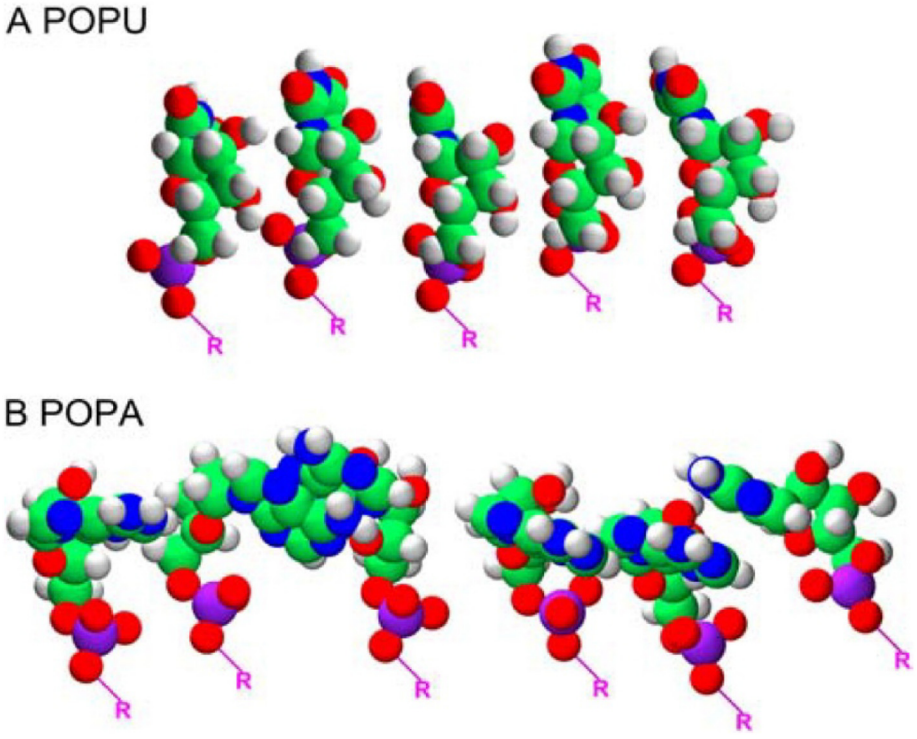

Fig. 6. Geometrical minimization for a group of POPU (A) and POPA (B) molecules performed with Hyperchem 5.1 using AMBER force field. The orientation of purinic rings is strongly altered by the interaction between bases, whereas the pyrimidinic bases keep, more or less, their original conformation. Reprinted with permission from [73]. Copyright 2006 Elsevier.

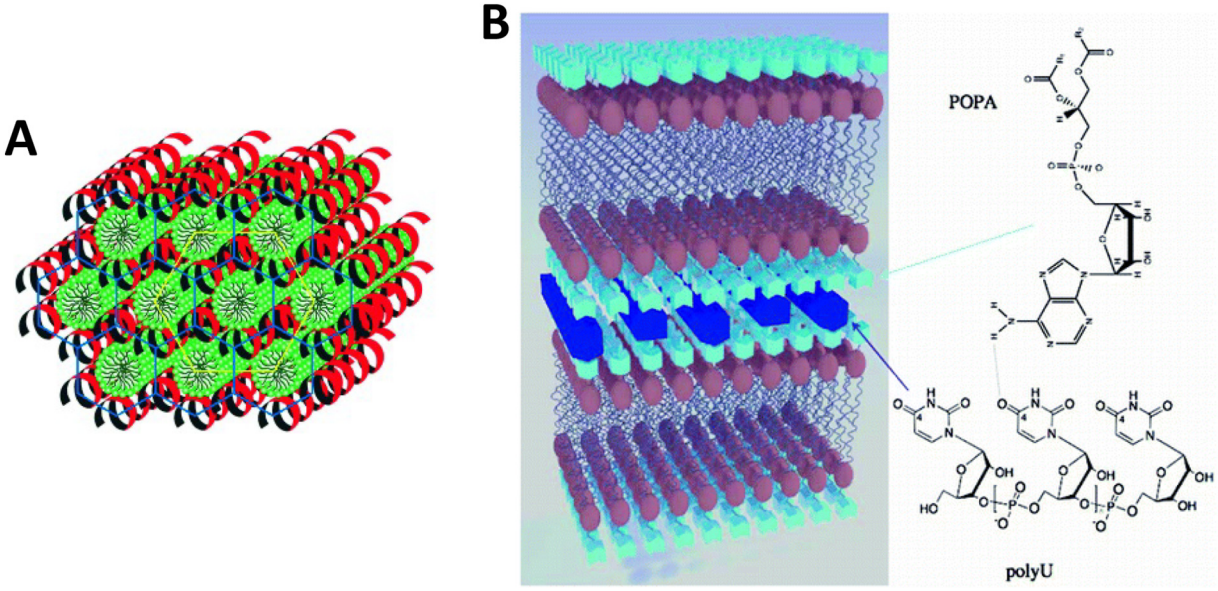

Fig. 7. Nucleolipoplexes formed by interactions between adenosine nucleolipid derivatives and polyU. (A) Hypothetical arrangement of a hexagonal phase formed by polyU (red helixes) and cylindrical diC_8PA micelles (green cylinders). Adapted with permission from [84]. Copyright 2007 John Wiley & Sons. (B) Hypothetical polyU arrangements between POPA membranes. Adapted with permission from [85]. Copyright 2007 American Chemical Society.

3.3.2. Adenosine nucleolipids nanoparticles for adenosine delivery

Other nucleolipids than adenosine ones are sometimes considered prodrugs, where the lipid moiety positively affects the drug biodistribution and leads to lowering toxicity and increasing pharmacological efficacy (*e.g.*, anticancer or antiviral agents). Surprisingly, although adenosine is often acclaimed for its potential as therapeutic molecule whereas its main limitation lies in its hydrophilicity and short half-life *in vivo*, adenosine nucleolipids have rarely been considered with the aim of delivering adenosine itself [13,14].

Squalene-adenosine (SQAd) is, to our knowledge, the only example of currently developed adenosine nucleolipid prodrug. The so-called "squalenoylation" technology consists in linking a derivative of squalene – a natural, biocompatible and biodegradable triterpene – to a hydrophilic active compound. The resulting bioconjugate showed the ability to self-assemble into stable nanoparticles (NPs) [91].

In the case of SQAd, two slightly different compounds were studied by Couvreur's team: a SQAd bioconjugate with a short squalene-based chain constituted of 5 isoprene units [61] and a SQAd bioconjugate with a longer squalene-based chain constituted of 6 isoprene units [62]. Both compounds were found to self-assemble as NPs into an aqueous phase after nanoprecipitation (consisting in the addition of an ethanolic solution of SQAd into a water phase). The SQAd bioconjugate with 5 isoprene units was shown to form sponge-like macrostructures [61], while the SQAd with 6 isoprene units self-assembled into lamellar or hexagonal phase NPs, depending on the water-to-ethanol ratio during the nanoprecipitation process [92].

The SQAd NPs with 6 isoprene units were used for *in vivo* pharmacological evaluation. Interestingly, the so-called "squalenoylation" of adenosine and its self-assembly into NPs dramatically affected adenosine pharmacokinetics and biodistribution in mice [93]. Importantly, adenosine plasma half-life was significantly increased after intravenous administration of SQAd NP, compared to adenosine injected free. Even though SQAd NPs did not show the ability to cross the blood-brain barrier, they displayed high neuroprotective pharmacological effect on cerebral ischemia in mice, as well as on spinal cord injury in rats [62]. Of note, free adenosine did not display any therapeutic activity in these models. Importantly, a complete toxicological study demonstrated the perfect innocuity of SQAd NPs in mice [62]. Nevertheless, disposing of an easy-to-handle formulation would be a step further in the preclinical development of this system.

On the other hand, the exact mechanism of action of SQAd NPs urgently deserves a deeper investigation, both at cellular and molecular levels. It was already shown that, after intravenous administration, the SQAd NPs disassembled before the bioconjugate inserted into low-density lipoproteins (LDLs), which explained the prolonged circulation time of SQAd in the blood [94]. The LDLs loaded with SQAd were further observed to be cell internalized through LDL receptor-dependent pathway [95]. But several questions remain: does the nanoparticle or SQAd bioconjugate itself act directly on adenosine receptors before internalization or is there a release in the extracellular space of free adenosine which binds to these receptors? If the second hypothesis is confirmed, how and where does this release happens? A better understanding would help to develop this promising technology further and extend its use to other therapeutic areas.

4. Conclusion and expert opinion

Adenosine and lipids form a fertile couple with many different offspring. ATP-liposomes seem to be the most advanced technology in terms of clinical applications, even though they are far from being FDA-approved yet and they are only sold as cosmetics. Covalent conjugation between adenosine and lipids generated completely unexpected materials with self-assembly abilities, but they probably still need several years to mature as gene delivery systems. Use of these adenosine nucleolipids as adenosine delivery systems may be an elegant way of developing this technology faster towards clinical applications, as the example of squalene-adenosine nanoparticles shows.

Bringing nanomedicines into the clinic is generally challenging [96], especially because they are generally developed at the pre-clinical stage in academic laboratories and in an environment far from the conditions for the pharmaceutical development rules. Most works cited in this review lack a comprehensive approach, with a rigorous reflection on chemistry, manufacturing and controls as well as thorough *in vitro* and *in vivo* evaluation. Extremely basic characterization such as size, size distribution, surface characterization, drug loading and drug encapsulation are missing in most studies, while animal testing, when

mentioned, generally focuses only on efficacy without describing detailed pharmacology and toxicology issues.

Another difficulty comes from the fact that both adenosine and nanomedicines may be considered as risky bets from an industrial point of view. Indeed, the development of adenosine agonists and antagonists has often been synonymous with important clinical failures so far [97], creating some misgivings from investors, while the development of nanomedicines was still at an early stage some decades ago. However, as the first generation of nanomedicines has been successfully commercialized for > 20 years now, industrials may become less reluctant to invest on these better-known technologies, under the condition that they can rely on solid preclinical studies. Hence, it is now time for them to fully exploit adenosine's therapeutic potential.

Declarations of interest

None.

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