Expert Opinion

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Sialic acid as a potential approach for the protection and targeting of nanocarriers

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Introduction: Nanocarriers are considered to be one of the most innovative drug delivery systems, owing to their high potential in drug protection, delivery and targeting to the diseased site. Unfortunately, their applicability is hampered mainly by their uptake, due to macrophagic recognition and lack of specificity, if not properly engineered.

Areas covered: Sialic acid (SA) and its derivatives have recently been studied in order to govern their stealthness as carriers and their effectiveness as targeting moieties. In this review, the most outstanding research (*in vitro* and *in vivo*) dealing with the use of SA or its derivatives to modify the surface carriers, in order to achieve targeted or stealth nanosystems, is summarized. Moreover, the application of SA or its derivatives as modifiers in cancer targeting and therapy, and in recognition purposes, is considered.

Expert opinion: The application of SA-based strategies for nanocarrier engineering represents one of the most stimulating challenges in drug delivery and drug targeting. Both *in vivo* and *in vitro* results on stealth or targeted nanocarriers, modified with different kinds of SA or SA derivative, have highlighted the great potential of this approach. These studies have drawn attention to both the advantages (stealth properties, targeting ability, cancer inhibition, viral and inflammation recognition, brain targeting) and the possible disadvantages (i.e., presence of possible multi-target side effect outputs) of this strategy, and overall suggests that further investigations on this strategy are required.

Keywords: nanocarriers, sialic acid, stealth, targeting

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1. Introduction

Nanoparticles (NPs) and liposomes (LPs) are two of the most studied and promising drug delivery systems as they are deputed to possess the ability to revolutionize the therapy of the difficult-to-treat diseases by means of spatially and temporally controlled drug delivery. The ability of these carriers to circulate in the bloodstream over a prolonged period of time is often a prerequisite for successful delivery. In addition, specific and selective delivery is needed in most of the diseases, as the off-target accumulation of active molecules could lead to undesired side effects [1].

For this reason, two main issues have been considered in relation to conventional and untargeted systems: rapid elimination from the bloodstream by the reticuloendothelial system (RES) due to opsonin proteins recognizing non-self nanocarriers [2,3]; and the lack of specific targeting, hampering high drug concentrations at the site of action.

In the last three decades, to prevent opsonization the nanosystem surface has been engineered with different hydrophilic molecules, as carbohydrates, in order to avoid immune system recognition. Among them, sialic acid (SA), the most abundant carbohydrate on the erythrocyte cell membrane, plays an important role in RES

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Article highlights.

- Nanotechnology-based systems display a tremendous potential in drug targeting, drug delivery and imaging.
- SAs and derivatives demonstrated several advantages in acting as targeting, recognition or detection moiety.
- SAs and derivatives as stabilizer have been exploited by modifying liposomes, achieving stimulating results and allowing stealth systems to be obtained.
- SAs and derivatives as targeting moieties have been used for brain and cancer targeting, inhibition of viral infection and inflammation.
- SAs and derivatives as recognition moieties have been applied in viral infection, inflammation and cancer detection.

This box summarizes key points contained in the article.

escape of these cells, allowing them to circulate into the bloodstream without any uptake by immune systems [4].

Naturally occurring SAs constitute a family of > 50 structurally distinct nine-carbons 3-deoxy-ulosonic acids [5-7], the most widespread derivative being 5-*N*-acetyl-neuraminic acid (Neu5Ac, **Figure 1**). Interestingly, SA is overexpressed on the cell surface of many cancer cells [8] and is a well-known ligand for selectins [9], the adhesion molecule responsible for spreading tumor metastasis and inflammation owing to its modulation of cell–cell interactions among leukocytes, platelets, cancer cells and endothelial cells [10,11].

Sialic acid is also present inside the CNS bound to mammalian neuronal cell adhesion molecule (NCAM), a glycoprotein present on the surface of glial cells, but also in skeletal muscle cells. This association enables SAs to participate in several neurological processes, such as the maintenance of plasticity during neuron development, fasciculation and axonal branching by regulating homophilic interactions [12].

Essentially, the homopolymers of SA, the polysialic acid (PSA) generally referred to Colominic acids present on the cell surface, are exposed to the external environment and involved in numerous physiological and pathological recognition phenomena [13,14]. As examples, to evade immune responses of the host, highly virulent bacteria, such as *Neisseria meningitidis* [15] and *Escherichia* coli K1 [16], adopted a PSA coating, taking advantage of structural similarity to endogenous PSA [17,18].

Thus, the sialylation of therapeutic formulations, leading to a PSA-induced 'watery cloud', has been proposed in order to improve pharmacokinetic parameters, protecting from proteolytic enzymes, clearance receptors, opsonine and even antibodies [19].

In the 1980s and 1990s, besides their use as potential targets for immune-chemotherapy [20], the use of gangliosides, namely glycosphingolipids with one or more SAs linked in different positions on the sugar chain, became one of the most applied strategies to stabilize carriers [11,21-23], unfortunately leading to a weak prolonged 'stealth' effect if compared with the polyethylene glycol (PEG) approach.

On the contrary, the surface-protecting/masking feature of PSA seemed to be more promising as preliminary experiments confirmed that long-chain PSA showed prolonged circulation half-lifes [24], mainly as a result of their high hydrophilicity [25]. Thus, polysialylation has been applied to a wide range of therapeutics, demonstrating significant improvements in pharmacokinetic parameters (increased half-life, decreased degradation, reduced metabolism and immunological reactivity) [25-28]. Indeed, the industrial application of polysialization (PolyXen® technology (Lipoxen PLC, London, UK)) was applied with regards to insulin (SuliXen® (Lipoxen PLC, London, UK), Phase I clinical trials), alfa2β-interferon (InferoXen®(Lipoxen PLC, London, UK)), granulocyte colony stimulating factor (StimuXen™ (Lipoxen PLC, London, UK)) and erythropoietin (ErepoXen® (Lipoxen PLC, London, UK), Phase I clinical trials). This review aims to summarize the most significant applications of SA or its derivatives to engineer the surface of nanocarriers, by exploiting SA as surface-ligand nanocarrier stabilization or for specific targeting.

2. Sialic acid to stabilize nanocarriers

In order to stabilize the nanocarriers once they had been administered in the bloodstream, several scientists applied polysialylation by using different compounds bearing one or more SA moieties, with the main aim of generating stealth nanocarriers able to avoid macrophage capture. Remarkably, most of the research focused on the LPs' surface coverage with SA, whereas there are only a few examples regarding polymeric NPs' surface coverage.

The use of ganglioside GM1 (**Figure 1**) as surface modifier represented one of the first attempts to stabilize nanocarriers; to this end, GM1 was incorporated in the LPs' bilayer, showing SA molecules on the nanocarrier surface. The first studies, performed by Gabizon and Papahadjopoulos at the end of the 1980s [11], after intravenous (i.v.) administration in mice, demonstrated a 3.4-fold decrease in LP-GM1 RES uptake in comparison with conventional carriers (PC:Chol/10:5). When phosphatidylcholine (PC) LPs were prepared with GM1 and sphingomyelin (SM), after i.v. administration in ICR mice, Allen and Chonn were able to increase further the RES-avoiding effect (from 17-fold for PC/GM1 LPs to 330-fold for PC/SM/GM1 LPs, in comparison with PC LPs) [29], probably owing to the achievement of optimum bilayer stability. In other research, the same authors compared the effect promoted by other kinds of ganglioside and glycolipid, and found that only GM1 PC/Chol LPs were able to increase significantly the blood/RES ratio (6.3-fold more than conventional LPs) after i.v. administration in female ICR mice [21].

Afterwards, Liu and co-workers demonstrated the ability of GM1 LPs at avoiding RES capture, depending on the LPs' size. In particular, after i.v. administration in Balb/c mice, LPs of size > 300 nm were able to avoid hepatic Kupffer cell capture, but displayed a rapid and greater accumulation in the spleen tissue. This effect would probably be due to prolonged LP

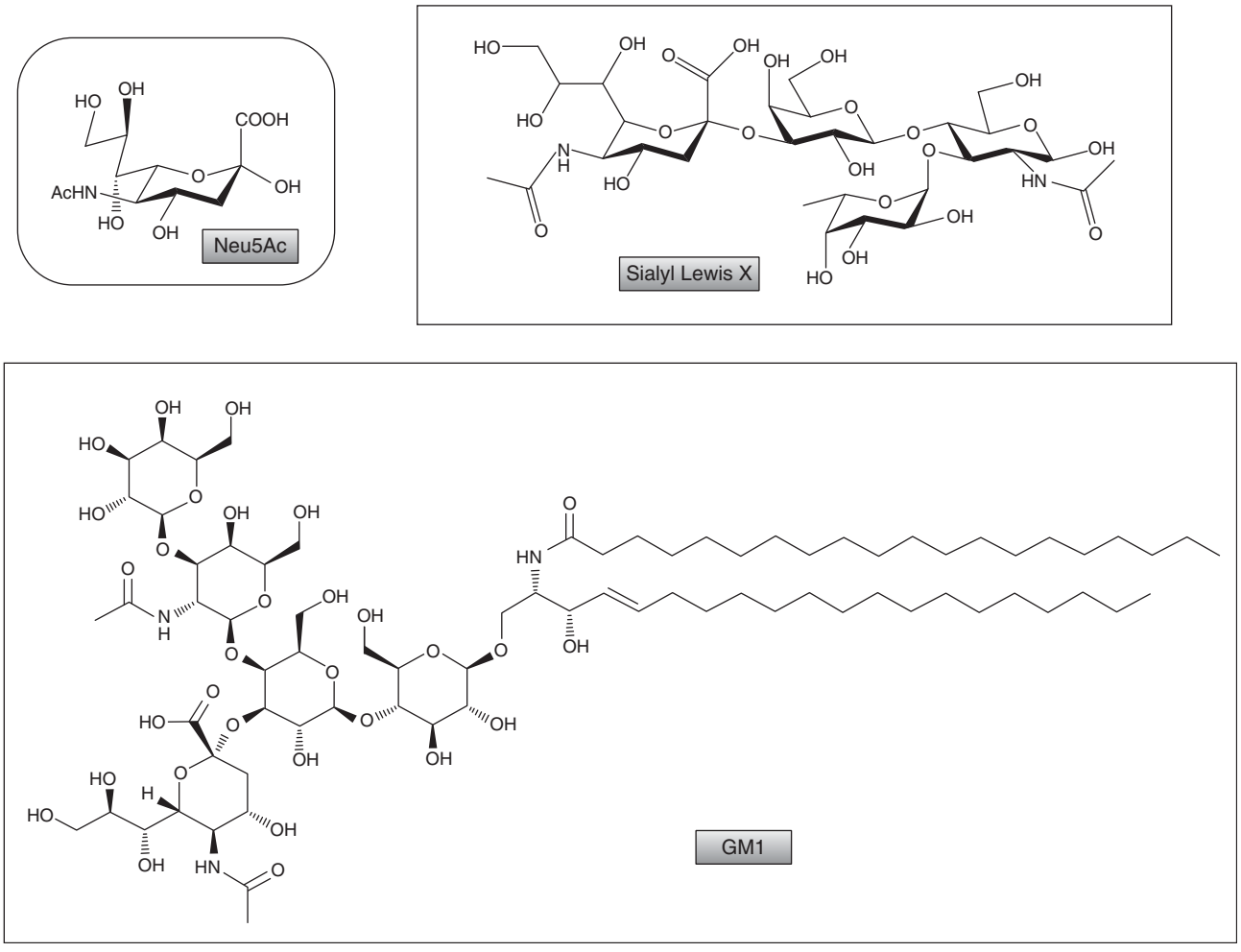

Figure 1. Chemical structures of 5-*N*-acetyl-neuraminic acid (Neu5Ac), sialyl Lewis X and ganglioside M1 (GM1).

residence in the bloodstream, promoted by the presence of GM1, allowing the carriers to encounter the spleen district much more readily than the LPs without GM1. In fact, if the GM1-containing LPs are dimensionally large enough (in this case > 300 nm), they will be retained by the spleen filter, resulting in an elevated LP spleen accumulation [30]. In another paper, the same research team tested GM1 LPs on Balb/c mice previously subcutaneously inoculated in the left leg with EMT6 tumor cells. This paper confirmed the size-dependent biodistribution of GM1 LPs, as LPs of size < 70 nm accumulated in the liver whereas LPs > 300 nm accumulated in the spleen. Moreover, the results showed that GM1 LPs of size between 90 and 200 nm accumulated in the tumor more readily than LPs with diameter < 40 nm or > 200 nm [31]. The same results were also confirmed by Gabizon and Papahadjopoulos [11] after i.v. administration in mice of GM1 LPs. Remarkably, this tumor accumulation should not be considered as real tumor targeting, but rather due to prolongation of permanence of the GM1 LPs in the bloodstream, allowing greater distribution in other tissues, tumor included [32].

Considering other ganglioside-based formulations, the blood concentration of LPs with glycophorin A and ganglioside GM3 was increased 3.3-fold 6 h after i.v. administration in Sprague-Dawley rats. Moreover, the authors declared that the presence of SM in combination with the other two sialo derivatives increased the blood concentration 3.7-fold in comparison with the control. The authors suggested that this increase in the blood concentration was due to the carbohydrate–carbohydrate interaction (through hydrogen bond) between glycophorin A/GM3 and the membrane of SM LPs, affecting the conformation of sugar residues [33].

Starting from the results obtained with the gangliosides, some scientists synthesized new compounds bringing SA moieties and characterized by a backbone chemically comparable to the ganglioside backbone. Indeed, some researchers incorporated a new compound obtained from the derivatization of palmitic acid (PA) with Neu5Ac (Neu5Ac β-PA) in LPs and compared it with GM1 [34], and studied in-depth the effect of increased concentration of Neu5Ac β-PA on the LPs' surface as well as the effect in two different rodent animal species (rats and

mice). The authors demonstrated a species-specific difference in the effect on RES avoidance, as GM1 was able to avoid RES in the mouse model, contrary to Neu5Ac β-PA, leading to a better stealth effect in rat. The different composition of opsonine proteins in the bloodstream, depending of rodent species, would probably be the reason for the different interaction with the two compounds [23].

To study the importance of SA position on the LPs' surface and the presence of other chemical moieties interfering with SA activity, Kojima and co-workers synthesized different SA-linked glycoproteins, using the albumin as base protein and binding different sugar residues [22]. The authors pointed out a remarkable difference when fucose residues were present: in the presence of fucose residues, after i.v administration in mice, SA residues lead to an increase RES uptake of glycoprotein-conjugated LPs, whereas in the absence of fucose residue and exploiting the masking galactose residue, a strong reduction in the uptake was found [22].

With the aim of comparing the long circulating effect promoted by PEG and GM1, Maruyama and colleagues demonstrated that both compounds effectively reduced RES uptake and prolonged the circulation time of PEG/GM1-bearing LPs, showing some peculiar differences. In particular, PEG and GM1 LPs were loaded with a model drug (Adriamycin) and i.v. administrated in DBA/2 mice: in comparison with loaded unmodified LPs, the Adriamycin blood levels (6 h after injection) were 2.3-fold and 2.9-fold higher when loaded into GM1 LPs and PEG LPs, respectively. On the contrary, considering GM1-modified LPs and PEG-modified LPs, the blood/RES uptake ratios of the drug were 4.6-fold higher and 7.3-fold higher, respectively [32].

GM1 and other related compounds were also used to stabilize nanocarrier systems, not only to improve the time of permanence in the bloodstream but also to ameliorate some technological aspects. As an example, in LP formulations, different types of glycophorin A, the major sialoglycoprotein of the human erythrocyte membrane, demonstrated an ability to stabilize the dioleoylphosphatidylethanolamine (DOPE) bilayer. To this end, to evaluate the importance of SA concentration in stabilizing the DOPE bilayer, Pinnaduwage and Huang used 5 types of glycophorin A (natural and seminatural), differing in SA content. The result of this study pointed out that the MM glycophorin, containing the greater quantity of SA residue (19 ± 2 per glycophorin molecules), was the only compound that allowed the maximum percentage of lipid recovery after sample purification by Sepharose 4B column separation. Besides, MM glycophorin generated DOPE LPs able to load a remarkable amount of drug, which remained stable on storage. Also, the use of wheat-germ agglutinin (WGA), which binds SA moiety, caused aggregation of MM glycophorin LPs with following destabilization of the systems, leading to rapid release of loaded drug [35].

GM1 was also used to stabilize *N*-palmitoylphosphatidylethanolamine (NPPE), another kind of fusogenic lipid in LP preparation: its use in the preparation of LPs in the presence of GM1 increased the bilayer rigidity, the LPs' size and the aqueous volume. Furthermore, GM1 determined a slow release of the loaded drug (8 – 9%) in different medium solutions (PBS and serum) independently of temperature (37 and 47°C) [36]. The same study evaluated the biodistribution of GM1 LPs after administration in Sparague-Dawley rats' carotid artery: the presence of the ganglioside seemed to enhance the brain delivery (14-fold higher accumulation of GM1 LPs with respect to control LPs), probably owing to a longer permanence of the carriers in the bloodstream and to brain NCAM receptors.

In another study, Taira and colleagues evaluated the stability of LPs containing GM1 at different pH values and in the presence of bile pancreatine and plasma. At 37°C and pH 7.4, in plasma and in the presence of bile and pancreatine, the concurrent presence of GM1 and SM determined the stability of the formulations and the controlled release of the loaded drug (30% over 3 h). On the contrary, at the same temperature and in the same medium, at pH 2, the liposomal formulation displayed a lower stability, leading to faster release (60%) [37].

In a unique study dealing with polymeric NPs, Olivier and colleagues prepared poly(isobutylcyanoacrylate) (PBCA) NPs with Orosomucoid protein adsorbed onto the NPs' surface [38]. This seric glycoprotein is highly glycosylated (42% carbohydrates (w/w)) and carries 15 SA residues per molecule [39]. In the presence of serum, simulating i.v. administration conditions, the stability of NPs was extremely low, probably owing to the rapid displacement from the NPs' surface of the Orosomucoid from other serum protein. This finding was confirmed by other experiments carried out with a lower amount of serum, allowing the orosomucoid layer to reduce the adsorption of serum proteins (in particular opsonin), in comparison with uncoated NPs (**Figure 2**) [38].

3. Sialic acid as recognition and targeting moiety

Sialic acids can be used as ligands or targeting moieties able to recognize and bind specific molecules, for example proteins, on the surface of the selected target. Alternatively, as SA moieties are present on the surface of cancer cells or virus, nanocarriers engineered with specific antibodies against SA or with WGA (able to bind SA) can be used to recognize and bind SAs.

3.1 Virus targeting and detection

Sialic acids as targeting moieties were first used to recognize hemagglutinin, a viral surface protein that causes red blood cell agglutination. Reichert and colleagues used a sialoside lipid previously synthesized [40] to prepare LPs able to recognize and colorimetrically detect the influenza virus. After SA binding with the viral SA hemagglutinin, the polydiacetylene backbone of the molecule provided a color transition when changes in the conjugation molecule length occurred: in this elegant approach, the specific virus–LP interactions altered

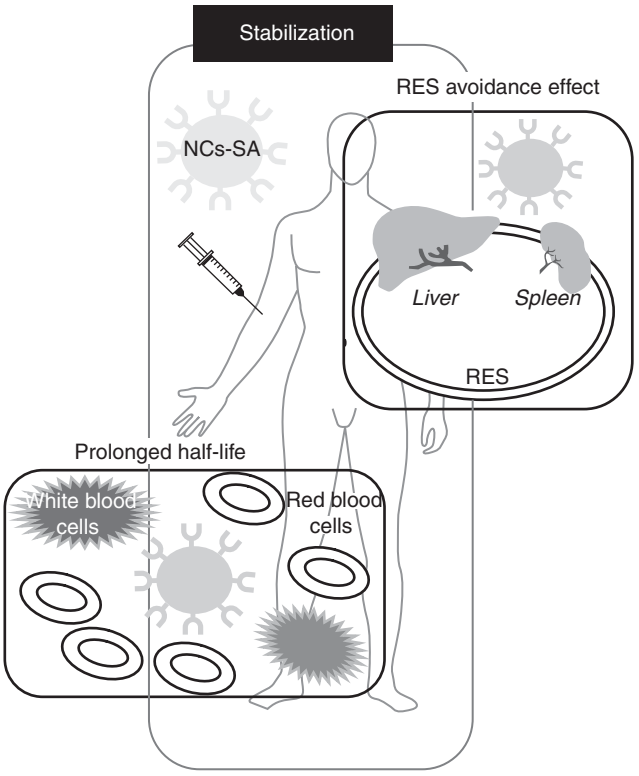

Figure 2. Sialic acid and derivative-based nanotechnology application in stabilization.
RES: Reticuloendothelial system.

the side-chain conformation, reducing the effective conjugation length of the ene-yne backbone, thus leading to color variation [41].

To exploit the potential of SA as a viral detection tool, Niikura and co-workers derivatized gold NPs with SA moiety. As previously pointed out, the SA moiety allowed the binding of hemagglutinin on the capside surface. Then the assembly of a remarkable number of gold NPs onto the virus capside led to a red shift in the peak wavelength of the surface plasmon resonance frequency [42].

Sialic acid was also used to detect β-amyloid in Alzheimer's disease. In an elegant experiment, the binding of β-amiloid with gold NPs derivatized with SA immobilized on a carbon electrode surface was detectable by the oxidation signals of tyrosine by differential pulse voltammery, as confirmed also by atomic force microscopy (AFM) analysis (**Figure 3**) [43].

3.2 Brain targeting

Another application of SA as a recognition and targeting moiety to functionalized polymeric NPs was recently applied at the Te.Far.T.I. Center at the University of Modena and Reggio Emilia [44] with the aim of brain targeting. As these carbohydrates could influence the biological and physical properties of biopharmaceutical proteins and living cells, Bondioli and colleagues [44] synthesized Neu5Ac-conjugated polymeric poly(DL-lactide-*co*-glycolide) (PLGA) NPs and tested them on human monocytes. The results demonstrated a high level of phagocytosis, apparently with a different mechanism from unmodified PLGA NPs. These results allowed the authors to suggest that, in order to avoid phagocytosis, the NPs' surface should be covered by a hydrophilic polymer of Neu5Ac, such as Colominic acid, with a proper conformation and a high molecular mass, as shown by literature data concerning PEG-decorated NPs [45,46].

Moreover, aiming to obtain new targeted NPs with a prolonged residence within the brain parenchyma, leading to long-lasting brain delivery of drugs, the same group prepared PLGA NPs modified with a glycopeptide (g7) for blood–brain barrier (BBB) crossing and with a SA residue for interaction with brain receptors [47]. After i.v. administration in rats, several pharmacological results demonstrated a significant long-lasting effect over 24 h (based on loperamide brain delivery and antinociceptive effect). Furthermore, biodistribution studies showed high NP localization (6% of the injected dose into the CNS) over a prolonged time (24 h) along with the qualitative evaluation of the NPs' visualization (confocal and fluorescent microscopy) within the brain, kidney, liver, spleen and lung tissue parenchyma. The authors suggested that the double-covered NPs (with SA and glycopeptide) crossed the BBB owing to the presence of glycopeptide on the NPs' surface, followed by endocytosis as the BBB crossing mechanism, as pointed out in previous papers [48-50]. Then, as a consequence of the presence of SA moiety on the NPs' surface, the double-covered NPs could interact with brain SA-specific receptors, thus explaining both the prolonged activity of loperamide delivered by NPs and the prolonged NP brain residence time (4 – 6% to 2% over 24 h). The putative mechanism of BBB crossing and brain cell interaction is summarized in **Figure 4**.

The biodistribution studies showed also that the double-covered NPs accumulated in liver and kidney to a greater extent than non-sialylated NPs, demonstrating uptake by macrophages and a strong rate of elimination. This accumulation could be due to receptor-mediated recognition by specific receptors for SA derivatives (sialoadhesins) located in the lung, liver and kidney. Thus, as sialoadhesin receptors are present in several RES organs (liver and lung), the authors considered it to be unreliable to apply a SA molecule as a PEG-like molecule conjugated to NPs.

3.3 Cancer detection

Moreover, SA can also be used as a target molecule on the cell surface for cancer detection. With this aim, Lee and colleagues prepared a dual-mode nanoparticulate probe (Rhodamine-dye-doped silica NPs and iron oxide NPs), fusing multiple fluorescent dyes and multiple magnetic NPs into a single nanoprobe [51]. With this interesting approach, they obtained superior fluorescence and MRI capabilities

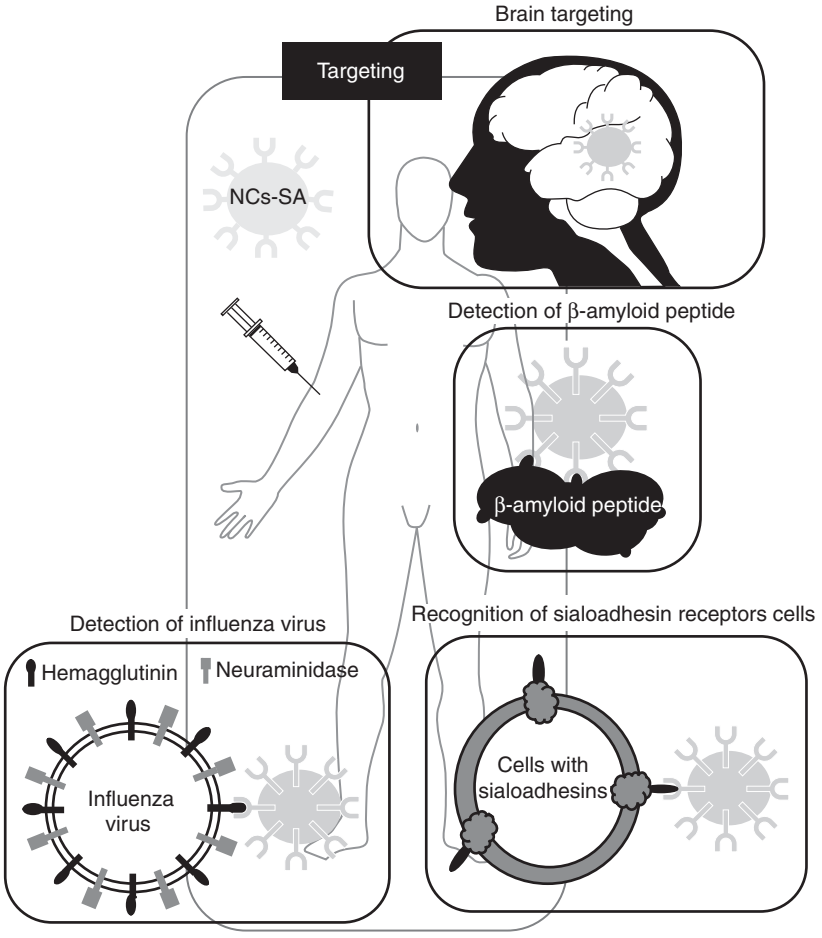

Figure 3. Sialic acid and derivative-based nanotechnology application in targeting and detection.

through the synergistic enhancement of its respective components and further NP engineering with HmenB1, an antibody known to specifically target cells with PSA. Thus, the authors demonstrated the ability of these systems to discriminate between CHP-134 cells, a neuroblastoma cell line overexpressing PSA, and a control cell line not displaying PSA on the surface [51].

In another paper, the same research group showed similar results by using PEGylated inorganic FePt-Au NPs superficially engineered with a dihydrolipoic acid derivatized with HmenB1 [52]. As another example of this application, Xie and colleagues prepared trifunctional nanospheres composed of a double core formed by fluorescent semiconductor quantum dots and Fe_2O_3 magnetic NPs covered with poly(styrene/acrylamide) copolymer. At the end of the process, the surface was modified with the introduction of WGA, finally to obtain trifunctional nanospheres. The authors showed the ability of the nanoprobes to bind, to separate and to collect cells overexpressing PSA. Afterwards, they demonstrated, through a Trypan blue assay, the low cytotoxicity of the system (the number of cells decreases only 5%) [53].

4. Sialic acid for cancer targeting

Among the most promising applications of SA as a targeting ligand, some evidence has shown a specific involvement of SAs in tumor targeting. The authors have already mentioned the importance of SA in lymphocyte activation, and the development of a tumor liposomal vaccine, due to the presence of specific sialylated proteins [54]. Recent studies have demonstrated the importance of SA moiety in the development of cancer as tumor cells are characterized by an increased expression of lectins, able to bind SA moiety [8]. Based on this finding, several studies reported surface engineering of liposomes with SA to target tumor cells, and the most important derivative used is sialyl Lewis X (SLEx) (**Figure 1**). After *in vivo* administration in mice of the modified LPs loaded with different kinds of antitumoral agent such as merphalan [55], methotrexate [56], or cisplatin [57], a remarkable decrease in side effects and in toxicity in comparison with the free drug [57] was demonstrated. Moreover, a significant increase of drug accumulation in the tumor area led to inhibition of tumor growth [55,57]. Other two very recent studies reported the use of different SA derivatives to target tumor. In particular, Zheng

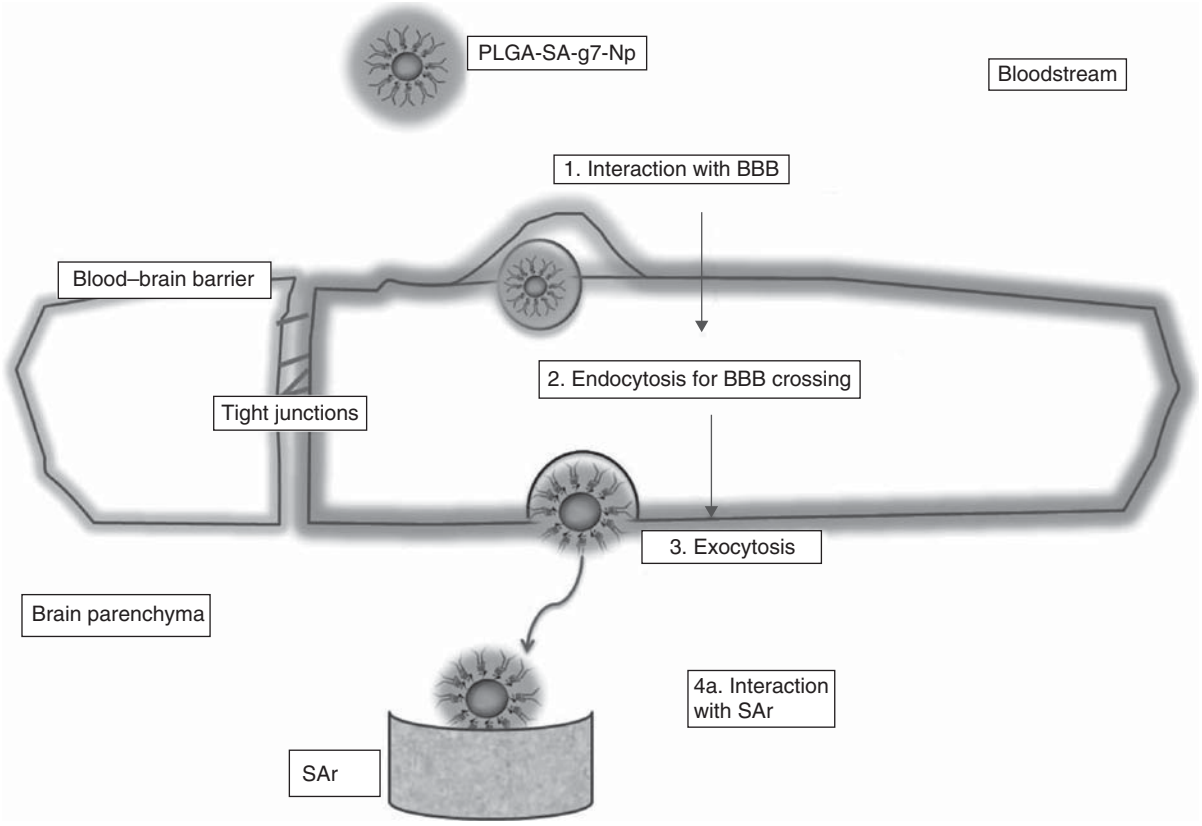

Figure 4. Putative mechanism of SA and glycopeptide engineered NPs in crossing the BBB and interacting with SA brain receptors. Step 1 represents the interaction of glycopeptides (g7) present on NPs surface, allowing BBB crossing. Step 2 represents the endocytotic mechanism of BBB passage. Step 3 represents the exocytotic mechanism for NPs release into the brain parenchyma. Step 4 represents the SAr interaction with SA present on NPs surface.
BBB: Blood–brain boundary; NPs: Nanoparticles; SA: Sialic acid.

and co-workers prepared selenium NPs modified with SA to study the effect on human cervical carcinoma cells (HeLa): SA-modified NPs demonstrated enhanced cancer cell uptake and cytotoxicity. On the contrary, a lower toxicity after SA-modified selenium NP incubation on non-cancer cells was recorded [58]. In another recent paper, Chen and colleagues took advantage of the presence of CD22 (a molecule belonging to the SIGLEC family of lectins) on B-cell lymphoma. *In vitro* studies on human blood samples incubated with LPs (loaded with doxorubicin and modified with sialylated glycan ligand) highlighted a significant toxicity on B cells compared with unmodified doxorubicin-loaded LPs. *In vivo* experiments on a murine model of human B-cell lymphoma showed prolonged half-life of the modified LPs [59].

Another activity against cancer promoted by SA is inhibition of metastasis formation. As is known, several cytokines produced by tumor cells (such as IL-1 and TNF) induce endothelial cells to express adhesion molecules, such as ELAM-1, which mediates the adhesion of malignant tumor cells to endothelium [60]. These adhesion molecules can therefore act as targets/receptors for the circulating/metastasizing tumor cells, expressing SA on their membranes and thus promoting the invasion and metastatic spread of the tumors [61]. As pointed out previously, SLEx was used for inhibiting cancer cell adhesion: in particular an *in vitro* study demonstrated that E-selectin present on tumor cells promoted cancer cell adhesion and that SLEx-modified LPs can hamper the adhesion by means of E-selectin inhibition [62]. Similarly, Saiki and colleagues showed the inhibition of B16-BL6 melanoma cell extravasation in lung after i.v. administration (mice) of SLEx-modified LPs [63].

Remarkably, metastatic tumor growth can be promoted by the platelets' aggregation with tumor cells, leading to the formation of micro-thrombi. As confirmed, Keil and co-workers demonstrated the presence of large aggregates of PEGylated SLEx-modified LPs with platelets and tumor cells, probably as a result of the presence of P- and E-selectin on the platelet and tumor cell surfaces, respectively. Based on this finding, after i.v. administration of the same LPs in HT29 cancer cells of mice, metastasis formation decreased in the lung, liver and intestine, but not in muscle, allowing one to hypothesize there is a difference in the microenvironment promoting the adhesion of the aggregate between modified LPs and tumor cells [64].

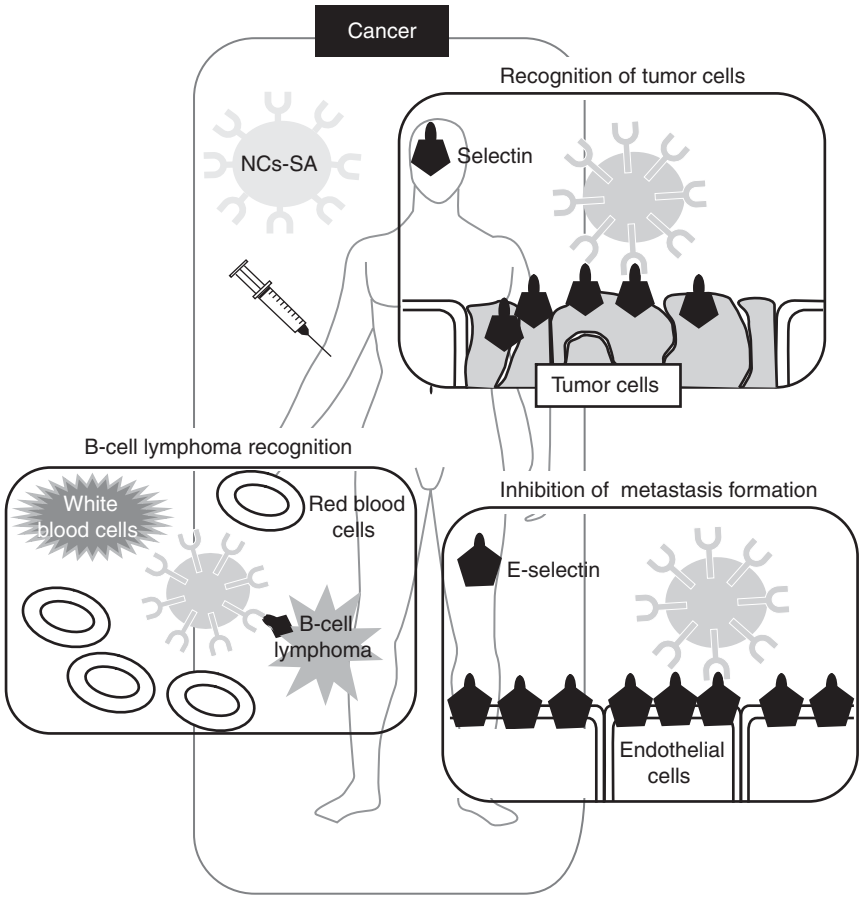

Figure 5. Sialic acid and derivative-based nanotechnology application in cancer detection and therapy.

Sialic acid moiety was also studied to create detection tools for tumors; in particular, SLEx was used to prepare a liposomal bio-imaging fluorescent tool, able to target selectine, for *in vivo* diagnosis of tumors and regions of inflammation [65]. Similarly, aiming for *in vitro* detection of cancer cells, a lectin-based biosensor was able to recognize and bind the SA present on the tumor cells membrane (**Figure 5**) [66].

5. Other effects promoted by sialic acid

Sialic acids concur in different physiological and biological pathways, depending on different prerogatives. In fact, the molecules to which SA is bound, the number of SA moieties present on the compound and the binding position of the residues represent important features that lead to discrimination between one activity and another promoted by SA.

Even if SAs could sometimes be applied to mask surface properties (such as, for example, with the use of GM1), they can also be used to recognize some proteins on the cell's surface, such as viral hemagglutinin.

5.1 Lymphocyte activation and inhibition of infection

Sunamoto and colleagues demonstrated extensively that SA is involved also in lymphocyte activation. As an example, the SA moiety was investigated for the production of a liposomal vaccine. For this study, a tumor surface antigenic protein (TSAP), derived from a leukemia cell line, was incorporated in dimyristoylphosphatidylcholine/1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DMPC/DDPC) LPs. The LPs were obtained either with the direct incubation of LPs with BALB RVD leukemia cells, thus allowing direct transfer of TSAP from cells to LPs (protein-transferred LPs), or with an organic (*n*-butanol) extraction of the proteins from the cells and following reconstruction of LPs in the presence of TSAP (reconstituted LPs). To evaluate immunization success and the prevention of tumor growth, the authors intravenously administered both formulations in mice that had been previously intradermally injected with BALB RVD leukemia cells. The results demonstrated great immunization activity of LPs. When mice were immunized by protein-transferred LPs, cytotoxic 'T' lymphocytes were largely activated and tumor growth was effectively prevented, in contrast to a decreased cytotoxic 'T'-lymphocyte induction

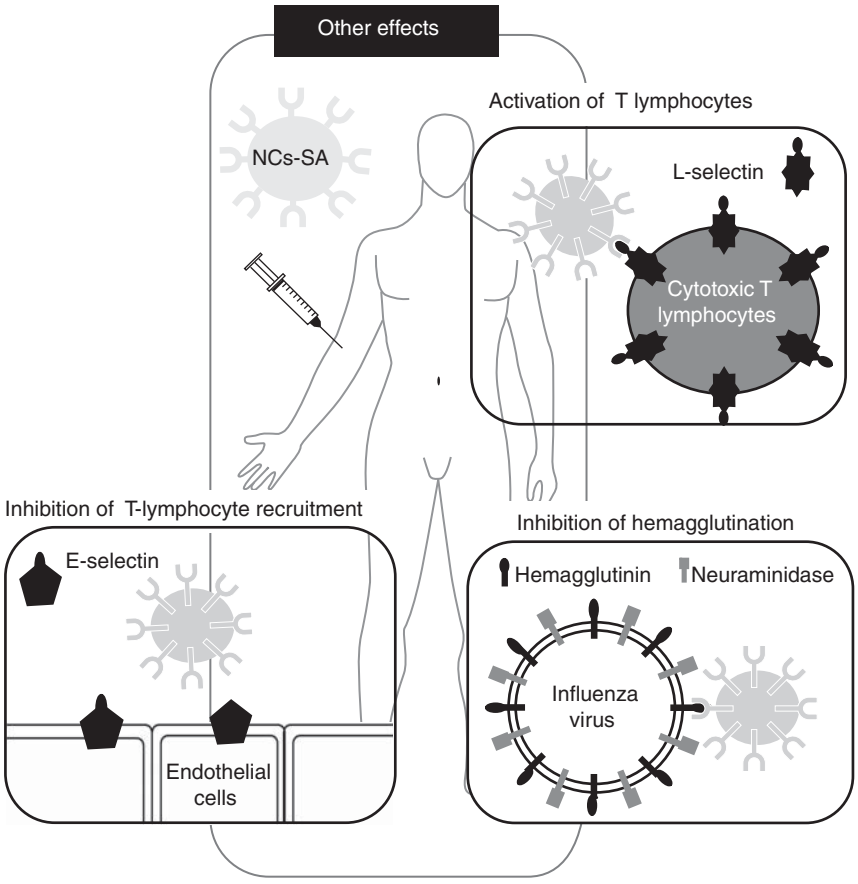

Figure 6. Sialic acid and derivative-based nanotechnology application in activation on T lymphocytes and inhibition of inflammation.

and the immunization effect obtained after reconstituted LP administration [54].

In the following research the authors substituted the TSAP with different kinds of ganglioside (GT1b or GQ1b) in LP preparation, showing an effective stimulation of the *in vitro* activation of 'T' lymphocytes, contrary to GM3 or GD3, which are almost ineffective. To reach an increased stimulation of rat lymphocytes, the authors claimed that the concentration of ganglioside on the LPs' surface was also pivotal, demonstrating the role of both the number of SA groups and the position of the linkage in influencing the efficiency of the T lymphocytes [67].

The same study was also conducted *in vitro* on human T lymphocytes, demonstrating GT1b and GD1a as the most active gangliosides in the stimulation of lymphocytes, contrary to GQ1b, which is less active. The significant difference in stimulation efficiency of lymphocyte cells by ganglioside may be caused by the configuration of the SA moiety on the LPs' membrane surface, recognized by a species-specific receptor on T lymphocytes [68].

To clarify the importance of the number and position of SA moiety on T cell stimulation activity, Kato and co-workers synthesized new SA-conjugated cholesterols as ganglioside analogues, namely SC2 (shorter spacer) and SC6 (longer spacer). In particular, SC6-based LPs were more effective than SC2 ones. The authors claimed that the monosialyl moiety of SA-derivatized cholesterol, with a flexible longer spacer, may display the correct spatial distance from the LPs' surface, thus being able to interact with the receptor on the cell surface and to be responsible for the stimulation of lymphocytes [69].

To clarify better the role of SA derivates in T-cell stimulation, some research was conducted on L-selectin, a cell adhesion molecule on the surface of T cells. As happens for E- and P-selectins, L-selectin specifically recognizes sulfated, sialylated and fucosylated glycoproteins, known as GlyCAM-1 and Sgp90, and plays a major role in activating cytolytic properties of cytotoxic 'T' lymphocytes [70].

With this aim, exploiting the potential of SLEx, usually exposed on the cells' surface and representing one of the most important blood group antigens, DeFrees and colleagues demonstrated that SLEx-modified LPs can promote inhibition of E-selectin, with the expected inhibition of the inflammatory process [71]. In particular, after i.v. administration in a cat

model, the cardioprotection of these systems was demonstrated after myocardial ischemia, in which an acute inflammatory tissue response and massive polymorphonuclear leukocyte recruitment occur [71].

Thus, Murohara and co-workers demonstrated that the i.v. administration of SLE LPs with SLEx in a cat animal model can significantly attenuate polymorphonuclear leukocytes' adherence to the ischemic reperfused coronary endothelium, leading to cardioprotection and to a final 70% reduction in necrotic myocardium. The mechanism of action was explained by the SLEx exposed to the LPs' surface interacting with the P-selectin (mediating lymphocyte recruitment) on the endothelial cell surface. This molecular binding inhibited the interaction with white blood cells, which were not yet able to extravase in the injury site. Unfortunately, as soluble SLEx possesses a short half-life in the bloodstream, incorporation or linkage to the LPs' surface allowed improvements in pharmacokinetic parameters [72].

In a more recent work, aimed at the same inhibition and reduction in necrotic and inflammation effect, Yamazaki and colleagues showed the pivotal role of a fucose residue beside the presence of a SA moiety [73].

5.2 Inhibition of viral infectivity

The authors have already pointed out that SA is important for the detection of influenza virus by recognizing specific capside proteins. Actually, this moiety is able not only to recognize particular capside protein, but also to inhibit hemagglutination and, in particular conditions, viral infectivity.

With this aim, Kingery-Wood and colleagues, using the hemagglutination assay (HAI assay), evaluated the hemagglutination inhibition effect promoted by the SA moiety present on the liposomal surface prepared with sialyl ganglioside analogues. For this test, after addition of LPs (including the new ganglioside analogues) to the chicken red blood cells, Kingery-Wood *et al.* incubated the cell line with influenza virus X-31. Their results established that SAs moieties on the surface of LPs were moderately more effective at inhibiting agglutination of red blood cells by influenza virus than SA groups linked to soluble molecules used as controls [74].

Moreover, with other HAI assays on Madin-Darby canine kidney cells, Spevak and colleagues, using LPs with an increasing concentration of synthesized sialoside lipids on their surface, established that the ability of sialoside LPs in inhibiting hemagglutination did not necessarily reflect their capacity to inhibit infectivity. In fact, a higher concentration of sialoside lipids on LP preparation (5 – 10%) was able to inhibit efficaciously hemagglutination, interestingly leading to only 46% of infectivity inhibition. On the contrary, a lower sialoside lipid concentration (1 – 5%) led to high infectivity inhibition (96%). These data indicate that, notwithstanding that synthetic sialosides are poor inhibitors of hemagglutination, they can effectively stop the infectivity (**Figure 6**) [40].

6. Expert opinion

The SAs could be considered as the first lead-molecules used by researchers to enhance bloodstream stability of carriers. Despite the presence of discordant papers, the overall research produced over the last 40 years has described in-depth the potential of SAs and derivatives (**Table 1**). The discrepancy in results and applications of SAs and derivatives in nanotechnology hampered the evolution of this field of research, and most of all the application of SAs and derivatives as surface modifiers of nanocarriers, differently from what happened for PEGylation technology.

One of the most stimulating fields of SA application is surely cancer detection and therapy. Despite the relevant number of papers on this topic rightly claiming the efficacy of this strategy, some possible drawbacks of this strategy could be related to the expression of SA receptors in other areas, organs and even cell types. Thus, if the therapeutic goal is almost fully reached (cancer targeting and tumor growth inhibition), side effects could be increased as a result of unwanted targeting effects.

Another pivotal SA application is the stabilization of nanocarriers and the attempt to create stealth carriers by mimesis with natural exogenous bacteria and living beings. The main issue regarding the failure of this technology for nanocarrier stabilization is focused on activity of SA and its derivatives in the immune system, with contrasting results leading to lymphocyte inhibition or stimulation.

Moreover, the huge variability in the newly synthesized SA derivatives, often structurally and conformationally different, led to a significant difference in research output, not allowing any comparison on activity and perspectives.

A common feature of all the research on this topic is surely the molecular mass of the surface modifier along with its spatial conformation on the nanocarrier surface (i.e., the activity as nanocarrier stabilizer is closely linked to PEG conformation during mushroom–brush transition). This issue should be strongly considered when planning a SA-based strategy, independently from the main aim (cancer targeting, viral detection or production of stealth systems). The conformation of SA shield on the nanocarrier surface should be thoroughly assessed because the nanoparticulate surface engineering is efficient only if the modifiers are clearly exposed on the surface. For this reason, the authors suggest that a single molecule (SA) without any spacer is not fully able to reach the final aim.

Moreover, an in-depth study on the effective inputs given by single or poly-molecules should be developed as a function of the final aim: in particular, the use of polysialylated molecules could answer the need for a stealth-inducer, also confirmed by the Phase I marketed products, mainly based on PSA-prodrugs.

Despite the limitations of SA as a stealth-inducer molecule, the application of SA as a targeting agent (to

Table 1. Summary of the most outstanding *in vivo* and *in vitro* results on SA and derivative-based nanomedicines.

Carrier	Composition	Aim	Drug loaded	Administration route	*In vivo*/*vitro* model	Effect	Ref.
LPs	PC:CHOL:GM1 (10:5:1)	Stabilizer	–	Intravenous	Balb/c female mice IM inoculated with J6456 lymphoma cells	Decrease in RES uptake 3.4-fold versus control LPs	[11]
LPs	PC:GM1 (1:0.07)	Stabilizer	–	Intravenous	ICR mice	Decrease in RES uptake 17-fold versus PC LPs	[29]
LPs	PC:SM:GM1 (1:4:0.035)	Stabilizer	–	Intravenous	ICR mice	Decrease in RES uptake 330-fold versus PC LPs	
LPs	PC: CHOL:GM1 (2:1:0.3)	Stabilizer	–	Intravenous	ICR mice	Decrease in RES uptake 6.3-fold versus control LPs	[21]
LPs	PC: CHOL:GM1 (10:5:1)	Stabilizer	–	Intravenous	Balb/c female mice subcutaneously inoculated with EMT6 tumor cells	Importance of LPs' size GM1 LP (< 70 nm) accumulation in liver GM1 LP (> 300 nm) accumulation in spleen GM1 LP (90 – 200 nm) accumulation in tumor	[31]
LPs	DPPC:CHOL:GP:GM3 (11.4:4.8:0.007:0.07)	Stabilizer	–	Intravenous	Sparague-Dawley rats	Increase in blood concentration 3.3-fold versus control LPs	[33]
LPs	SM:CHOL:GP:GM3 (11.4:4.8:0.007:0.07)	Stabilizer	–	Intravenous	Sparague-Dawley rats	Increase in blood concentration 3.7-fold versus control LPs	[33]
LPs	DPPC:CHOL:Neu5Acβ-Pa (10:10:1)	Stabilizer	–	Intravenous	Rats and mice	RES avoidance effect on rats	[23]
LPs	DPPC:CHOL:GM1 10:10:1)	Stabilizer	–	Intravenous	Rats and mice	RES avoidance effect on mice	[23]
LPs	DPPC:CHOL:DCP: SA-linked glycoproteins (35:40:5:15)	Stabilizer	–	Intravenous	Mice	Fucose residue unable to avoid RES uptake Galactose residue produced strong reduction in RES uptake	[22]
LPs	GM1:DSPC:CHOL (0.13:1:1) PEG:DSPC:CHOL (0.13:1:1)	Stabilizer	Adriamycin	Intravenous	DBA/2 Mice	2.3-fold higher blood concentration for GM1 LPs 2.9-fold higher blood concentration for PEG LPs	[32]
LPs	DOPE:GPs (5 types of GP) (1:0.5)	Stabilizer	–	–	*In vitro*	Effect of SA residues on lipid recovery and WGA aggregation test	[35]
LPs	DMPC:CHOL:NPPE:GM1	Stabilizer	–	–	*In vitro* (PBS and serum)	GM1 increases the stability of LPs and determined a slow drug release	[36]
LPs	DMPC:CHOL:NPPE:GM1	Stabilizer	–	Intra-arterial	Sparague-Dawley rats	Brain accumulation 14-fold higher versus control LPs	[36]

AD: Alzheimer's disease; CHOL: Cholesterol; DAP: Diacetyl phosphate; DCP: Dicetyl phosphate; DDPC: 1,2-Dimyristoylamido-l,2-deoxyphosphatidylcholine; DMPC: Dimyristoylphosphatidylcholine; DMPE: Dimyristoylphosphatidylethanolamine; DOPE: Dioleoylphosphatidylethanolamine; DPPC: Dipalmitoyl phosphatidylcholine; DPPE: Dipalmitoylphosphatidylethanolamine; DSPC: Distearoylphosphatidylcholine; DSPE: Distearoylphosphatidylethanolamine; GM1: Ganglioside 1; GM3: Ganglioside 3; GP: Glicophorine; LPs: Liposomes; ^{BPC}NeuAc: 9-*N*-biphenylcarboxyl-NeuAcα2-6Galβ1-4GlcNAc; Neu5Ac β-PA: Palmitic acid (PA) with Neu5Ac; NPs: Nanoparticle; NPPE: *N*-palmitoylphatidylethanolamine; PBCA: Poly(butyl-cianoacrylate); PC: Phosphatidylcholine; PEG: Poly(ethylene glycol); PI: Phosphatidylinositol; PLGA: Poly(lactic-*co*-glycolic acid); QDs: Quantum dots; RES: Reticuloendothelial system; SA: Sialic acid; SC2 (shorter spacer) and SC6 (longer spacer): Ganglioside analogues; SLEx: Sialyl Lewis X; SM: Sphingomyelin; TSAP: Tumor surface antigenic protein; VLP: Virus-like particles; WGA: Wheat-germ agglutinin.

Table 1. Summary of the most outstanding *in vivo* and *in vitro* results on SA and derivative-based nanomedicines (continued).

Carrier	Composition	Aim	Drug loaded	Administration route	*In vivo*/*vitro* model	Effect	Ref.
LPs	PC:CHOL:SM:GM1 (1:1:1:0.14)	Stabilizer	–	–	*In vitro* (plasma, addition of bile pancreatine)	GM1 and SM affect LPs' stability and drug release at different temperatures and pH values	[37]
NPs	PBCA with orosmucoid protein adsorbed on surface	Stabilizer	–	–	*In vitro* (serum)	Effect of Orosomucoid protein on NPs' stability and protein adsorption	[38]
NPs	NPs-SA and LPs-SA	Stabilizer	–	–	*In vitro* (human monocytes)	Lack of stealth properties	[45,46]
LPs	Polymerized LPs bearing SA	Viral detection	–	–	*In vitro* (influenza virus)	Viral hemagglutinin detection Colorimetrical detection	[41]
NPs	Gold NPs bearing SA	Viral detection	–	–	*In vitro* (VLP)	Viral hemagglutinin detection Gold NP-based detection	[42]
NPs	Gold NPs bearing SA	AD detection	–	–	*In vitro*	Binding of β-amyloid to SA-gold NPs	[43]
NPs	NPs (SiO_2-Fe_3O_4)-Rhodamine-HmenB1	PSA detection on cancer cells	–	–	*In vitro* (neuroblastoma cells)	Effective recognition of neuroblastoma cells expressing PSA	[51]
NPs	NPs FePt-Au-HmenB1	PSA detection on cancer cells	–	–	*In vitro* (neuroblastoma cells)	Effective recognition of neuroblastoma cells expressing PSA	[52]
NPs	NPs (QDs-Fe_2O_3) polystyrene-acrylamide coated, modified with WGA	PSA-expressing cell detection	–	–	*In vitro* (*Staphylococcus aureus* and DU-145 cells)	Effective recognition of PSA-expressing cells	[53]
NPs	PLGA modified with SA and BBB-crossing glycopeptide	Brain targeting	Loperamide/ Rhodamine 123	Intravenous	Rats	Prolonged and long-lasting brain residence (6% of injected dose over 24 h)	[47]
LPs	PC:PI:SA-vector (8:1:20%)	Tumor targeting	Merphalan	Intravenous	BLRB mice injected subcutaneously with BLRB-Rb tumor cells	Significant decrease of tumor growth Increase in animal survival time	[55]

AD: Alzheimer's disease; CHOL: Cholesterol; DAP: Diacetyl phosphate; DCP: Dicetyl phosphate; DDPC: 1,2-Dimyristoylamido-l,2-deoxyphosphatidylcholine; DMPC: Dimyristoylphosphatidylcholine; DMPE: Dimyristoylphosphatidylethanolamine; DOPE: Dioleoylphosphatidylethanolamine; DPPC: Dipalmitoyl phosphatidylcholine; DPPE: Dipalmitoylphosphatidylethanolamine; DSPC: Distearoylphosphatidylcholine; DSPE: Distearoylphosphatidylethanolamine; GM1: Ganglioside 1; GM3: Ganglioside 3; GP: Glicophorine; LPs: Liposomes; ^{BPC}NeuAc: 9-*N*-biphenylcarboxyl-NeuAcα2-6Galβ1-4GlcNAc; Neu5Ac β-PA: Palmitic acid (PA) with Neu5Ac; NPs: Nanoparticle; NPPE: *N*-palmitoylphatidylethanolamine; PBCA: Poly(butyl-cianoacrylate); PC: Phosphatidylcholine; PEG: Poly(ethylene glycol); PI: Phosphatidylinositol; PLGA: Poly(lactic-*co*-glycolic acid); QDs: Quantum dots; RES: Reticuloendothelial system; SA: Sialic acid; SC2 (shorter spacer) and SC6 (longer spacer): Ganglioside analogues; SLEx: Sialyl Lewis X; SM: Sphingomyelin; TSAP: Tumor surface antigenic protein; VLP: Virus-like particles; WGA: Wheat-germ agglutinin.

Table 1. Summary of the most outstanding *in vivo* and *in vitro* results on SA and derivative-based nanomedicines (continued).

Carrier	Composition	Aim	Drug loaded	Administration route	*In vivo*/*vitro* model	Effect	Ref.
LPS	PC:PI:SA-PEG-LipoAnchor (8:1:20%)	Tumor targeting	Methotrexate	–	*In vitro* (M3 melanoma cells)	Cytotoxicity 2.5 higher versus control LPs	[56]
LPs	DPPC:CHOL:DCP:GM (30:40:15:5:5)	Tumor targeting	Cisplatin	Intravenous	Balb/C mice injected subcutaneously with A549 tumor cells	Decrease of cisplatin toxicity and side effects Significant increase of drug accumulation in the tumor region (sixfold versus free drug) Decrease of tumor growth	[57]
NPs	Selenium-SA	Tumor targeting	Selenium	–	*In vitro* (HeLa cells)	Increased cellular uptake Increase in cytotoxicity via apoptosis induction	[58]
LPs	DSPC:CHOL:^{BPC}NeuAc-PEG-DSPE	Tumor targeting	Doxorubicin	Intravenous	Mice i.v.-injected with Daudi Burkitt lymphoma cells *In vitro* (human blood sample)	Prolongation of survival time of 1.5 time versus naked LPs Target and kill human B cells	[59]
LPs	PC:DMPE:DSPE-PEG-SLEx (93:3.5:3.5) PC:DMPE:DSPE-PEG (93:3.5:3.5)	Inhibition of tumor cell adhesion	–	–	*In vitro* (14 tumor different cells lines)	Only 4 cells line express high quantity of SLEx PEG chain is necessary to allow macrophages escape SLEx-PEG LPs show high inhibition of adherence	[62]
LPs	PC:CHOL:SLEx (2:1)	Inhibition of metastasis formation	–	Intravenous	C56BL/6 mice injected intravenously with B16-BL6 melanoma cells	Decreased lung extravasation of melanoma cells	[63]
LPs	PC:CHOL:DMPE-PEG-SLEx (63:30:3.5)	Inhibition of metastasis formation	–	Intravenous	NMRI mice injected intravenously with HT29 colon carcinoma cells	Formation of large aggregates with platelets and tumor cells Inhibition of metastasis formation in lung, liver and intestine	[64]

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Table 1. Summary of the most outstanding *in vivo* and *in vitro* results on SA and derivative-based nanomedicines (continued).

Carrier	Composition	Aim	Drug loaded	Administration route	*In vivo*/*vitro* model	Effect	Ref.
LPs	DMPC:CHOL:DAP:GM1:DPPE (17:10:2:15:2)	Detection of tumor and inflammation region	Cy5.5	Intravenous	ddY mice injected with EAT tumor cells or Chondorex to induce arthritis	Increase of accumulation of LPs in tumor or inflammation region	[65]
LPs	PC:DDPC coated with TSAP	Immunization and prevention of tumor growth	–	Intravenous	Mice intradermal injected with BALB RVD leukemia cells after immunization with LPs	T-lymphocyte activation Tumor growth decrease	[54]
LPs	DPPC:CHOL and different ganglioside (GT1b, GQ1b, GM3, GD3)	Lymphocyte activation	–	–	*In vitro* (rat T lymphocytes)	GT1b LP and GQ1b LP stimulation of T lymphocyte GM3 and GD3 inefficacy	[67]
LPs	DPPC:CHOL and different ganglioside (GT1b, GQ1b, GM3, GD3)	Lymphocyte activation	–	–	*In vitro* (human T lymphocytes)	GT1b LP and GQ1b LP stimulation of T lymphocyte GM3 and GD3 inefficacy	[68]
LPs	DPPC:CHOL:SC2 or SC6 (37:7:6)	Lymphocyte activation	–	–	*In vitro* (rat T lymphocytes)	Importance of number and position of SA moiety	[69]
LPs	PC:CHOL:DSPE-PEG-SLEx (11:8:1)	Inhibition of inflammatory processes	–	–	*In vitro*	Efficacious inhibition of E-selection (5000-fold versus free SLEx) Inhibition of inflammatory processes	[71]
LPs	PC:CHOL:DSPE-PEG-SLEx	Inhibition of inflammatory processes in myocardial ischemia	–	Intravenous	Cat myocardial ischemic model	Cardioprotection 70% reduction in necrotic myocardium	[72]
LPs	Polymerized LPs bearing SA	Inhibition of viral infection	–	–	*In vitro* (chicken red blood cells with influenza virus X-31)	Inhibition of agglutination and viral infection	[74]
LPs	Polymerized LPs bearing SA	Inhibition of viral infection	–	–	*In vitro* (Madin-Darby canine kidney cells with influenza virus)	Evidence on the percentage of SA on LPs' surface able to produce the higher infectivity inhibition	[40]

AD: Alzheimer's disease; CHOL: Cholesterol; DAP: Diacetyl phosphate; DCP: Dicetyl phosphate; DDPC: 1,2-Dimyristoylamido-l,2-deoxyphosphatidylcholine; DMPC: Dimyristoylphosphatidylcholine; DMPE: Dimyristoylphosphatidylethanolamine; DOPE: Dioleoylphosphatidylethanolamine; DPPC: Dipalmitoyl phosphatidylcholine; DPPE: Dipalmitoylphosphatidylethanolamine; DSPC: Distearoylphosphatidylcholine; DSPE: Distearoylphosphatidylethanolamine; GM1: Ganglioside 1; GM3: Ganglioside 3; GP: Glicophorine; LPs: Liposomes; ^{BPC}NeuAc: 9-*N*-biphenylcarboxyl-NeuAcα2-6Galβ1-4GlcNAc; Neu5Ac β-PA: Palmitic acid (PA) with Neu5Ac; NPs: Nanoparticle; NPPE: *N*-palmitoylphatidylethanolamine; PBCA: Poly(butyl-cianoacrylate); PC: Phosphatidylcholine; PEG: Poly(ethylene glycol); PI: Phosphatidylinositol; PLGA: Poly(lactic-*co*-glycolic acid); QDs: Quantum dots; RES: Reticuloendothelial system; SA: Sialic acid; SC2 (shorter spacer) and SC6 (longer spacer): Ganglioside analogues; SLEx: Sialyl Lewis X; SM: Sphingomyelin; TSAP: Tumor surface antigenic protein; VLP: Virus-like particles; WGA: Wheat-germ agglutinin.

SA-specific receptors in the brain or cancer delivery) appears promising, with good results both *in vitro* and *in vivo*, opening the way to a new vista and concept on SA as a target or moiety.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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