RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Journal Name

COMMUNICATION

Novel bivalent spermine-based neutral neogalactolipids for modular gene delivery systems

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

E.A. Ivanova,[a] A.V. Filatov,[b] N.G. Morozova,[a] M.A. Zenkova[b] and M.A. Maslov[a]

New bivalent spermine-based neutral neogalactolipids have been synthesized to develop an effective modular gene delivery systems (MGDS) targeted to hepatocyte asialoglycoprotein receptors. MGDS composed of new neogalactolipids, cationic gemini-amphiphile, and DOPE were agglutinated in the presence of lectin *RCA*$_{120}$ and provided the efficient delivery of fluorescent-labeled oligodeoxyribonucleotides into HepG2 cells.

One of the problems of gene therapy is the development of stable and safe non-viral transport systems for the delivery of therapeutic nucleic acids (NA) into the target cells with efficiency equal to that of viral vectors. Using cationic liposomes as delivery systems is one of the promising approaches of gene therapy.[1,2] Positively charged complexes composed of cationic liposomes and NA (called "lipoplexes") are able to adsorb on plasmatic membrane of cells and enter inside them by means of endocytic pathway.[3] However, the wide application of cationic liposomes is limited by their low efficiency and unspecific NA delivery into target cells.[4] These drawbacks are related to the presence of different biological barriers for lipoplexes such as the instability in biological fluids, the interaction with blood serum proteins and plasmatic and nucleic membranes, and endosomal degradation. A concept of modular gene delivery systems (MGDS) was introduced ten years ago as an appropriate structural paradigm for synthetic non-viral vector.[5–8] In MGDS, nucleic acid is condensed into cationic liposomal formulations consisting of different lipophilic modules that mediate environmental and cellular receptor interactions and intracellular trafficking to overcome limiting barriers.

Genetic damages of liver cells lead to serious diseases such as hemophilia, hypercholesterolemia, cancer and other.[9,10] It is known that hepatocytes express the asialoglycoprotein receptors (ASGPr), which recognize D-galactose or *N*-acetyl-D-galactosamine terminal residues;[11,12] therefore, the targeting of therapeutic NA to hepatocytes can be achieved by MGDS containing neogalactolipids (*neo* means non-natural) as a targeting module. ASGPr as a multidomain binding protein exhibits significantly different affinities for ligands depending on their structures. ASGPr bind monovalent ligands (free D-galactose, lactose, and simple galactosides) with millimolar dissociation constants (K_d ~10^{-3} M), which are far below those needed to achieve glycotargeting under physiological conditions. In comparison, bivalent galactose-terminated oligosaccharides (compounds with two carbohydrate ligands) bind with affinities that are three-orders-of-magnitude higher (K_d ~10^{-6} M).[13] To construct multivalent carbohydrate-containing conjugates suitable for multiple molecular interactions with ASGPR, a number of different polyfunctional core molecules [tris(2-aminoethyl)amine, tris(hydroxymethyl)-aminometane, pentatreitole derivatives, heterocyclic bases, amino acids, oligopeptides, and carbohydrates[14–19]] are used.

Previously, to develop MGDS targeted to the hepatocytes, we designed and synthesized bivalent galactose-containing neutral lipids on the basis of L-glutamic acid as core molecule.[20] These neoglycolipids were successfully incorporated into liposomal formulations composed of polycationic gemini amphiphile (as the condensing module) and zwitterionic lipid DOPE (as the helper module). Specific binding of galactose-decorated liposomes was demonstrated using the ricin agglutination test. Here we described a convenient approach for the synthesis of novel bivalent spermine-based neutral neogalactolipids **1a,b** (Fig. 1) as well as physicochemical characteristics and biological activity of targeted MGDS composed of them.

Natural polyamines, spermine and spermidine, play an important role in the vital activity of cells and can pack DNA to toroidal and rod-like structures. Lipophilic derivatives of polyamines condense DNA more efficiently than natural polyamines and can be used as non-viral NA delivery systems.

[a] *Lomonosov Moscow University of Fine Chemical Technologies (MITHT), 86 Vernadskogo ave., Moscow, 119571 Russian Federation. Fax: +7(495)9368901; Tel: +7(495)9368903; E-mail: mamaslov@mail.ru*
[b] *Institute of Chemical Biology and Fundamental Medicine SB RAS, 8 Lavrentieva ave., Novosibirsk, 630090, Russian Federation. Fax: +7(383)3635153; Tel: +7(383)3635160; E-mail: marzen@niboch.nsc.ru*

Electronic Supplementary Information (ESI) available: NMR- and mass-spectra of compounds synthesised and cytotoxicity of liposomes. See DOI: 10.1039/x0xx00000x

On the basis of spermine, various amphiphilic molecules were synthesized to deliver oligonucleotides, DNA and siRNA into eukaryotic cells.[21—26] Also it was found that HepaRG cells (progenitor cells which are capable to differentiate into hepatocyte-like cells) could be easily transfect by cationic liposomes composed of lipophosphonates, lipophosphoramidates and betaine-type lipids.[27]

To construct spermine-based neutral neogalactolipids **1a,b**, cholesterol and 1,2-di-*O*-tetradecyl-*rac*-glycerol were used as hydrophobic domains for the incorporation of neogalactolipids into a liposomal bilayer. Hexamethylene and triethylene glycol spacers were chosen to distance the galactose moieties from hydrophobic domain and to provide flexibility and independent orientation of carbohydrate units in the space.

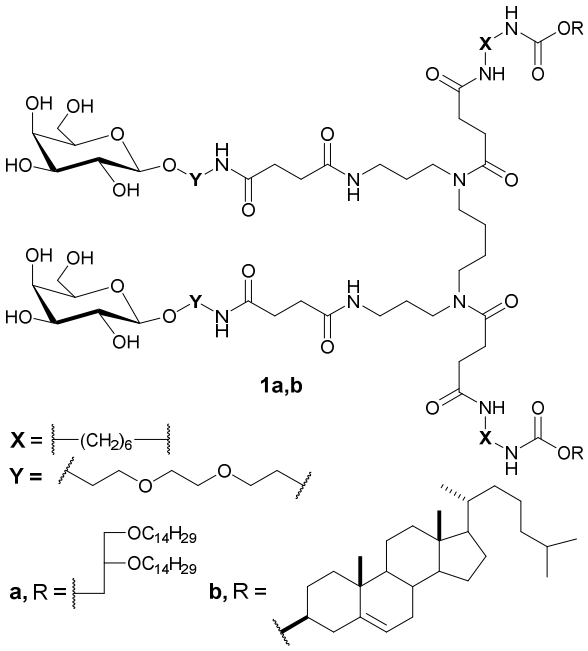

Figure 1. Bivalent spermine-based neutral neogalactolipids **1a,b**.

For the synthesis of neogalactolipids **1a,b**, sets of building blocks, hydrophobic **2a,b** and **3a,b** and carbohydrate **4a,b** precursors, which contain various spacers and terminal functional groups (Fig. 2), were prepared. Compounds **2a,b** and **3a,b** with terminal amino or carboxyl groups were synthesized as previously described.[20] Carbohydrate precursor **4b** was obtained by the treatment of galactoside **4a**[20] with succinic anhydride in the presence of Et_3N in 69% yield.

Due to the symmetry of spermine (**5**) and the presence of four functional amino groups, different arrangement of hydrophobic and carbohydrate domains can be achieved. We suggested to introduce firstly the hydrophobic precursors **3a,b** on spermine secondary nitrogen atoms followed by the connecting of glycoside **4b** on primary amino groups (Fig.3). Spermine (**5**) was treated with ethyl trifluoracetate to give bis-amine **7** in 95% yield. It is known that the synthesis of N^4,N^9-diacylspermines can be done by an acylation of the protected spermine derivatives with fatty acids in the presence of DCC and HOBt.[28] Another effective approach utilizes acid chlorides or mixed anhydrides as acylating agents. Chloroanhydrides and mixed anhydrides[29] of compounds **3a,b** were prepared. Unfortunately, the treatment of bis(trifluoroacetamide) **6** with these anhydrides led to the formation of cyclic amide of compounds **3a,b** but not to the desired products **10a,b**. The condensation of compounds **3a,b** and **6** in the presence of HBTU, *N*-methylmorpholine, and HOBt[30] was also ineffective. Therefore, for the synthesis of neogalactolipids **1a,b**, a new approach was implemented, which involved the initial introduction of succinic residue into spermine molecule **6** and subsequent condensation with hydrophobic precursors **2a,b** containing a terminal amino group.

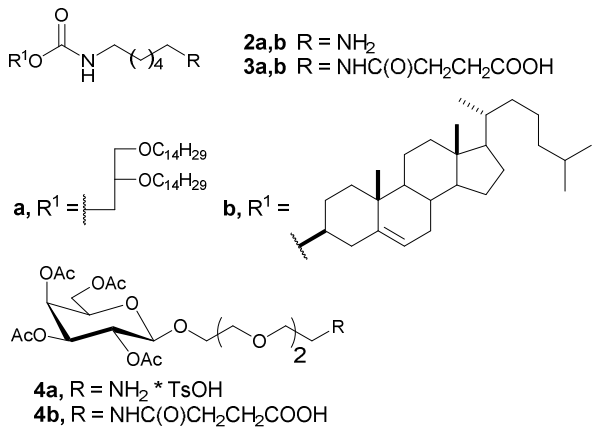

Figure 2. Hydrophobic and carbohydrate precursors with terminal amino and carboxylic groups.

Bis(trifluoroacetamide) **6** was treated by the chloride of succinic acid monobenzyl ester[31,32] in the presence of catalytic amount of DMAP at 0°C for 60 min. After column chromatography on silica gel, spermine derivative **7** was obtained in 37% yield. Benzoyl protective groups were removed by catalytic hydrogenation on Pd/C in the presence of an excess of ammonium formate to give compound **8** with terminal carboxyl groups. Further coupling of hydrophobic components **2a,b** and compound **8** was carried out in the presence of HBTU and DIPEA in anhydrous DMF to afford compounds **9a,b** in 76% and 90% yields, respectively. After the unblocking of primary amino groups in conjugates **9a,b** by sodium hydroxide in methanol spermine derivatives, **10a,b** containing two hydrophobic domain were obtained. The attachment of galactoside **4b** to conjugates **10a,b** promoted by HBTU led to the formation of di-substituted glycoclusters **11a,b** in 43% and 32% yields, respectively. The deacetylation of compounds **11a,b** under the Zemplen reaction condition yielded the desired bivalent neutral neogalactolipids **1a,b** in 72% and 90% yields, respectively. The structures of the synthesized neogalactolipids **1a,b** were confirmed by ^1H and ^{13}C NMR spectroscopy as well as $^1H^1H$-COSY 2D-NMR spectroscopy and mass spectrometry (see Supplementary information).

Targeted MGDS composed of polycationic lipid 2D3,[33,34] zwitter-ionic lipid DOPE, and neogalactolipid **1a** (2.5%, 5%, and 10% mol.) were prepared as previously described.[23] The particle size and ζ-potential measurements were carried out to assess the influence of neoglycolipid molar ratio on the physicochemical characteristics of MGDS (Table 1). All targeted MGDS were positively charged (ζ-potentials are within the range +45 to +50 mV) and characterized by monomodal size distribution. The increasing of neogalactolipid **1a** amount in liposomal formulations from 0% to 10% resulted in the dramatical decrease of the average particle diameters from 114 nm to 44 nm. Additionally, the PDI values also decreased, indicating the stabilizing effect of compound **1a** on liposomal formulations.

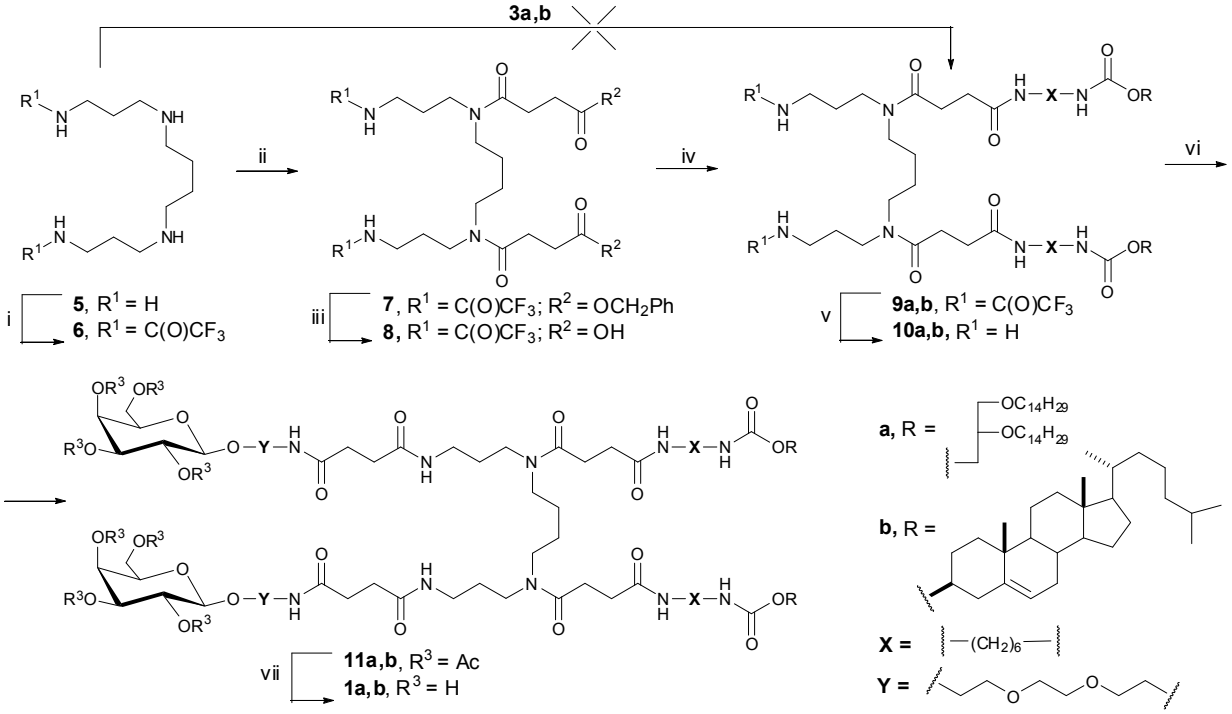

Figure 3. Synthesis of bivalent neogalactolipids based on spermine **1a,b.** Reagents and conditions: (i) $CF_3C(O)OEt$, H_2O, MeCN, 100°C; (ii) $BnOC(O)CH_2CH_2C(O)Cl$, Py, DMAP, Et_3N, 24°C; (iii) $NH_4{\cdot}HCO_2$, 10% Pd/C, MeOH, 60°C; (iv) compound **2a,b**, HBTU, DIEA, DMF, 0°C→24°C; (v) K_2CO_3 or NaOH, MeOH, 50 or 24°C; (vi) compound **4b**, HBTU, DIEA, DMF, 0°C→24°C; (vii) 0.04 *N* MeONa/MeOH, MeOH, 24°C.

Table 1 Physicochemical characteristics of MGDS modified by neogalactolipids **1a**.

MGDS	Module composition (molar ratio, %)			Diameter ±S.D., nm	PDI[a]	ζ-potential, mV
	2D3	DOPE	1a			
L0	50	50	-	114.3 ± 21.9	0.313	+ 57.3
L1	48.75	48.75	2.5	95.1 ± 18.3	0.315	+ 50.0
L2	47.5	47.5	5	83.6 ± 23.8	0.282	+ 47.4
L3	45	45	10	43.7 ± 7.2	0.233	+ 45.9

[a] P.I. - polydispersity index

To estimate the accessibility and affinity of galactosyl ligands exposed on MGDS surface toward ASGPr, a ricin agglutination test was performed. *Ricinus communis* lectin (*RCA_{120}*) promotes the aggregation of liposomes that exhibit D-galactose moieties on their surface.[25] The addition of *RCA_{120}* (1 mg/mL) to targeted MGDS **L1**, **L2**, and **L3** resulted in the increasing of dispersion optical density and the precipitation of liposomes (Fig. 4). Conventional liposomes **L0** were unaffected. The agglutination level depended on the molar ratio of neogalactolipid **1a** into liposomes. When solution of free D-galactose (100 mg/mL) was added to a mixture of **L3** and *RCA_{120}*, a dramatic decrease of the optical density of the solution was observed, indicating the binding specificity of galactose-containing MGDS toward *RCA_{120}*.

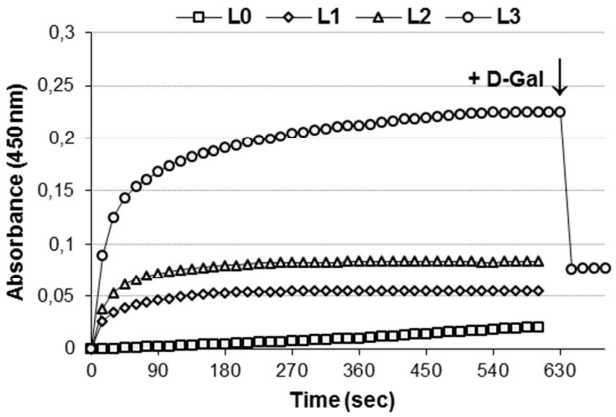

Figure 4. Agglutination of the targeted MGDS containing neogalactolipids **1a** after the addition of *RCA_{120}*: square–**L0**; rhomb–**L1**; triangle–**L2**; circle–**L3** liposomes.

To estimate the cytotoxic effect of MGDS, HEK 293 cells were incubated with the liposomes at concentrations ranging from 5 to 80 μM for 24 h under the serum-free conditions, and MTT test[26] was performed. It was found that all the studied MGDS were not toxic for cells; at concentration 80 μM, the IC_{50} values are not achieved (see Supplementary information).

To estimate the capability of the targeted MGDS to transfer NA into eukaryotic cells, the uptake of a 21-mer 5'-fluorescein isothiocyanate-labeled oligonucleotide (FITC-ODN) mediated by one of the targeted (**L2**) and conventional (**L0**) liposomes was studied (Fig. 5). Transfection was performed using Hep G2 cells, which overexpressed ASGPr, and the efficiency of FITC-ODN accumulation was determined by the percentage of fluorescent cells (Fig. 5A) in the population and fluorescence intensity of cells (Fig. 5B).

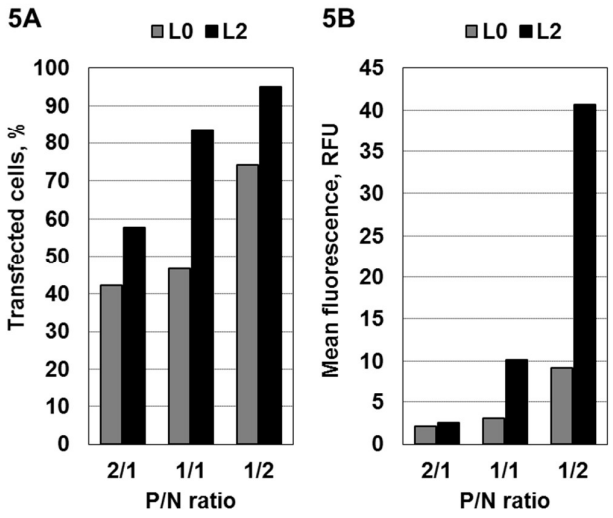

Figure 5. . Accumulation of FITC-ODN (1 μM) in the Hep G2 cells mediated by targeted **L2** (black bars) and conventional **L0** (gray bars) MGDS in the presence of 10% FBS. The percentage of FITC-positive cells (A) and the mean fluorescence intensities of the cells in a population (B) were determined by flow cytometry after 4 h of cell incubation with FITC-ODN/MGDS complexes. Standard deviations were within 8%.

Complexes between MGDS and FITC-ODN were formed at various P/N (phosphate to nitrogen) ratios (2/1, 1/1 and 1/2) and incubated with cells within 4 h. A number of FITC-positive Hep G2 cells as well as fluorescence intensity values were affected by the presence of neogalactolipids **1a** in the MGDS and by the P/N ratio (Fig. 5). Only liposomes **L2** mediate cellular delivery of FITC-ODN at reasonably high levels, which 1.3-1.8 fold exceeds the one observed for conventional liposomes **L0** (Fig. 5A). Moreover, at P/N = 1/2 the observed accumulation of FITC-ODN mediated by targeted MGDS **L2** was significantly higher than that of liposomes **L0**, which lacked addressing component (Fig. 5B). Typical images (see Supplementary information) showed the accumulation of FITC-labelled oligonucleotide in both the cytoplasm and the nucleus of Hep G2 cells after 4 h of incubation with the lipoplexes composed of FITC-ODN and **L2**.

Conclusions

We have elaborated the synthetic route for the preparation of the new branched neogalactolipids based on spermine containing two hydrophobic and two targeting domains for the development of modular gene delivery systems (MGDS). The investigations of physicochemical characteristics of MGDS showed that the addition of neogalactolipids resulted in the stabilization of liposomal formulations and decrease their diameters twice. Ricin agglutination test indicated that the targeted MGDS obtained were bound specifically with RCA_{120}, and the efficiency of this process depended on the molar ratio of neogalactolipids in the liposomal formulations. Low toxicity and efficient delivery of short oligonucleotides into HepG2 cells allow to consider MGDS decorated by new neogalactolipids as promising objects for further *in vitro* and *in vivo* studies.

This research was supported by Russian Foundation for Basic Research (research project No. 13-04-40183 сомfi and 13-04-40181 сомfi).

Notes and references

1 *Non-viral Vectors for Gene Therapy (Part 1)*, ed. L. Huang, M. C. Hung and E. Wagner, Elsevier: Amsterdam, 2005.
2 M. A. Maslov and M. A. Zenkova, in *Non-Viral Gene Therapy*, ed. X. Yuan, InTech: Rijeka, 2011, 349-380.
3 R.L. Juliano, X. Ming and O.Nakagawa, *Bioconjug. Chem.*, 2012 **23**, 147.
4 S. Djurovic , N. Iversen, S. Jeansson, F. Hoover and G. Christensen, *Mol. Biotechnol.*, 2004, 28, 21.
5 L. De Laporte, J. Cruz Rea and L. D. Shea, *Biomaterials*, 2006, **27**, 947.
6 K. Kostarelos and A. D. Miller, *Chem. Soc. Rev.*, 2005, **34**, 970.
7 V. P. Torchilin, *Nat. Rev. Drug Discov.,* 2005, **4**, 145.
8 T. Nakamura, H. Akita, Y. Yamada, H. Hatakeyama and H. Harashima, *Acc. Chem. Res.*, 2012, **45**, 1113.
9 J. Prieto, R. Hernandez-Alcoceba, G. Gonzalez-Aseguinolaza, G. Mazzolini, B. Sangro and G. Kramer, *Expert Opin. Biol. Th.*, 2004, **4**, 1073.
10 K. Kunath, A. von Harpe, D. Fischer and T. Kissel, *J. Control. Release*, 2003, **88**, 159.
11 G. Ashwell and J. Harford, *Annu. Rev. Biochem*, 1982, **51**, 531.
12 M. Spiess, *Biochemistry*, 1990, **29**, 10009.
13 T. K. Lindhorst, in *Carbohydrate-Modifying Biocatalysts*, ed. P. Grunwald, CRC Press: Boca Raton, 2011, 119-182.
14 T. K. Lindhorst, *Top. Curr. Chem.*, 2002, **218**, 201.
15 R. T. Lee, P. Lin and Y. C. Lee, *Biochemistry*, 1984, **23**, 4255.
16 E. M. V. Johansson, E. Kolomiets, F. Rosenau, K. E. Jaeger, T. Darbrea and J. L. Reymond, *New J. Chem.*, 2007, **31**, 1291.
17 I. Vrasidas, S. André, P. Valentini, C. Böck, M. Lensch, H. Kaltner, R. M. J. Liskamp, H. J. Gabius and R. J. Pieters, *Org. Biomol. Chem.*, 2003, **1**, 803.
18 M. Dubber, A. Patel, K. Sadalapure, I. Aumüller, T. K. Lindhorst, *Eur. J. Org. Chem.*, 2006, **16**, 5357.
19 D. Deniaud, K. Julienne and S. G. Gouin, *Org. Biomol. Chem.*, 2011, **9**, 966.
20 E. A. Ivanova, M. A. Maslov, N. G. Morozova, G. A. Serebrennikova and V. V. Chupin, *RSC Advances*, 2012, **2**, 4600.
21 J.-P. Behr, B. Demeneix, J.-P. Loeffler and J. Perez-Mutul, *Proc. Natl. Acad. Sci. USA,* **86**, 6982.

22 F. Lamarche, M. Mével, T. Montier, L. Burel-Deschamps, P. Giamarchi, R. Tripier, P. Delépine, T. Le Gall, D. Cartier, P. Lehn, P.-A. Jaffrès and J.-C. Clément, *Bioconjug. Chem*., 2007, **18**, 1575.
23 M. Mével, N. Kamaly, S. Carmona, M. H. Oliver, M. R. Jorgensen, C. Crowther, F. H. Salazar, P. L. Marion, M. Fujino, Y. Natori, M. Thanou, P. Arbuthnot, J.-J. Yaouanc, P. Alain Jaffrès and A. D. Miller, *J. Control. Release*, 2010, **143**, 222.
24 N. Niyomtham, N. Apiratikul, K. Suksen, P. Opanasopit and B. Yingyongnarongkul, *Bioorg. Med. Chem. Lett.*, 2015, **25**, 496.
25 I. A. Petukhov, M. A. Maslov, N. G. Morozova and G. A. Serebrennikova, *Russ. Chem. Bull.*, 2010, 59, 260.
26 M. A. Maslov, T. O. Kabilova, I. A. Petukhov, N. G. Morozova, G. A Serebrennikova., V. V. Vlassov, M. A. Zenkova, *J. Control. Release*, 2012, 160, 182.
27 V. Laurent, A. Fraix, T. Montier, S. Cammas-Marion, C. Ribault, T. Benvegnu, P.-A. Jaffres and P. Loyer, *Biotech. J.*, 2010, **5**, 314.
28 H. M. Ghonaim, S. Li and I. S. Blagbrough, *Pharm. Res.*, 2009, **26**, 19.
29 M. M. Joullié and K. M. Lassen, *ARKIVOC*, 2010 (viii), 189.
30 P. Neuner and P. Monaci, *Bioconjugate Chem*., 2002, **13**, 676.
31 J. Winkler, E. Urban, D. Losert, V. Wacheck, H. Pehamberger and C. R. Noe, *Nucleic Acids Res.*, 2004, **32**, 710.
32 N. S. Bodor, *US Pat.* 4 264 765, 1981.
33 E. A. Ivanova, Yu. I. Tokareva, M. A. Maslov, N. G. Morozova and V. V. Chupin, *Vestnik MITHT*, 2012, **7**, 54.
34 O. V. Markov, N. L. Mironova, M. A. Maslov, I. A. Petukhov, N. G. Morozova, V. V. Vlassov and M. A. Zenkova, *J. Control. Release*, 2012, **160**, 200.
35 S. R. S. Ting, G. Chen and M. H. Stenzel, *Polym. Chem*., 2010, **1**, 1392.
36 J. Carmichael, W. G. De Graff, A. F. Gazdar, J. D. Minna and J. B. Mitchell, *Cancer Res.*, 1987, **47**, 936.