A model of targeted stealth nanoparticles that are stable and biocompatible for RNA interference *in vivo* to macrophages

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ABSTRACT

Liposomes have been shown to be useful non-viral vectors for siRNA delivery to cells. However, ensuring maximum delivery in vivo is limited by biocompatibility and delivery difficulties.

We propose a model, using the ABCD construct, of a stealth nanoparticle based on a liposome which incorporates stability, biocompatibility, and high encapsulation efficiencies, using a novel lipid to target mannose receptors. The particles are to be tested against THP-1 cell lines to confirm knockdown of the GAPDH expression. If shown to have high transfection ability, such particles could be used to target mannose-receptor-presenting cells such as macrophages, and be useful in reducing their activity in autoimmune diseases such as rheumatoid arthritis.

1 INTRODUCTION

Knockdown inhibition of genes associated with cancer using RNA interference is currently a topical area of research [1]. Effectiveness of small interfering RNA (siRNA) in targeting cancer cells *in vivo* has so far been hindered by the difficulty in getting the RNA molecules to the malignant cells.

1.1 Liposomes

Liposomes are easily generated vesicular nanoparticles, and recognised as one of the most powerful mechanisms in bottom-up engineering[2]. They are small spheroidal lipid-based bodies, usually generated from phospholipids, forming a bilayer around central vesicle in the presence of water.

Liposomes have already been shown to be ideal for the delivery of drugs, such as doxorubicin liposomal[3] as a chemotherapeutic agent for various cancers, with promising results for other dermatological[1][4][5] and respiratory conditions[6].

Liposomes have been suggested as ideal vectors for gene transfer[7], however, modifications have been made to the naive liposome to improve its delivery to the cell.

1.2 ABCD nanoparticle

Kostarelos *et al.* have previously suggested a structural paradigm for the generation of self-assembly nanoparticles based on liposomes as non-viral vector agents[8]. This ABCD *nanoparticle* consists of a central core (A), containing the nucleic acids to be delivered. The core is surrounded by a lipid envelope layer (B), in turn surrounded by a stealth/biocompatibility layer (C) and a bio-recognition layer (D) – figure 1.

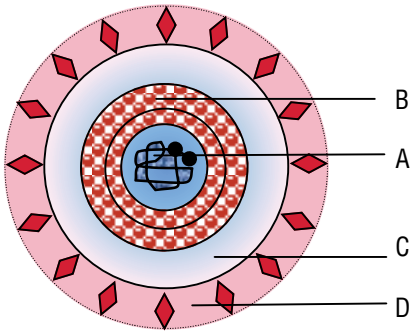


Figure 1: Structural representation of an ABCD nanoparticle, constituting

A – core containing nucleic acid (e.g. siRNA)
B – lipid envelope bilayer
C – biocompatibility layer (e.g. formed by PEG)
D – biological recognition layer (containing targeting moieties such as mannose)

We have suggested a novel biocompatible nanoparticle that could be used in the targeted delivery of siRNA to macrophages, in order to achieve knockdown of gene expression. This method could be used in formulating new disease-modifying agents in autoimmune diseases such as rheumatoid arthritis.

1.3 Biocompatibility

Lipoplexes consisting of A & B layers are well-established as potential gene delivery agents *in vitro*, with the encapsulation of the nucleic acid core improving considerably when cationic lipids are used[9]. However,

addition of a polyethylene glycol (PEG) layer to the surface – thus generating an ABC nanoparticle – has been shown to improve bioavailability. This has already proved to be clinically effective as a drug delivery technology[3]. More recent work has also shown it to be a useful method for siRNA delivery *in vivo*, as an anti-hepatitic agent comparable to lamivudine in reducing viral load in hepatitis B infection[10].

1.4 Targeting

Modification of this ABC type nanoparticle by the addition of a targeting molecule would improve the specificity of cell transfection. Antibodies have been suggested as targeting molecules[11], however targets such as folate receptors have also shown promise[12].

More recently, the targeting of the mannose receptor on phagocytes has already had some success in animal models. These receptors have been shown to be a potentially useful targets for drug delivery (e.g. ciprofloxacin) to alveolar macrophages[13], and provide a ligand for lipoplexes within the liver[14].

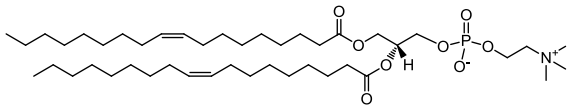
This is also a potential route for macrophage transfection, and therefore our aim is the generation of mannosylated PEGylated lipoplexes as a method for transfection of macrophage-based cells.

2 SYNTHESIS

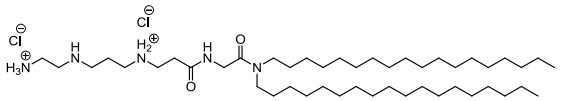
We aim to generate liposomes using a mix of cationic and neutral lipids – figure 2. The use of the cationic lipid N′,N′-dioctadecyl-N-4,8-diaza-10-amino decanoylglycine amide (DODAG) with a neutral lipid such as 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) or 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) has shown encouraging encapsulation of nucleic acid ability, and transfection ability to hepatic cells, with minimal *in vitro* cytotoxicity effects[15].

To generate the stealth layer, the lipid 1,2-Diostearpyl-*sn*-glycero-3-phosphoethanolamine (DSPE) modified with a PEG molecule is used. This PEGylated DSPE (PEG-DSPE) would provide the biocompatibility (C) layer within the nanoparticle.

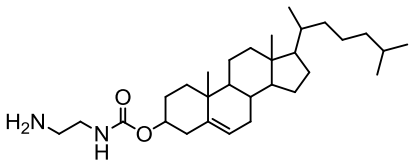
Work on characterisation of cationic liposomes (currently awaiting publication) has shown a mix of DODAG, DOPC and cholesterol to provide small, stable liposomes suitable for encapsulation and modification to generate a viable ABC nanoparticle.



DOPC: 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine



DODAG: N′,N′-dioctadecyl-N-4,8-diaza-10-amino decanoylglycine amide



Chol: Cholesterol

Figure 2: Chemical structure of lipids used in the generation of a lipoplex

2.1 Mannosylated lipid

Generating an ABCD nanoparticle involves the synthesis of the targeting lipid by mannosylation of a cholesterol molecule. This was achieved using a bridge molecule, aminoxyacetic acid, in a three step process – figure 3. The amine group of the bridge molecule was protected using a *tert*-butyloxycarbonyl (Boc) group, ensuring the acetic acid moiety reacted first (as described below).

Step 1 - This is the reaction of the amine group on the cholesterol molecule to the acetic acid group on the bridge molecule, and purified.

Step 2 - The amine group on the bridging molecule was then deprotected by removal of the Boc group.

Step 3 – the now-free amine group on the bridging molecule was then coupled to the C_1 atom on the mannose molecule, and the product purified.

This structure was confirmed using nuclear magnetic resonance and high pressure liquid chromatography.

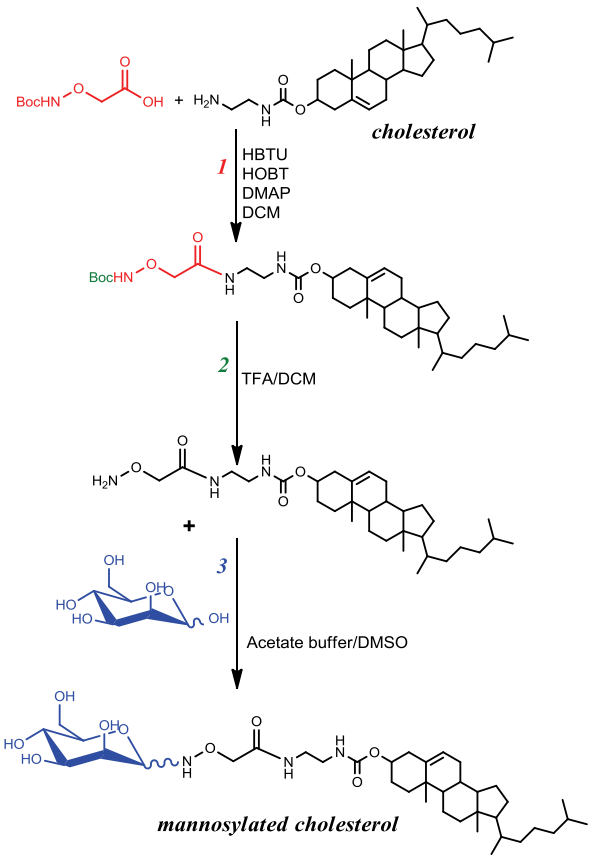


Figure 3: three step reaction used to generate a mannosylated cholesterol using a bridging molecule

The mannosylated cholesterol is subsequently used alongside the DODAG, DOPC, cholesterol and PEGylated DSPE for the generation of a BCD-layered liposome, and then an ABCD-lipoplex nanoparticle.

2.2 Liposomes

The proportions of each lipid to generate the liposome were derived from previous work in our lab on siRNA lipoplexes. Several formulations were devised, and were tested establish maximum efficacy of encapsulation, stability and minimal size.

DODAG of 20% molar ratio or 50%molar ratio was used with 30% molar ratio cholesterol (Chol). PEGylated DSPE (PEG-DSPE) would constitute either 5% or 1% (along with a third control liposome without PEGylated DSPE). DOPC constituted the remainder of the liposome.

Liposomes were generated by mixing lipids at required concentrations into 1ml chloroform, evaporating *in vacuo* to a film in a pear flask, and reconstituted in 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffer (pH 7.0), generating multilamellar liposomes. Sonication of the liposome suspension was then undertaken for 2-3 hours at 40°C with the aim of reducing size to below 100nm.

3 CHARACTERISATION

Liposomes were analysed by Photon Correlation Spectroscopy (PCS). Size distribution and polydispersity index were measured by light scattering at a 90° angle and at 20°C. Results are shown in table 1. The ζ-potential (zeta-potential) of the particles is also to be characterised.

Molar ratio[a]	Mean size (nm)	PDI[b]
20/50/30/0	84.5	0.563
20/49/30/1	103.3	0.379
20/45/30/5	127.3	0.197
50/20/30/0	76.8	0.457
50/19/30/1	87.5	0.403
50/15/30/5	80.9	0.441

[a] Molar ratio is the ratio of lipids used in generation of liposome in order DODAG/DOPC/Chol/PEG-DSPE respectively

[b]Polydispersity index

Table 1: Results of photon correlation spectroscopy measurements for BC liposomes of various compositions

Amongst the liposome compositions generated, we then aimed to identify that which gave maximum encapsulation efficiency– figure 4. This was done by mixing liposomes with siRNA at various charge ratios; *i.e.* 4 parts of liposomes to either 1, 2, 4 or 8 parts of siRNA (or no siRNA as control). Encapsulation was assessed by adding the dye propidium iodide, and using fluorimetry to monitor the relative fluorescence. Given that propidium iodide fluoresces in presence of an oligonucleotide, any unencapsulated siRNA will show increased fluorescence, therefore indicating poorer encapsulation by the liposome in question.

Once the best liposome compositions are identified with respect to liposome size, polydispersity index, ζ-potential and encapsulation efficiency, more analogous liposomes will be generated. This time, however, liposomes will comprise 1% of mannosylated cholesterol and 29% of naive cholesterol (instead of the 30% used previously). Liposomes with 5% mannosylated cholesterol and 25% naive cholesterol will also be generated.

These liposomes will again be characterised with respect to size, polydispersity index, zeta-potential, and encapsulation efficiency.

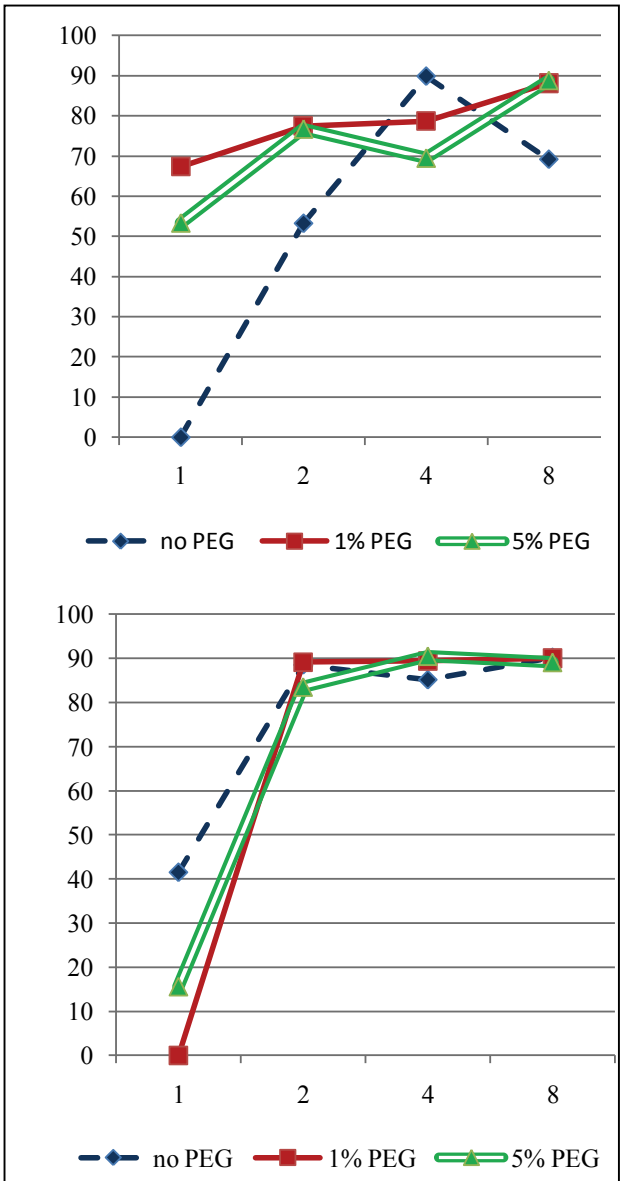


Figure 4 : Graphs showing lipoplex siRNA charge ratio (x-axis) against relative percentage encapsulation of siRNA (y-axis) with 20% DODAG liposomes (top) and 50% DODAG (bottom)

Following this we aim to target these ABCD lipoplexes against THP-1 cell lines, using siRNA to knock down the expression for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a multifunction enzyme involved in glycolysis.

4 CONCLUSION

We present here a model for a stable, biocompatible stealth nanoparticle based on a liposome, with moieties targeting the mannose receptor found on macrophages. The nanoparticles exhibit a good size distribution, and high encapsulation efficacies. Once a satisfactory knockdown is achieved in cell studies, studies could be undertaken to identify the ability of these targeted lipoplexes to achieve RNA inhibition *in vivo*.

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