Organising Peptide Ligands on the Surface of Gene Vectors

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Abstract

Through the considerate organisation of surface ligands, we have developed a lipid-based vector for the targeted delivery to cancer cells. Our ligand of interest is a short peptide with high affinity for the Urokinase Plasminogen Activator receptor (uPAR), a receptor overexpressed on the surface of many tumours. We modified the surface of high-charge and low-charge liposomal vectors with uPAR-specific peptides, by either inserting peptide-lipid conjugates onto the surface, or by chemical modification of preformed nanoparticles. We conclude that the nature of the liposomal platform (stability, zetapotential) with the presentation of surface ligands, contribute to the maximisation of a vector's targeting effect.

Keywords: liposome, peptide, cancer, targeting, receptor-mediated endocytosis, nanoparticle assembly.

1 Introduction

For successful drug delivery into solid tumours, therapeutic nanoparticles must be designed to exhibit certain characteristic to aid their accumulation at the specified tissues. In the case of cancer targeting, qualities such as the size and surface chemistry are all factors to be considered. The diameters of the nanoparticles must also be under 150nm when reaching the diseased tissue in order to fit through the fenestration in the tumour epithelial cells. [1] The surface chemistry of the nanoparticles can be adapted to prevent their aggregation in blood. To resist aggregation, a therapeutic nanoparticle must be neutrally charged, to prevent electrostatic interaction with negatively charged serum proteins, and must be protected by a stealth layer, such as a Polyethylene glycol (PEG) polymer. To improve site-specific delivery, ligands with specific affinity can be used to decorate the nanoparticles' surface, through either covalent or non-covalent attachment.

The urokinase plasminogen activator receptor (uPAR) is over-expressed on a variety of cancer cells such as those of the prostate and the breast. [2, 3] Urokinase plasminogen activator (uPA) interacts with its cognate receptor, uPAR, to form an uPAR-uPA conjugate that enters cells by clathrin-coated, receptor-mediated endocytosis. [4] The work of Appella *et al* described the binding sequence within the uPA which docks into the binding region of the uPAR. [5] Derived from this sequence is a 11mer peptide with which previous studies in our group have shown to have high binding affinity for the uPAR. Here, we intend to organise this 11mer peptide sequence onto the surface of our nanoparticles, for site-specific gene delivery to cancer cells.

Through the considerate organisation of surface ligands, we have developed a lipid-based vector based on the ABCD paradigm [6]. In this study, we investigated and optimised nanoparticle parameters such as charge and ligand organisation for improved receptor bio-recognition and targeting.

2 Materials and Methods

All cell lines were obtained from ATCC. Lipids were purchased from Avanti Polar Lipids. All amino acids and peptide synthesis reagents were from Novabiochem. Cell culture reagents were purchased from Invitrogen.

2.1 DNA nanoparticle preparation

The liposomal platform consisted of lipids at different molar ratios. These include cationic lipids CDAN and DODAG (Imuthes), fusogenic (DOPE), PEGylated plus the targeting lipids. Solutions of the lipids in chloroform were added to a glass round-bottomed flask and dried *in vacuo* to form a lipid film. The film was then hydrated with H_2O and sonicated at 40°C for 30mins. pDNA solution in H_2O (encoding luciferase gene) was then added to the vector solution under heavy vortex at a ratio of 1:12 (lipid:pDNA, w/w).

2.1.1 Post-modification of nanoparticle vectors

On assembly of DNA-encapsulated nanoparticles, a solution of 11mer peptide-lipid amphiphiles in H_2O was added and incubated at 37% for 1 hr. Unincorporated amphiphiles were then removed by ultracentrifugation (MWCO=30k).

2.1.2 Post-conjugation of 11mer peptide onto surface of nanoparticles

The second method of ligand incorporation was that by *post-conjugation.* In this case, the PEGylated lipid in the formulation was changed to one that was functionalised by a maleimide group at the distal eng of its PEG chain. After vector assembly and before pDNA encapsulation, the vector solution was stirred with a solution of a Cysteine-functionalised 11mer peptide (u11). pDNA was then encapsulated followed by removal of unconjugated Cys-u11 peptide by centrifugal purification.

NSTI-Nanotech 2008, www.nsti.org, ISBN 978-1-4200-8504-4 Vol. 2

2.2 Nanoparticle cell uptake and transfection

All cell lines were maintained in DMEM (plus 1% penicillin and streptomycin and 10% Fetal Calf Serum). Luciferase transfections were carried out on cells in Optimem for 4hrs before removal of transfection media and replacement with growth media. Cells were then grown for 48 hrs before lysis and analysed for relative light units and total protein content. For fluorescence uptake studies, nanoparticles were labelled with 1 molar % of DOPE-Rhomadmine, and applied to cells in the manner of transfection. After 60mins incubation, the cells were lysed and analysed for cell-associated fluorescence by fluorescence-activated correlation spectroscopy (FACS).

2.3 Photocorrelation spectroscopy (PCS) and zetapotentials

Nanoparticle diameters and zetapotentials were measured on a Malvern ZS zetasizer at room temperature. All nanoparticles were prepared at 0.5mgml^{-1} (of total lipid mass) in H_2O.

3 Results and Discussion

High Charge Vectors

The first generation nanoparticles were prepared using a combination of a high molar percentage of cationic lipids (50%), fusogenic lipids (48.5%), plus 0.5% of DSPE-PEG^{2000} lipids. The initial aim was to verify the amount of PEGylated lipid required for the exhibition of the surface peptide's targeting effect. An amount of PEGylated lipid is required to increase colloidal stability in serum and buffered solutions, but high molar percentages could lead to steric shielding of the surface peptides. The targeting peptide was incorporated through self-insertion (as a peptide-lipid amphiphile) into the hydrophobic layers of the particle. The molar percentage of the peptide-lipid amphiphile must also be adapted as over-loading can lead to inter-colloidal aggregation caused by the hydrophobic interactions between the surface peptides. Our data shows that the optimum amount of PEGylated lipids was at a 0.5% of total lipid molarity, enough to prevent aggregation in serum and in high salt solutions, but not too much to inhibit the exhibition of the surface ligands. Fig 3 shows that incorporating PEG lipids over 0.5 molar % is detrimental on the display of the targeting effect of the ligand. It was also found that a maximum loading of 1 molar % of the peptide-lipid amphiphile could allow the display of the ligand's targeting effect but any more would induce the vector's aggregation.

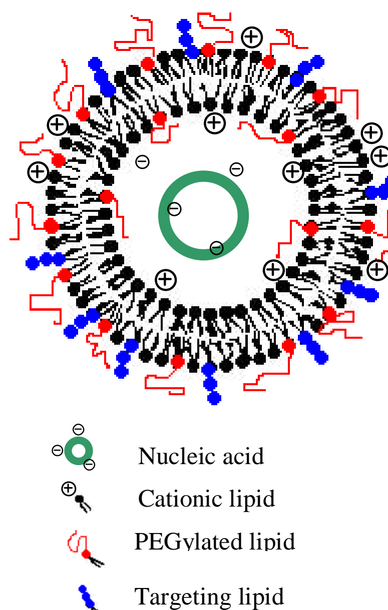

Fig 1- Representation of the targeted delivery system for gene delivery, as surface-modified by the u11 peptide-lipid amphiphile. The liposomal compartment consists of cationic, fusogenic and PEGylated lipids, which directly encapsulates the nucleic acids within the core. The surface is decorated with targeting ligands (blue).

Initial screenings of the secondary structure of the surface peptides were carried out using circular dichroism, where data suggests their secondary structure to be of a β-hairpin configuration. This is advantageous as the β-turn structure is similar to that of the epitope of the natural ligand, indicating optimum configuration for receptor-binding.

Transfections on the uPAR-overexpressing prostate cancer cell line, DU145, showed an increase in transgene expression by delivery of the optimised targeted vector compared to its non-targeted counterparts (Table 1). It is likely that the increased uptake of targeted vectors is due to receptor-mediated endocytosis as the diameter of the particles are <150nm, hence small enough to enter the cell via such an uptake mechanism (Fig 2). On the other hand, transfections on HEK293 cells (uPAR negative) showed lower levels of transfection by targeted delivery, due to non-specificity of the targeting peptide for surface membrane receptors. Fluorescence microscopy also indicated a time-dependent and increased uptake of rhodamine-labelled targeted vectors. To improve the delivery efficiencies of the targeted vectors, we developed nanoparticles with lower overall surface charge within the liposomal platform. Low-charged nanoparticles can be used to reduce non-specific cell membrane-vector interactions.

Vector surface charge	Molar % of cationic lipid	Method of ligand incorporation	% RLU of non-targeted control
High	50	post-insertion of peptide-lipid amphiphile	400
Low	20	post-insertion of peptide-lipid amphiphile	2,000
Low	20	post-coupling of peptide onto distal end of functionalised PEG lipids.	3,000

Table 1- Summary of luciferase transfection on DU145 cells by targeted vectors (with 1 molar % of ligand) of various preparations. The high charged vectors showed the lowest increase in transfection, 4-fold in comparison to its non-targeted counterparts, where as the low charged vectors gave up to a 20-fold increase. The transfection level of the targeted low charge vectors were increased further to 30-fold when the targeting ligand is presented at an extended length from the vector surface.

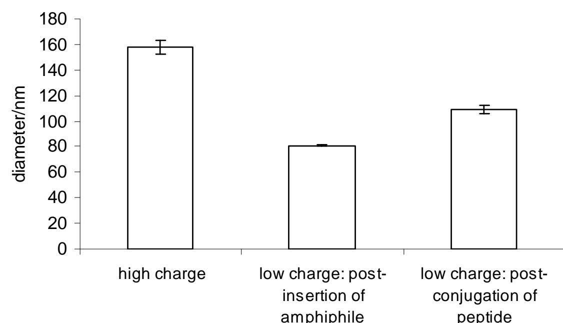

Figure 2- Diameter sizes of targeted nanoparticles in H_2O, as measured by PCS. All particles have diameters under 150nm, important for *in vivo* transportation plus receptor-mediated endocytosis.

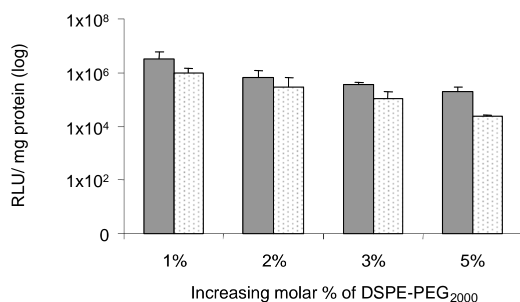

Fig 3- Transfection data of targeted high charge vectors (with 1 molar % of targeting peptide-lipid amphiphile) on DU145 cells. Increasing percentages of PEG lipids in the liposomal platform prohibits the exhibition of the targeting effect of the targeting peptide, as shown by the lower levels of transfection by the targeted vectors (dotted) compared to that by the non-targeted vectors (grey). A lower percentage of PEG lipids was therefore needed, where an optimal percentage was found to be at 0.5%.

Low Charge Vectors

Due to the presence of several negatively charged membrane proteins such as the proteoglycans, the cell membrane surface can be highly anionic. Electrostatic interaction with positively charged nanoparticles is the root of non-specific interactions. Non-specific uptake of the vectors by the cells can therefore be minimised on the reduction of the vector's zetapotential. Here, decreasing the amount of cationic lipid in the formulation lowered the overall charge of the vectors. In the high charge formulation, the cationic lipids were incorporated at a 50% molar ratio. Here, we reduced the amount of such cytofectins to 20%, whilst maintaining the lipid to pDNA ratio. The zetapotential of such vectors was reduced to 30% of that of the high charge vector, therefore decreasing the amount of non-specific uptake due high vector charge. Another advantage of low charged vectors is the reduction in nanoparticle size. Whilst the diameter of the particles of the high charge formulation was measured to be approximately 150nm, the low charge nanoparticles showed an average diameter of 81nm (Fig 2). Accompanying the decrease in vector charge is an even further increase in transfection by the targeted vectors, up to 20 times higher than non-targeted vectors. To prove that the increase in transfection is a result of increased uptake, FACS studies were performed on the same cell line, using rhodamine-labelled vectors. The data clearly indicates that the uptake of vectors is higher in the case of the targeted nanoparticles (Fig 4). To maximize the display of the targeting ligand, we continued by conjugating the peptide onto the distal ends of the extended PEG lipids.

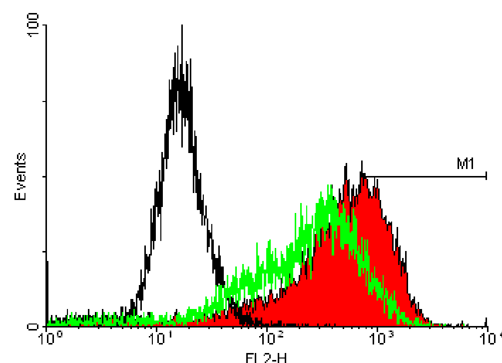

Fig 4- FACS data of the uptake of low charged vectors, decorated with (red) or without (green) 1 molar % of peptide-lipid amphiphile at 60mins post-transfection. On DU145 cells, the targeted vectors show greater cell-associated fluorescence, indicating increased uptake of vectors due to receptor-selectivity. Black= non-treated cells.

Distal Peptide Conjugation

One of the major drawbacks of the u11 peptide-lipid amphiphile is the limited extension of the ligand on the vector surface. To improve receptor recognition, the presentation of the ligand should extend further from the vector surface, hence allowing increased docking into the target receptor's binding site (Fig 5). In our second method

of targeted-vector preparation, we covalently conjugated the u11 peptide onto the distal end of functionalised PEGylated lipids, which are extended into the space surrounding the vector. In this method known as *post-coupling,* the distal ends of the PEGylated lipids were functionalised with a maleimide group. The maleimide functional group reacts readily with thiols, which required the modification of the u11peptide with an extra Cysteine amino acid before conjugation.

Again, transfection levels by targeted nanoparticles were higher, 30 times greater than that of the non-targeted particles, indicating the superior targeting efficiency of a low-charged nanoparticle with extended ligand presentation.

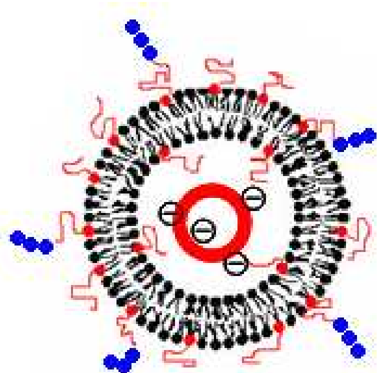

Fig 5- Representation of the targeted delivery system for gene delivery, as surface-modified by post-conjugation of Cys-peptide (blue) onto the maleimide-functionalised PEG-lipids (red). The liposomal compartment consists of cationic (20 molar %), fusogenic and maleimide PEGylated lipids (1 molar%), which directly encapsulates the nucleic acids within the core.

4 Conclusions

The results from our study shed light onto the factors contributing to the maximisation of a nanoparticle delivery vehicle's targeting effect. We conclude that a vector's stability, plus its surface zetapotential, along with the surface ligands' secondary structure and presentation, needs to be considered when developing a site-specific gene vector.

5 References

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Acknowledgements

We thank the EPSRC and the Royal Society for providing financial support for the project.