Novel Cleavable PEGylated Nanoparticles for Enzyme Triggerable Release of Nucleic Acid in Tumours

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Abstract

A PEGylated peptide lipid sensitive to enzymatic cleavage has been designed for triggerable release of therapeutic nucleic acid in solid tumours. The peptide linker is a short peptide sequence, Ala-Ala-Pro-Val, which is highly sensitive to elastase, the proteolytic enzymes present in tumours. High and low charged nanoparticles were modified by addition of the synthetic PEG-AAPV-lipid. *In vitro* transfection study showed that PEG-AAPV-lipid significantly improved transfection efficiency. In the absence of peptide the enzymatic cleavage does not occur and lower transfection efficiency was observed. Fluorescence microscopy confirmed the transfection result. It was demonstrated that PEG-AAPV-lipid can be triggered under enzyme environment, leading to dePEGylation and increase in transfection efficiency.

Keywords: liposomes, elastase, enzyme, triggering, fusion

1. Introduction

A successful lipid-based delivery system requires a stealth surface modification of the lipid bi-layer, normally made from polyethylene glycol; PEG. The advantages of the stealth layer are providing stability to the nanoparticles and preventing their aggregation *in vivo* [1]. However, PEG layer appears to be problematic at a target site as it blocks intracellular uptake of the nanoparticles, and results in limited cell uptake and release of encapsulated agent [2]. The subsequent dePEGylation once the nanoparticles reach the target site could be a strategy to improve endocytosis and release of therapeutic agent into the cell.

The dePEGylation approach in this study relies on enzymatic activity associated with tumour cells. High levels of proteolytic enzyme, elastase, have been found to promote tumour invasion and metastasis by degrading basement membrane and extracellular matrix barrier [3-5]. The enhanced elastase activity can be employed to design and develop an ABC lipid-based vector [6] modified with an enzyme cleavable PEG-AAPV-lipid. In our lab we use the ABC nanoparticle paradigm in which the letters code for the therapeutic and/or the functional layer. For example, in the current work, A is the plasmid DNA, B is the lipid bilayer, and C is the stealth layer. In this study we investigate if dePEGylation of the ABC nanoparticle can be activated by the tumour proteases and hence enhance liposomal fusion with the cell membrane, leading to efficient delivery of therapeutic agent to tumours.

2. Materials and Methods

All amino acids and peptide synthesis reagents were purchased from Novabiochem. PEG polymer bearing activated ester group was purchased from RapPolymere. Lipids, Human Leukocyte Elastase, and other chemical used in the synthesis were purchased from Sigma. All cell lines were from ATCC. Cell culture medium and other cell culture reagent were purchased from Invitrogen.

2.1 Synthesis of PEGylated peptide lipid

The peptide linker, AAPV, was synthesized using solid phase peptide synthesis (SPPS). HBTU and HOBt were used as coupling agents. After cleavage from the resin (using TFA solution), Fmoc-AAPV-OH was purified by recrystallization in ether. The peptide was then reacted with lipid in the presence of DMAP, HBTU under N_2 overnight. The reaction was quenched with diluted citric acid and extracted with DCM. The product was purified by flash column chromatography and was then deprotected in 20% piperidine, dry DCM for 4 h. AAPV-lipid was finally coupled to PEG polymer in DIPEA and dry DCM, affording PEGylated peptide lipid, after purification, in good yield (68%). PEG-lipid was synthesized following the procedure described above. After purification, the compound was obtained in excellent yield (98%). PEG-lipid, with the absence of the peptide linker, was used as a control for *in vitro* study. The compounds were characterized using NMR, mass spectroscopy and HPLC.

2.2 Photocorrelation spectroscopy (PCS) and ζ potential

Diameter, polydispersity index, and zetapotential of the nanoparticles were determined by Malvern ZS Zetasizer at 25 ^{o}C. The nanoparticles were prepared at 0.5 mg/ml lipid concentration in 4 mM HEPES buffer, pH 7.

2.3 Preparation of the ABC nanoparticles

Liposomal nanoparticles were prepared by dehydration-rehydration method. $CHCl_3$ solution of the lipids of interest were added to a round bottom flask and evaporated to obtain a lipid film. The lipid film was then rehydrated with H_2O and sonicated at 40 ^{o}C for 40 min. pDNA encoding luciferase gene was added to the liposomes solution at 12:1 (lipid: DNA w/w) and the mixture was vortexed.

2.4 *In vitro* transfection

All cell lines were grown in DMEM, supplemented with 10% Fetal Bovine Serum and 1% penicillin and streptomycin. 10 µl of elastase (0.5 Unit/ml) was added to the nanoparticles. The mixture was then incubated at room temperature for 10 min before transfection. Luciferase transfection was performed on OVCAR cell lines in the presence of 10% serum at 37 ^{o}C for 24 h before removal of transfection media and replacement with complete growth media. The cells were incubated for further 24 h before lysis and analysed for the luciferase activities (RLU/mg protein). The transfection experiment conducting with MCF-7 cell lines were performed using different serum concentration (1%, 5%, 10% and 20%) at 37 ^{o}C for 6 h before removal of transfection media and replacement with growth media.

2.5 Fluorescence microscopy

The nanoparticles were labeled with 1% DOPE-Rhodamine before an addition of pDNA. The fluorescence-labeled nanoparticles were added to the cells in the same manner as the transfection. After 6 h incubation, the cells were analysed for cell uptake by fluorescence microscope.

3. Result and Discussion

3.1 Synthesis of PEG-AAPV- lipid

PEG-AAPV-lipid was synthesized using amide coupling reaction. Fmoc-AAPV-OH obtained from SPPS was coupled to N- terminus of the lipid overnight, yielding Fmoc-AAPV-lipid. Fmoc deprotection was then performed in 20% piperidine for 4 h. After that the compound was conjugated with the PEG polymer in the presence of base, DIPEA. Analytical High Pressured Liquid Chromatography (HPLC) was used to monitor the coupling reaction. PEG-AAPV-lipid was afforded, after purification by flash column chromatography, in good yield. PEG-AAPV-lipid was then employed for nanoparticles formulation (Figure 1).

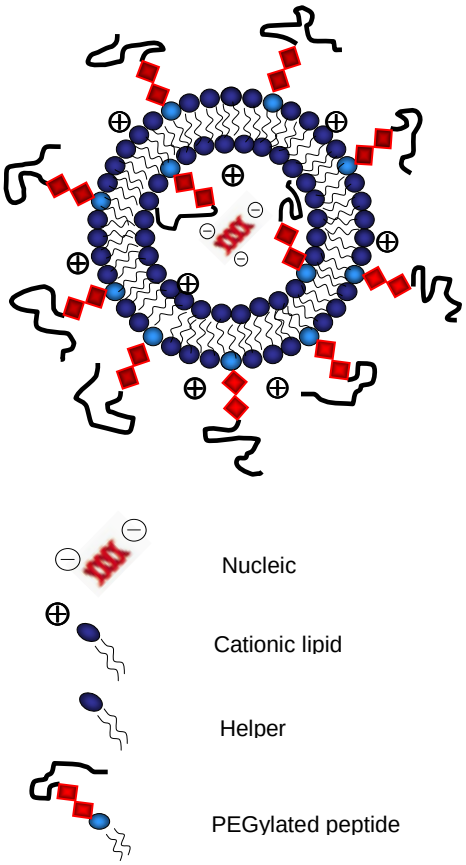

Figure 1: Representation of cleavable PEGylated nanoparticles. The liposomal nanoparticles are composed of cationic lipid, helper lipids and PEGylated peptide lipid, which encapsulate nucleic acid inside.

3.2 High Charged ABC nanoparticles

To evaluate the potential of PEG-AAPV-lipid, the compound was added to the nanoparticles, as cleavable PEG. The formulation was composed of high molar percentage of cationic lipid (50%), fusogenic lipid, DOPE (49% and 45%) and PEG-AAPV-lipid (1% and 5%). The transfection was performed on OVCAR cell lines in 10% serum. The nanoparticles containing 1% PEG-AAPV-lipid in the presence of elastase showed an increase of 23% in luciferase protein expression, compared to the nanoparticles in the absence of enzyme. In the presence of elastase, nanoparticles with PEG-lipid (not cleavable) showed a 56% decrease in transfection level, compared to the nanoparticles with the cleavable PEG, PEG-AAPV-lipid. The result suggested that PEG-AAPV-lipid can be a cleavable PEG in the presence of the proteolytic enzyme. Raising mol% of PEG-AAPV-lipid to

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5 % decreased the transfection efficiency of the nanoparticles. High percentage of PEG can block the cell uptake due to its steric hindrance and make it difficult for the enzyme to approach the linker.

3.3 Low Charged ABC nanoparticles

Low charged nanoparticles can reduce electrostatic interaction between the nanoparticles and cell membranes, which is the root of non-specific uptake. 50 mol % of cationic lipid was reduced to 20 mol %. Fusogenic lipids, DOPC and Chol, and 1% and 4% PEG-AAPV-lipid were used in this formulation. Transfection study was performed on MCF-7 cell line. As the concentration of the serum was increased (1% to 20%), the transfection level of 4% PEG-AAPV-lipid nanoparticles, in the presence of the elastase, was increased. The transfection efficiency was significantly higher than that of the nanoparticles in the absence of the elastase and the nanoparticles containing PEG-lipid.

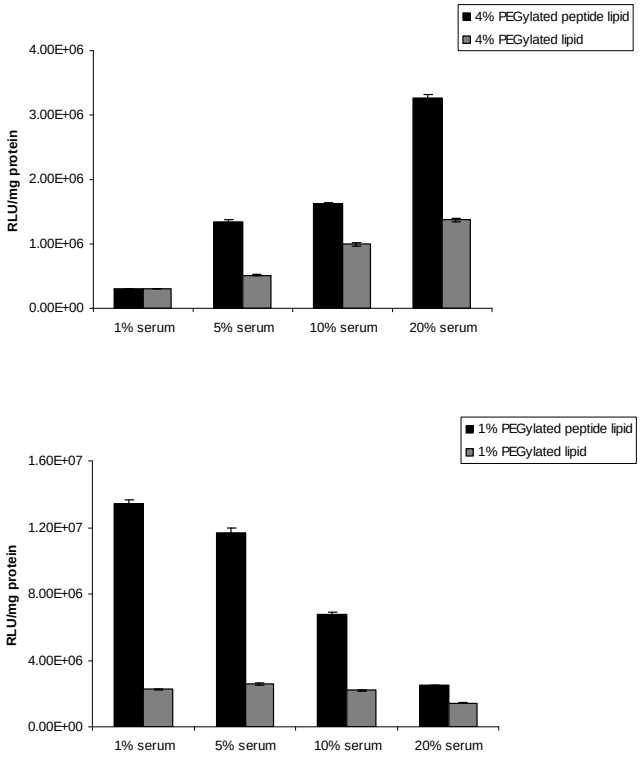

Figure 2: Transfection data of low charged formulation on MCF-7 cell line in different serum concentration. In comparison to PEG-lipid in the presence of the enzyme, nanoparticles containing specific peptide sequence remarkably enhanced transfection efficiency. In higher serum concentration 4% PEG-AAPV-lipid showed an increase in transfection level where as 1% PEG-AAPV-lipid lower the level of transfection.

However, 1% PEG-AAPV-lipid does not seem to be stable in high serum concentration as can be seen by the reduction in transfection level (Figure 2). The result was supported by stability data of ABC nanoparticles in 50% and 80% serum that only 4% PEG-AAPV-lipid was found to be stable at 37 ^{o}C over 4 h, in contrast to 1% PEG-AAPV-lipid. The transfection study in OPTIMEM, serum-free media, did not show improvement in transfection efficiency. It was more likely that enzyme activity was enhanced by high salt concentration in serum. The fluorescence microscopy results were in agreement with the transfection data. nanoparticles with PEG-AAPV-lipid showed high level of cell uptake. This suggests that the peptide cleavage resulting in dePEGylation has been catalyzed by elastase, and this facilitate the entrance of the nanoparticles in the cell.

4. Conclusion

Herein it was demonstrated the potential of enzymatic triggerable system for nucleic acid delivery. It is important that PEG stealth layer is removed once the nanoparticles are at the target site in order to enhance endocytosis. High level of transfection can be achieved by an effective design of the peptide linker. The future work will focus on *in vivo* investigation of the enzyme triggerable release of ABC nanoparticles.

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