Pharmacophore

ISSN-2229-5402

Journal home page: http://www.pharmacophorejournal.com

DEVELOPMENT AND CHARACTERIZATION OF DICER SUBSTRATE SIRNA-LOADED CHOLIC ACID-POLYETHYLENIMINE MICELLES FOR GENE SILENCING IN THE TREATMENT OF CANCER

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ARTICLE INFO

Received:
22th Mar 2018
Received in revised form:
15th Aug 2018
Accepted:
18th Aug 2018
Available online:
28th Aug 2018

Keywords: *DsiRNA, Cholic Acid, Polyethylenimine, Micelles*

ABSTRACT

Newly, Dicer-substrate small interfering RNA (DsiRNA) has gathered interest due to its better efficacy in comparison to small interfering RNA (siRNA). Yet, DsiRNA's use has been constrained due to its quick degradation. The micelles of cholic acid-polyethylenimine (CA-PEI) copolymer, as carriers of DsiRNA were developed in this study. CA is an amphiphilic steroid molecule made naturally from cholesterol, and has the potential to arrange itself into micelles. PEI is a cationic molecule mostly used to deliver genes because of its potential to condense nucleic acid, nuclear localization capability, endosome escaping ability and effective transfection ability. The mean particle size and zeta potential of DsiRNA-loaded CA-PEI micelles were studied and were discovered to be ranging from 125-150 nm, with zeta potential in the range of +4-+12 mV. The morphology of DsiRNA-loaded CA-PEI micelles was investigated under the transmission electron microscopy, and the micelles were found to be spherical but aggregated. The DsiRNA release from CA-PEI micelles was also studied using the dialysis membrane bags, and the results showed an initial burst release followed by a sustained release. The DsiRNA-loaded CA-PEI micelles had a high loading efficiency, and DsiRNA was strongly bound. The DsiRNA-loaded CA-PEI micelles were found to be nontoxic to the normal (V79) and cancer (DLD-1) cells. The study showed the potential of CA-PEI micelles as the promising carriers of DsiRNA.

To Cite This Article: Muhammad Wahab Bin Amjad, Maria Abdul Ghafoor Raja, Khidir A.M. Hassan, (2018), "Development and Characterization of Dicer Substrate siRNA-Loaded Cholic Acid-Polyethylenimine Micelles for Gene Silencing in The Treatment of Cancer", ***Pharmacophore, 9(4),*** *36-40.*

Introduction

Carriers used for the drug delivery have made tremendous assistances to the progress of therapeutic effects by improving the efficiency of already recognized as well as new drugs [1–4]. An important breakthrough in nano-medicine was the novel carriers development that have been capable to deliver drugs in the substantial amounts to sites of action [5, 6]. The majority of the research in this field has been focusing on the technologies including nanoparticles, micelles and liposomes [7–9].

Polymeric micelles have a core-shell structure, including a hydrophilic shell and a hydrophobic core [10, 11]. The hydrophobic core of micelles can encapsulate a range of hydrophobic drugs and agents used for the diagnostic purposes. This encapsulation considerably improves their bioavailability, and enhances their biodistribution and pharmacokinetics. The micelles' size allows the extravasation and buildup in numerous pathological areas where vascular endothelium's permeability is increased, including tumors. This offers a chance for physiology-based drug targeting and/or drug-loaded nano-carriers including micelles, to these sites via the enhanced permeation and retention (EPR) effects [12, 13]. Micelles can easily be prepared on larger scale, offering an extra practical benefit.

The latest results from the clinical trials of siRNAs for the respiratory syncytial virus (RSV) and age-related macular degeneration (AMD) infection have revealed their potentials [14–16]. In spite of the huge prospective of these quick improvements, clinical RNAi-based therapy application to human beings has been considerably restricted by the poor cellular uptake and short serum half-life of these molecules [17]. Recently, DsiRNA has gained attention owing to its greater potency over the conventional siRNA.

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Cholic acid (CA) is a natural amphiphilic molecule derived from cholesterol, which can arrange itself into micelles above critical micelle concentration (CMC). Cholic acid (bile acid), and phospholipids have the potential to alter the cell membrane permeability [18]. Few bile acids have the ability to make hydrogen-bonded aggregates with drugs, leading to the alterations in the bioavailability of drug [19]. Polyethyleneimine (PEI) is a cationic carrier mostly used for the delivery of genes as it has the nuclear localization capability, the ability to escape endosomes, the high potential of condensing nucleic acid [20, 21] and the efficient *in vitro* and *in vivo* transfection efficacy [20].

To discover the prospective of cholic acid-polyethylenimine (CA-PEI) copolymer micelles, their morphology was investigated using a transmission electron microscope. Other characterization studies were also carried out to assess their potential as DsiRNA carriers.

Materials and Methods

Chemicals and Reagents

CA, PEI (average molecular weight [MW] approximately 1300 g/mol), N, N'-Dicyclohexylcarbodiimide (DCC), N-Hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). DsiRNA was targeting the vascular endothelial growth factor (VEGF).

Cell Culture

The human colorectal adenocarcinoma (DLD-1) and Chinese hamster lung fibroblast (V79) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). RPMI-1640 medium, DMEM, 10% FBS, 1% penicillin-streptomycin were also used in this study.

Synthesis of CA-PEI polymer

Carbodiimide-mediated coupling was used to synthesize CA-PEI. Initially, CA was activated using DCC and NHS for 8 hours at 25ºC. The activated CA and PEI were conjugated by the incubation in dichloromethane for 15 hours. The polymer was dried and mixed in dil HCl, followed by the cold acetone precipitation. Finally, the polymer was suspended in the deionized water followed by the filtration and freeze-drying. The zeta potential and mean particle diameter of CA-PEI micelles were determined by using the dynamic light scattering technique.

Transmission electron microscopy (TEM)

The morphology of micelles was investigated using the transmission electron microscopy (accelerating voltage 120 kV). Onto a copper grid coated with carbon film, a small drop of aqueous polymer solution was put. Filter paper was used to wipe off the excess copolymer solution, and the grid was let till dry under the room temperature.

DsiRNA loading

DsiRNA (35 mg) was loaded onto CA-PEI micelles. The mixture was vortexed for 20 seconds, and then kept at the ambient temperature for 30 minutes without light to facilitate the strong binding between DsiRNA and micelles. DsiRNA LE (%) of CA-PEI micelles was determined after finding out the quantity of free DsiRNA in the supernatant after the centrifugation. UV-vis spectrophotometer at a wavelength of 260 nm was used to determine the concentration of DsiRNA. As a reference, the supernatants taken from blank CA-PEI micelles were used. DsiRNA LE (%) was calculated.

Binding efficiency of DsiRNA

The binding efficiency of DsiRNA to the micelles was studied by adding 20 µL of DsiRNA-loaded CA-PEI micelles into the wells of a 4% (w/v) precast agarose gel with SYBR Green. The positive control used in the study was free DsiRNA, whereas the blank CA-PEI micelles were negative controls. The duration of electrophoresis was 26 min. The DsiRNA bands were then visualized.

In vitro release study of BSA and siRNA

DsiRNA release study was carried out *in vitro* at pH 5 and 7. The DsiRNA-loaded CA-PEI micelles were put into dialysis membrane bags. The bags were then placed in 100 mL release medium (PBS) in beakers at pH 5 and 7 separately. The release medium was stirred at 37ºC at 100 rpm speed. At specific time intervals, the samples were drawn from the release medium and replaced with the equal volume of fresh medium. The quantity of DsiRNA released from the micelles into the medium was analyzed using spectrophotometer.

In vitro cytotoxicity assay

The cells (V79 and DLD-1) were seeded in 96-well cell culture plates and treated with DsiRNA-loaded CA-PEI micelles for 24 hours at 37ºC. After 24 hours, a final dilution of 1/10 per cell volume of alamarBlue® reagent was added for 4 hours followed by the analysis on a microplate reader.

Results and Discussion

Carbodiimide mediated coupling technique was used to link CA to PEI. The micelles were prepared by vortexing CA-PEI copolymer in the aqueous medium. The average diameter of all CA-PEI molar ratios was in the range of 125-150 nm. The micelles had a positive zeta potential. Micelles with the high CA to PEI ratio (i.e. 1:3) exhibited the highest positive zeta potential (+16.5$\pm$1.3) and vice versa (Table 1).

Table 1: Zeta potentials and LE (%) of CA-PEI micelles

Micelles	zeta potential (mV, n = 3)			LE (%, n = 3)		
	1:1	1:3	3:1	1:1	1:3	3:1
CA-PEI	+12.4 ± 1.1*	+16.5 ± 1.3*	+9.7 ± 1.2*			
DsiRNA-loaded CA-PEI	+6.6 ± 0.5	+4.1 ± 0.6**	+8.1 ± 1.1	94± 0.81*	96± 0.79*∞	93.4 ± 0.68∞

The morphology of micelles was investigated using the transmission electron microscope (TEM) (Figure 1). While looking at the TEM images, it was found that the average diameters of all CA-PEI micelles ranged from 125 to 150 nm at pH 7. The micelles looked spherical and often seen to be present in aggregates.

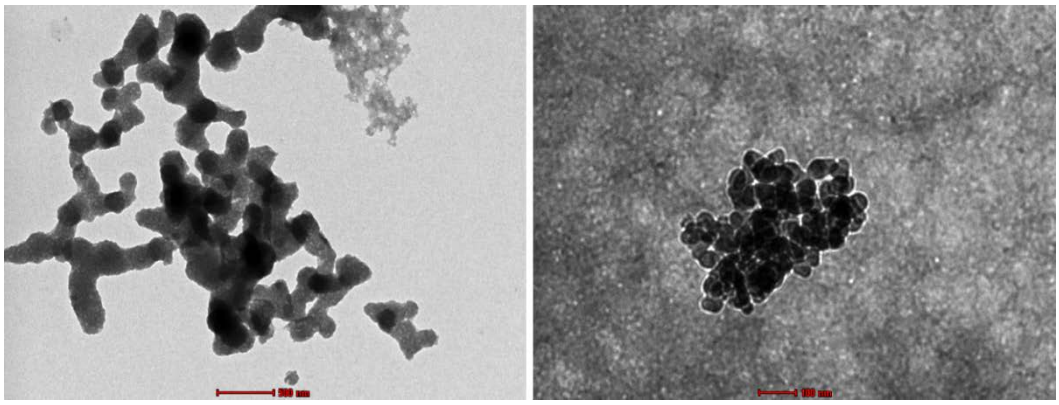

Figure 1. TEM images of the CA-PEI micelles

DsiRNA was loaded onto CA-PEI micelles. Post DsiRNA loading, the zeta potential of CA-PEI (1:3) micelles decreased to +4.1±0.6. This reduction was found to be the highest amongst all the molar ratios. As CA-PEI (1:3) micelles had higher PEI in comparison to others, more amine groups were accessible for binding. Therefore, more DsiRNA interacted with this molar ratio of CA-PEI leading to the neutralization and therefore, the reduction in zeta potential. The LE (%) of DsiRNA-loaded CA-PEI micelles was found to increase from 94%±0.81 to 96%±0.79 when PEI was increased, the difference in DsiRNA LE onto the molar ratios 1:1 and 1:3 was statistically significant. This showed that CA-PEI ratio influenced DsiRNA LE (%). Almost all DsiRNA bound strongly to micelles, and formed stable complexes. High LE (%) was attained owing to strong electrostatic attraction between PEI and DsiRNA, resulting in the formation of tight complexes [22, 23].

The binding efficiency of DsiRNA to the micelles was studied. As shown in Figure 2, it was evident that DsiRNA strongly attached to the CA-PEI micelles, as no DsiRNA trailing band could be seen in the channels loaded with DsiRNA-loaded CA-PEI micelles. The absence of DsiRNA trailing band showed that no DsiRNA migration occurred, indicating the strong interaction between DsiRNA and CA-PEI. These results showed that on the interaction, the negative DsiRNA phosphate groups interacted with the positively charged PEI amine groups, attaching strongly, resulting in the tight complexes [22, 23].

Figure 2. DsiRNA Binding Efficiency Test

DsiRNA release experiments were carried out *in vitro* at pH 5 and pH 7 (Figure 3). The innermost layer of DsiRNA was attached to the surface of the micelle by the electrostatic interaction. The adsorption of ensuing DsiRNA layers was primarily caused by the inter-molecular hydrophobic interactions, since the electrostatic interactions were reduced as the thickness of surface DsiRNA increased. Due to this phenomenon, various layers of DsiRNA adsorbed onto the surface of CA-PEI micelles were loosely bound [24]. The early fast DsiRNA release from the surface of micelles was primarily because of the detachment of the loosely-attached DsiRNA. The DsiRNA release was found to increase with lowering in pH of the medium. The equilibrium was established between the DsiRNA released in the medium, and DsiRNA was adsorbed on the surface of the nanoparticle. The late release of DsiRNA was primarily governed by the dissolution of the particles,

releasing the tightly-bound molecules of DsiRNA. The H+ concentration increased as the pH of the medium decreased. This increase shifted the dissolution equilibrium towards the right, enabling the micelles' dissolution. Both DsiRNA release and micelle dissolution occurred at a faster rate at pH 5, in comparison to pH 7.

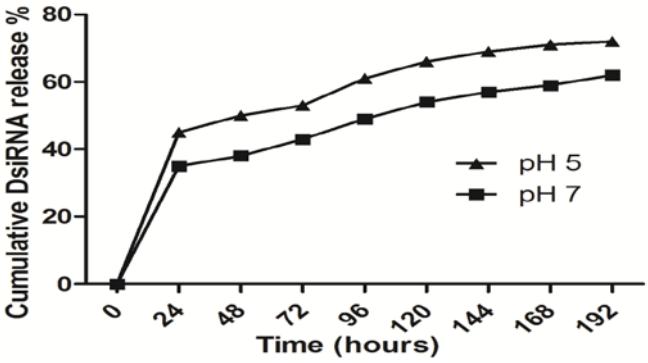

Figure 3. In Vitro DsiRNA release experiment from micelles at pH 5 and 7

The effect of DsiRNA-loaded CA-PEI micelles (Figure 4) on the viability of Chinese hamster lung fibroblast (V79) and human colorectal adenocarcinoma (DLD-1) cells was studied by alamarBlue® assay. The goal of using both DLD-1 (colorectal adenocarcinoma) and V79 (lung fibroblast) cell lines was to get an idea regarding the effect of DsiRNA-loaded CA-PEI micelles against cancer and normal cells. Only a minor decrease in cell viability was found when DLD-1 and V79 cells were exposed to free DsiRNA and DsiRNA-loaded CA-PEI micelles (Figure 4). A statistically significant decrease in cell viability was seen in V79 cells exposed to DsiRNA-loaded CA-PEI (1:3) micelles, in comparison to the untreated cells. The reason behind this may be the higher entrapment efficiency of DsiRNA in CA-PEI (1:3) micelles, along with a better transfection efficiency linked with the higher ratio of PEI. The results suggested that CA-PEI micelles were nontoxic and had a great potential as the carriers of DsiRNA.

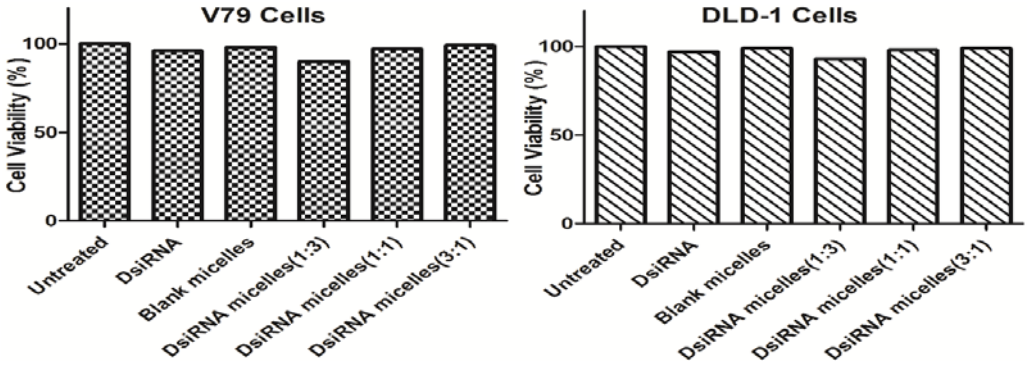

Figure 4. Investigation of cell viability after treatment with DsiRNA micelles at 24 h

Conclusion

CA-PEI micelles can be effectively loaded with DsiRNA. The integrity of DsiRNA while it was bound to CA-PEI micelles was conserved after processing and its subsequent release. The release of DsiRNA from the micelles was discovered to be faster at lower pH in comparison to pH 7. Furthermore, at both pHs, an initial burst release followed by the sustained release was seen. The CA-PEI micelles were non-cytotoxic to V79 and DLD-1 cells. The results showed the significant potential of CA-PEI micelles as a nano-carrier of DsiRNA.

Acknowledgements

The authors gratefully acknowledge the approval and the support of this research study by the grant no. 7060-PHM-2017-1-7-F from the Deanship of Scientific Research at Northern Border University, Arar, KSA.

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