Research article

SYNTHESIS OF NANOPARTICLES OF POLY (*ETHYLENEIMINE*) AND THEIR CHARACTERIZATION BY TRANSMISSION ELECTRON MICROSCOPY THIN LAYER CHROMATOGRAPHY, AND INFRARED SPECTROSCOPY

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Received date: 25 January 2022, Accepted date: 10 May 2022

ABSTRACT

Intracellular gene delivery alters the expression of a gene and corrects a defective gene that may be the cause of a disease or a disorder. Nonviral gene delivery is more appropriate than viral-mediated due to their low cytotoxicity and immunogenicity. Amongst these, polycationic nanoparticles i.e. Polyethyleneimine (PEI) were used most successfully. The PEGylation of such cationic polymer reduces its cytotoxicity. Different molecular weight poly (ethylene glycol) was used for the PEGylation of such cationic nanoparticles which have an individual effect. For this purpose, the PEG is esterified, which was then reacted with a cationic polymer. Four different molecular weights of PEG were used. The size of nanoparticles so formed depends upon the molecular weight of PEG. So formed nanoparticles were dialyzed, lyophilized, and then characterized by IR and TEM. The nanoparticles so formed are directly affected by the different molecular weights of PEG. Higher the molecular weights of PEG smaller size of nanoparticles so formed but only up to a limited extent. The decreasing order of nanoparticles as an increment of molecular weight of PEG was found as a -0.85 coefficient of correlation. The smaller-sized nanoparticles have higher transfection efficiency than larger-sized nanoparticles. So, the higher the molecular weight of PEG higher will be the transfection efficiency.

Keywords: Polyethyleneimine, PEGylation, IR, TEM

INTRODUCTION

Polyethylenimine (PEI) polymers have been extensively tested in vitro and in vivo and have been found to be one of the most efficacious non-viral agents. Most of PEI formulations studied to date have been prepared (Boussif,1996). Polyplexes from higher molecular weight branched PEIs (70-800 KDa) were found to be more efficient in vitro, but on intravenous administration the smaller and linear PEIs seem in general to be more efficient than branched PEI (Branden,1999). Poor solubility of polycation complexes with DNA is one drawback. PEGylation often can improve the solubility of the complexes; minimize their aggregation and reduced interaction with proteins in the physiological fluid (Erbacher, 1999 ; Fischer,1999). In vitro studies, PEI conjugate with a low degree of PEG grafting is able to reduce the size of polymer DNA complexes, prevent the aggregation of complexes, and enhance the transfection efficiency, although not significant in affecting the complex formation and reducing in vitro cell toxicity of PEI. PEGylation often reduces the immunogenicity of molecules to which PEG is attached (Godbey , 1999 ; Zanta, 1997).

Moreover, most cells whose genes are introduced in vitro are transformed or immortalized cell lines that are able to grow indefinitely given the proper culture conditions(Kichler, 2004). Cell lines are usually easier to transfect than primary cells because they are continuously dividing. The use of non-viral delivery methods has become routine for cell lines grown in vitro due to simplicity, robustness, and relatively high efficiency of gene transfer (Putnam, 2001). Primary cell lines are generally more difficult to transfect due to the absence of cell division and corresponding nuclear envelope breakdown. Viral vectors frequently provide the best options for gene delivery into primary cells in vitro (Boussif, 1995). The unique property of absorbing protons by PEI protects the PEI-based nucleic acid complex from lysosomal degradation and enhances its transfection efficiencies (Jere, 2009). PEI has been studied as non-viral carriers of nucleic acids but the high molecular weight PEI demonstrated a superior capability to form compact and stable PEI/DNA complexes in addition to enhanced transfection efficiency compared to lower molecular weight PEI derivatives (Kircheis et al.,2001). Although PEI with molecular weight ranging from 600 Da to 800,000 Da has been studied for gene

transfection, PEI with molecular weight between 600 Da to 1800 Da didn't show any significant transfection efficiency (Godbey et al., 1999). The average transfection efficiencies for solutions using the PEI of molecular weight 70,000 were higher than those seen for solutions using the PEI of molecular weight 10,000.

This study aimed to synthesize nanoparticles of Poly (ethyleneimine) by crosslinking it with activated PEG of different molecular weights and to characterize nanoparticles for their size and morphology by Transmission electron microscopy (TEM) and for structure by TLC and IR.

MATERIALS AND METHODS

This study was carried out at Dolphin Institute of Biomedical and Natural Sciences, Dehradun, and Indian Institute of Technology (IIT), Roorkee, India in May-July 2005.

Reaction of activated PEG (400Da) with Polyethyleneimine (PEI) (60,000 Da)

For the 10% cross-linking reaction of activated PEG (400Da) with 200mg of polyethyleneimine (PEI 60,000Da), a solution of 0.307gm PEG with water was prepared. 200mg PEI ie.400µl was dissolved in water on an RB flask & PEI solution was added dropwise with vigorous stirring. The reaction was kept on stirring for about 5 hrs till the reaction had been completed. The reaction mixture was then dialyzed in 5% Sodium Bicarbonate solution till the sample became colorless. The PEG-PEI complexes were then Rota evaporated & the dried solution of nanoparticles was synthesized.

The reaction of activated PEG (4000 Da) with Polyethyleneimine (PEI 60,000 Da)

For the 10% cross-linking reaction of activated PEG(4000Da) with 50mg of polyethyleneimine(PEI 60,000Da), a solution of 0.361g PEG with water was prepared.50mg PEI ie.100µl was dissolved in water on an RB flask & PEI solution was added dropwise with vigorous stirring. The reaction was kept on stirring for about 5hrs till the reaction had been completed. The reaction mixture was then dialyzed in 5% Sodium Bicarbonate solution till the sample became colorless. The PEG-PEI complexes were then Rota evaporated & the dried solution of nanoparticles was synthesized.

The reaction of Activated PEG (4000 Da) With Polyethyleneimine (PEI 60,000 Da)

For the 10% cross-linking reaction of activated PEG (8000Da) with 50mg of polyethyleneimine (PEI 60,000Da), a solution of 0.721g PEG with water was prepared. 50mg PEI ie.100µl was dissolved in water on an RB flask & PEI solution was added dropwise with vigorous stirring. The reaction was kept on stirring for about 5hrs till the reaction had been completed. The reaction mixture was then dialyzed in 5% Sodium Bicarbonate solution till the sample became colorless. The PEG-PEI complexes were then Rota evaporated & the dried solution of nanoparticles was synthesized.

The reaction of activated PEG (20,000 Da) with Polyethyleneimine (PEI 60,000 Da)

For the 10% cross-linking reaction of activated PEG (20,000Da) with 50mg of polyethyleneimine (PEI 60,000Da), a solution of 1.69g PEG with water was prepared. 50mg PEI ie.100µl was dissolved in water on an RB flask & PEI solution was added dropwise with vigorous stirring. The reaction was kept on stirring for about 5hrs till the reaction had been completed. The reaction mixture was then dialyzed in 5% Sodium Bicarbonate solution till the sample became colorless. The PEG-PEI complexes were then Rota evaporated & the dried solution of nanoparticles was synthesized.

Characterization of activated PEG by TLC & IR

The reaction mixture was monitored on TLC using EDC: Methanol (7:3). For this 14ml of EDC & 6ml of Methanol had taken. Then the reaction mixture was partitioned between EDC & water. The lower fraction of EDC in the separating funnel of EDC was collected & concentrated on the Rota evaporator to get the activated PEG. IR (Infra-red spectroscopy) is also used to characterize nanoparticles, the 0.2mg of the sample of activated PEG was taken and further mixed with 20mg KBr in order to get the pellet. The pellet was then further characterized with the help of IR spectroscopy

Characterization of nanoparticles for their structure, size, and morphology

IR (Infra-red spectroscopy) was used to characterize nanoparticles for their structure. 0.2mg of the sample of nanoparticles was taken and further mixed with 20mg KBr in order to get the pellet. The pellet was then further characterized with the help of FTIR spectrum BX series.

TEM (Transmission Electron Microscope) Lyophilized powder (3mg) was dispersed in 20 ml distilled water to have a clear solution and samples for TEM were prepared using this clear solution. The sample solution was put on a formvar-coated grid (1% solution of formvar was prepared in spectroscopic grade chloroform). A clean glass slide was dipped in the formvar solution to make a formvar film on the stars, the glass slide was scratched on the edges and the formvar film was floated on distilled water on a spherical container, the 2090 mesh copper grids were placed upside down on the floating plastic film. In this way, the plastic-coated grids were prepared. On this grid, a drop of the sample solution (containing dispersed nanoparticles) was placed and allowed to air dry.

RESULTS

Characterization of PEG/PEI Nanoparticles By TLC

The result showed that 4-Nirrophenyl Chloroformate has the highest affinity for the mobile phase and PEG/PEI nanoparticles have low affinity. PEI contains the NH2 group and whose peak was somewhat longer than PEG/PEI nanoparticles as the NH2 group reacted to activated PEG.

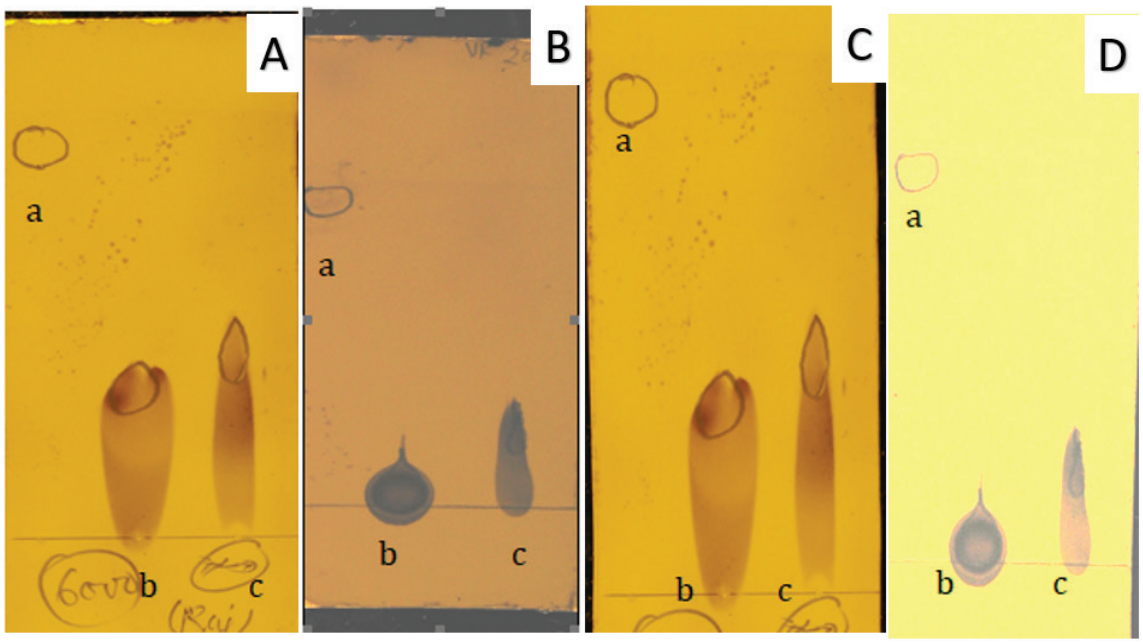

Figure 1. TLC spots of PEI/activated PEG at different molecular weights in Solvent system methanol:EDC (7:3) A. 732 Da nanoparticles B. 4332Da nanoparticles C. 8332Da nanoparticles D. 20332Da nanoparticles

Note: The letters in figures indicate: a. Activated PEG, b. PEG/PEI nanoparticles, c. PEI

Characterization of activated PEG/PEI Nanoparticles by IR spectroscopy

All the nanoparticles were characterized by Infrared spectroscopy. The results are shown below. In activated PEG the ester bond was found which corresponds to C=O at1768 cm^{-1} and C-O at 1011cm^{-1}, but in the case of PEG/PEI extra bond C-N at 1099 and N-H bond stretching at 3435cm-1 and bending at 841-961cm^{-1} were found as well as C=O and C-O peak. This indicates that their reaction was completed between PEG and PEI.

Characterization of Activated PEG/PEI Nanoparticles by TEM

All the nanoparticles were also characterized by Transmission electron microscopy.

The results are shown below-

Transmission Electron Microscopy (TEM) revealed that different size of nanoparticles was formed. The nanoparticles formed by PEGylation with 400Da. PEG was found as 250-280nm. Similarly, PEGylation with 4000Da, 8000Da, and 20000Da resulted in the 100nm, 50-60nm and 20-50nm respectively. From this result, it is clear that as the molecular weight of PEG increases , and the size of nanoparticles decreases.

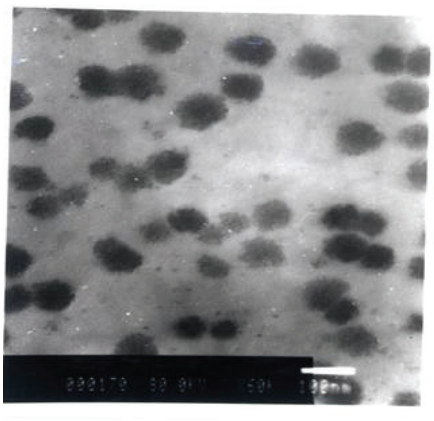

Figure 2. TEM picture of PEI/activated PEG 20,332 (10%)
Size: 20-50nm
Morphology: Round and oval

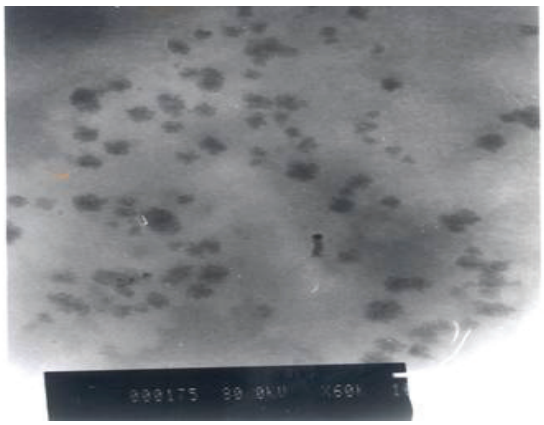

Figure 3. TEM picture of PEI/activated PEG 8332 (10%)
Size: 40-60nm
Morphology: Round and Spherical

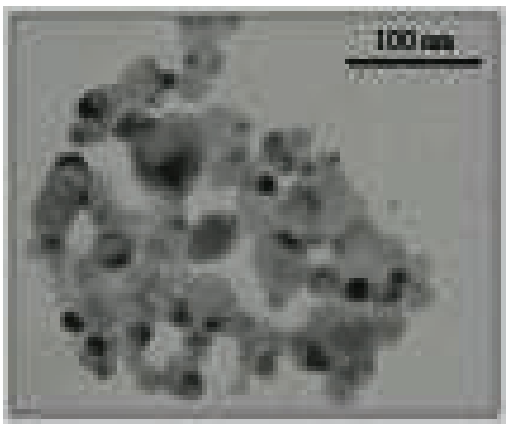

Figure 4. TEM picture of PEI/activated PEG 4332 (10%)
Size: 80-100nm
Morphology: Round and oval

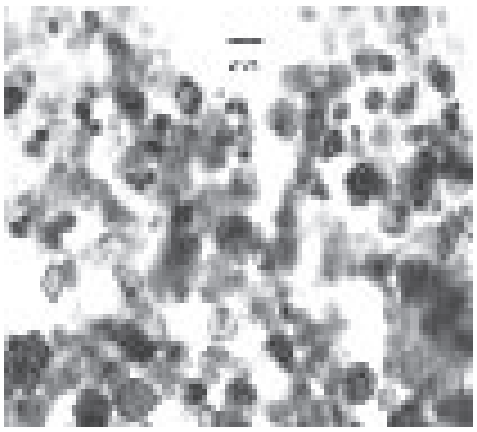

Figure 5. TEM pictureof PEI/activated PEG 732 (10%)
Size: 250-280nm
Morphology: Round

DISCUSSION

Viral vectors for gene delivery have high transfection efficiency but due to their high toxicity and immunogenicity non-viral vectors like chitosan, liposome, and PEI nanoparticles are used. Among them, PEI is the most popularly used. The PEGylation of PEI reduces it's cytotoxicity and immunogenicity (Branden, 1999). The cross-linking between PEG/PEI reduces the chain length of the PEI. As the Molecular weight of PEG is increased the cross-linking will be greater, so the size of nanoparticles will decrease. The transfection efficiency is directly dependent on the size of nanoparticles (Jere, 2009). The smaller the size of nanoparticles

higher will be its transfection efficiency because the smaller size nanoparticles can easily cross the cell membrane and are escaped from mononuclear phagocyte uptake as compared to the larger size nanoparticles. So, finally, it was concluded that as the Molecular weight of PEG increases the transfection efficiency will be higher. Tatum E.L 1996 modified PEI copolymers having relatively high molecular mass can be effective for drainage and pitch control. The branched form of PEI contains primary, secondary & tertiary amines, each with the potential to be protonated. This gives PEI the attribute of serving as an effective buffer through a wide range of pH ranges, with nitrogen appearing as one out of every three atoms in the PEI backbone, any benefits of branching and portability quickly accumulate in relation to the overall polymer size. PEI is obtained by acid-catalyzed polymerization of aziridine, (Dick et al., 1970) yielding a highly branched network with a high cationic charge-density potential that can ensnare DNA. The branched nature and exceptionally high charge of pure PEI make it an excellent choice for the treatment of highly anionic furnish, furnish that has high electrical conductivity or thick-stock furnish. The branched structure is believed to provide some resistance to penetration of the molecule into very small slit-like pores in the fiber cell wall (Fischer, 1999). The colloidal charge, filtrate turbidity, and drainage characteristics of the furniture should be monitored when selecting or optimizing PEI dosage. The modified PEI copolymer is usually added relatively late in the thin-stock cycle in order to achieve a significant boost in first-pass retention and the rate of drainage (Putnam, 1999). Neither additive is expected to be cost-effective in furnishing having pH values in excess of about 8, since the amine groups rapidly lose their charged character above that point. Likewise, strongly varying pH values are expected to cause variations in the performance of PEI products. PEI is a branched synthetic polymer that presents a high cationic charge-density potential since every third atom is amino nitrogen that can be protonated. (Suh, 1997)

CONCLUSION

From the result of TLC, it can be concluded that 4-Nirrophenyl Chloroformate has highest affinity for mobile phase and PEG/PEI nanoparticles have low affinity. The IR spectroscopy showed that in activated PEG the ester bond was found which corresponds to C=O at1768 cm^{-1} and C-O at 1011cm^{-1}, but in case of PEG/PEI extra bond C-N at 1099 and N-H bond stretching at 3435cm^{-1} and bending at 841-961cm^{-1} were found as well as C=O and C-O peak. This indicates that their reaction was completed between PEG and PEI. TEM report shows as the Mol.Wt. of PEG increases , the size of nanoparticles decreases.

ACKNOWLEDGMENTS

The author would like to thank all the laboratory staffs of Dolphin Institute of Biomedical Science, Dehradun and Indian Institute of Technology, India for their support in laboratory works.

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