ORIGINAL ARTICLE

Effect of Polyethylene Glycol Chain Length on PEGylation of Dendrimers

Hemant Khambete, Nishi P. Jain, C. P. Jain

Department of Pharmaceutical Sciences, Mohanlal Sukhadia University, Udaipur, Rajasthan, India

Abstract

Aim: To check the effect of polyethylene glycol (PEG) chain length on PEGylation of dendrimers. **Materials and Methods:** In the present work, we have synthesized different PEGylated polyamidoamine dendrimers using six different PEG chains, i.e., PEG - 200, 300, 400, 600, 2000, and 6000. The PEGylated dendrimers were evaluated for color reaction ultraviolet, infrared, and nuclear magnetic resonance studies and compared with standard data. **Results and Discussion:** The plain dendrimers give violet color due to free NH2 groups. The intensity of violet color of 4.0 G dendrimers decreases on PEGylation, due to attachment of PEG chain on free NH2 groups which is responsible for violet color. The change in λmax values from 283 to 353 nm was observed, which shows the change in structure of dendrimers. On comparing, it was found that PEG 400 and 600 shows value near their expected values, i.e., 19,150 and 19,780, respectively. **Conclusion:** From the results of the present study, it can be concluded that PEGylation using PEG 400 and 600 gives a considerable level of attachment of PEG to dendrimers as compared to other PEG chains.

Key words: Dendrimers, epichlorohydrin, polyamidoamine, polyethylene glycol, PEGylation

INTRODUCTION

PEGylation defines the modification of a protein, peptide, or non-peptide molecule by the linking of one or more PEG chains. This polymer is non-toxic, non-immunogenic, non-antigenic, and highly soluble in water and FDA approved. The polyethylene glycol (PEG)-drug conjugates have several advantages: A prolonged residence in body, a decreased degradation by metabolic enzymes, and a reduction or elimination of protein immunogenicity.[1,2]

"PEGylation," the covalent coupling of PEG chains to drugs, has been the trailblazing innovation of the past few years. Important pioneering work in this field was performed by Abuchowski *et al*.,[1] laying the cornerstone for the commercial success of this technology. PEGylation increases the hydrodynamic radius of a biopharmaceutical and shields its surface toward the periphery. Thus, the stability of these conjugates against proteases is increased, their immunogenicity is reduced, and their renal excretion is decelerated. Consequently, PEGylation secures a prolonged half-life of the biopharmaceutical, reduces side effects, and finally increases the efficiency of the therapy.

Even though many attempts have been undertaken to develop new polymers with improved properties, none of these new substances has been able to compete with PEG for this application. This can be explained by the biocompatibility of PEG and the good experience with PEG as a low-cost additive for the pharmaceutical and cosmetic industry over the past decades.[3,4]

Since PEGylation is a permanent modification of the biopharmaceutical, the relevant national and international authorities for drug approval make high demands on the PEG reagents and the final PEGylated product. Major requirements are the specification of the degree of PEGylation, analysis of the dispersity index, and determination of the PEGylation sites. Thus, an ideal PEG reagent fulfills at least the following criteria:

- Monodispersity or at least a dispersity index close to 1.00, to assure a reproducible high quality
- Availability of one single terminal reactive group for the

Address for correspondence:
Hemant Khambete, Department of Pharmaceutical Sciences, Mohanlal Sukhadia University, Udaipur, Rajasthan, India. Phone: +91-9893670420.
E-mail: khambete.hemant@gmail.com

Received: 11-11-2016
Revised: 07-12-2016
Accepted: 24-02-2017

coupling reaction, to avoid cross-linking between drug molecules
- Non-toxic and non-immunogenic, biochemically stable linker
- Branching for optimal surface protection
- Options for site-specific PEGylation.

More and more, polydispersity of PEG comes to the fore as a quality problem for PEGylated drugs. As a consequence of the production process, long and linear PEG chains used for PEGylation today are only available as a mixture of PEG chains with different chain lengths. But now, efforts are under way to solve this problem using monodisperse starting material.[5]

Many efforts have been undertaken to achieve an efficient and stable coupling of PEG chains to the biopharmaceutical. Very successful developments have been achieved with regard to the variability of the coupling chemistry and the availability of specialized linkers.

Site-specific mono-PEGylation is of significant relevance to provide highly reproducible products maintaining maximum activity. In the majority of cases, high molecular weight PEG chains (10-40 kDa) are used for the mono-PEGylation of proteins. At best, it is possible to attach one single PEG chain to the N-terminal amino group of a protein by reductive amination. Especially with small proteins such as cytokines, it is possible to apply genetic methods to introduce rare amino acids, which then can be used for the coupling of PEG.[4] Preferred for this purpose is a cysteine residue which can be specifically PEGylated at the thiol group by maleimide coupling.[6,7]

Some examples have also been published in which the site-directed PEGylation has been achieved by an enzyme-catalyzed reaction with a transglutaminase. Several conjugation strategies are now available, such as alkylation, which maintains the positive charge of the starting amino group because a secondary amine is formed, or acylation, accompanied by loss of charge.[8-10]

The synthesis and application of PEGylated dendrimers have also been published. In these, the synthesis of PEGylated dendrimers and importance of PEGylation were discussed.[11-14]

MATERIALS AND METHODS

Synthesis of polyamidoamine (PAMAM) dendrimer was performed by divergent method. Construction of an energy design assistance (EDA) core PAMAM dendrimers consists of consecutive steps: Michael addition of primary amine (EDA in very first step) to methyl acrylate followed by amidation of formed multiester (tetra ester at very beginning) of EDA.

The conjugation of PEGylation was done using epichlorohydrin as a cross-linking agent. 100 mg (6.3 µM) of lyophilized 4.0G PAMAM dendrimer was dissolved in methanol. 16 molar times of PEG - 600 was mixed with epichlorohydrin in separate container and stirred vigorously for 2 h and incubated for 36 h at room temperature in dark; now in this mixture, the 4.0 G dendrimer solution was added and shaken properly and kept a side for 24 h which facilitate the linking of PEG with 4.0 G dendrimer using epichlorohydrin as a linker. The final product was dialyzed to remove byproducts [Figure 1].

Identification of dendrimers was done by first subjecting the plain and PEGylated dendrimers to reaction of copper sulfate aqueous solution (1%w/v) in (0.1%w/v) methanol.

Change in structure of dendrimers from plain to PEGylated system were analyzed by ultraviolet (UV)/ visible spectrophotometer. The sample was taken as 0.01% w/v concentration in distilled water and scanned in the range of 200-500 nm against distilled water. The changes in λmax values were analyzed.

The formed 4.0 G dendritic system and PEGylated system were subjected to infrared (IR) spectroscopy analysis; various peaks were interpreted for different groups.

The sample was analyzed by nuclear magnetic resonance (NMR) spectroscopy. The 4.0 G and PEGylated dendrimers were solubilized in D_2O using methanol as cosolvent and analyzed at 300 MHz. Various shifts in the peaks were observed, which were interpreted for different groups present in PEGylated system.

The plain dendrimers give violet color due to free NH_2 groups. The intensity of violet color of 4.0 G dendrimers decreases on PEGylation, due to attachment of PEG chain on free NH_2 groups which is responsible for violet color.

The changes in λmax values were analyzed by UV/Visible spectrophotometer (Shimadzu-1700). The change in λmax values from 283 to 353 nm was observed, which shows the change in structure of dendrimers [Figures 2 and 3].

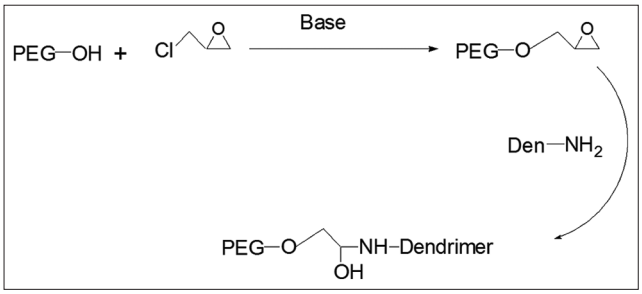

Figure 1: Scheme of PEGylation of dendrimers using epichlorohydrin as linking agent

The formed 4.0 G and PEGylated system were subjected to IR spectroscopy analysis by Fourier transform IR - 470 Plus, Jasco, Japan. The IR peaks confirmed the progress of PEGylation on dendrimers. The important peaks in IR spectra of 4.0 G dendrimers were of N-H stretch of primary amine at 3310.21/cm, N-H stretch of anti-symmetric substituted primary amine at 3021.87/cm, and C-H stretch at 2947.66/cm. In IR spectra of PEGylated dendrimer, peak of C-O at 1100/cm for ether linkage appears predominantly in the spectrum of 4.0 G PEGylated species. IR spectra show major change in peaks of carbonyl resonating symmetric and antisymmetric peaks at 3021.87/cm on linking by amide linkage at dendritic end. These two major changes in C-O linkage in dendrimers prove that dendrimers have been well PEGylated. The results obtained are given in Tables 1 and 2. The IR spectra of 4.0 G dendrimers and PEGylated dendrimers are given in Figures 4 and 5, respectively.

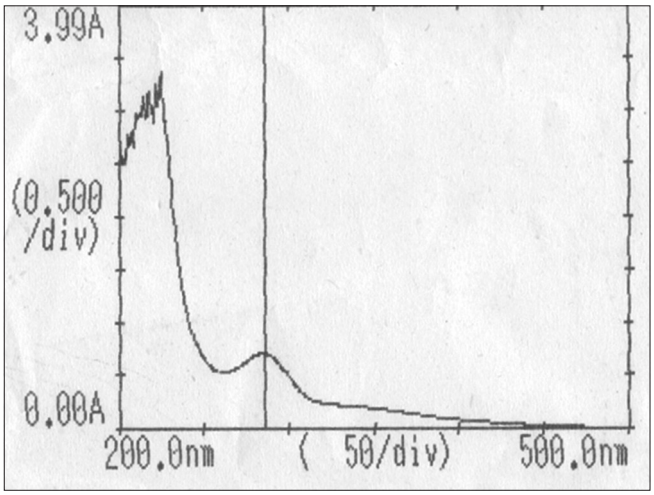

Figure 2: Ultraviolet spectra of 4.0 G dendrimers

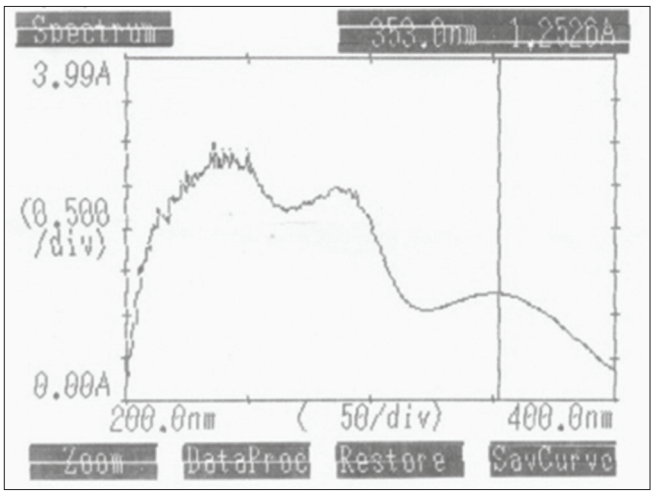

Figure 3: Ultraviolet spectra of PEGylated dendrimers

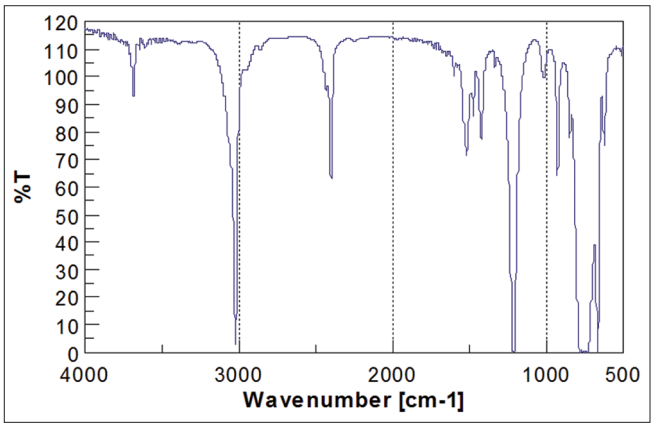

Figure 4: Infrared spectra of 4.0 G polyamidoamine generation dendrimers

NMR spectra further confirm the PEGylation of dendrimers. The sample was analyzed by NMR spectroscopy by Bruker DRX-300. The 4.0 G dendrimers and PEGylated dendrimers were solubilized in D_2O using methanol as cosolvent and analyzed at 300 MHz. Important shifts in NMR spectra of 4.0 G dendrimers were 2.401-2.425 ppm for carbonyl – (CH_2C=O), 2.539-2.921 ppm for amide - N-H, 3.164-3.434 ppm for –CH_2NH_2 terminal group, and 4.82 for -OH methanolic group. The NMR spectra and shifts of PEGylated dendrimer as compared to simple dendrimers

Table 1: IR interpretation of 4.0 G dendrimers

Wave no. (peak) in cm^{-1}	Interpretation
3310.21	N-H stretch of primary amine
3022.87	N-H stretch antisymmetric of sub. primary amine
2947.66	C-H stretch
1668.12	C=O stretch of carbonyl group
1511.92, 1417.42	N-H bending of N-substituted amine
1215.90	C-C bending

IR: Infrared

Table 2: IR interpretation of PEGylated dendrimers

Wave no. (peak) in cm^{-1}	Interpretation
3434.6	N-H stretch of primary amine
3022.87	Carbonyl symmetric and antisymmetric peaks
2399.98	Carboxylic acid C=O and O-H stretch unconjugated
1473.25	CH-NH_C(=O) amide bending
1211.08	Ester unconjugated C=O and C-O stretching
1103.08	C-O stretch ether linkage strong and sharp
767.53	Aromatic C-H bending

IR: Infrared, PEG: Polyethylene glycol

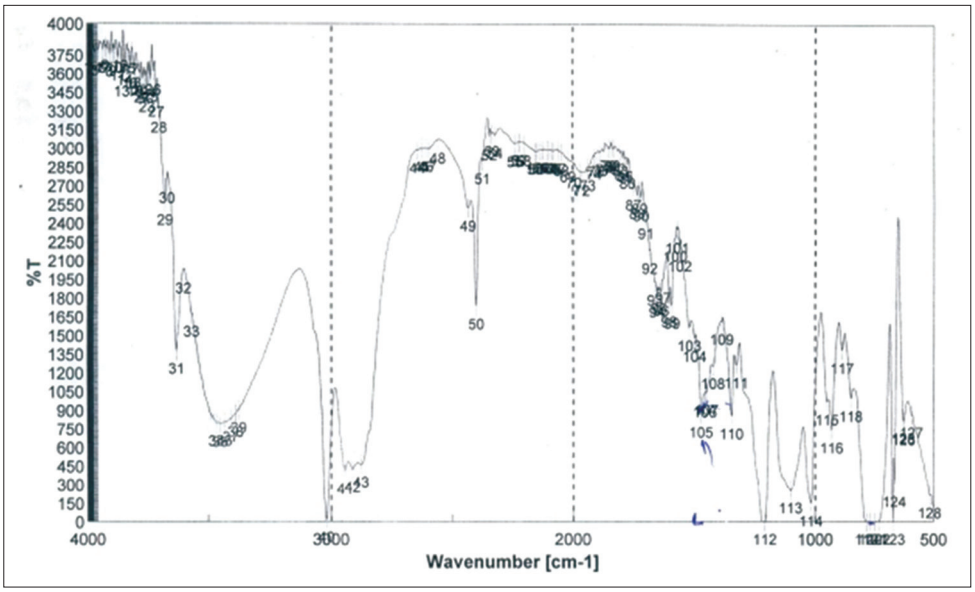

Figure 5: Infrared spectra of 4.0 PEGylated dendrimers

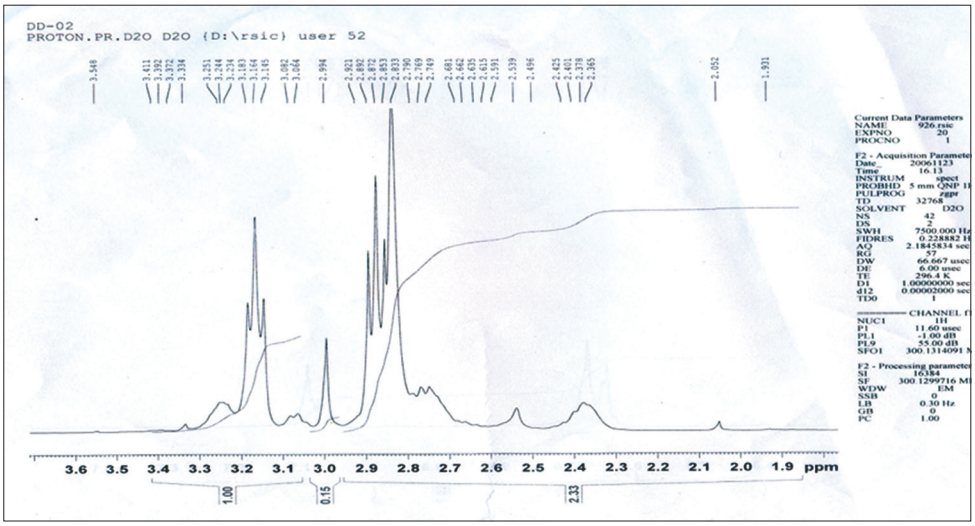

Figure 6: Nuclear magnetic resonance spectra of 4.0 G dendrimers

Table 3: NMR shifts and interpretation of the spectrum of 4.0 G PAMAM dendrimers

δ values range (in ppm)	Interpretation
2.401-2.425	Carbonyl ($CH_2C=O$)
2.539-2.921	Amide-NH
3.164-3.434	$-CH_2NH_2$ terminal group
4.822	-OH (methanolic)

PAMAM: Polyamidoamine, NMR: Nuclear magnetic resonance

provide the proof of PEGylation. Drastic change in integral values and shifts of secondary - CH_2 group was noticed on PEGylation. Similarly, newer peaks of ether linkage at 3.2-3.5 ppm unremarkably increase amount. The results obtained are given in Tables 3 and 4. The NMR spectra of 4.0 G and PEGylated dendrimers are given in Figures 6 and 7 respectively.

Table 4: NMR shifts and interpretation of the spectrum of PEGylated dendrimers

δ values range (in ppm)	Interpretation
1.165-1.677	R_2 CH_2 (secondary)
2.041-2.274	($-CH_3$ C=O) carbonyl
3.583-3.880	Ether linkage
3.224-3.463	$-CH_2$-NH_2 (remaining free amines)
4.895-5.127	Amide (-C=O-NH)

NMR: Nuclear magnetic resonance, PEG: Polyethylene glycol

The main difference between PEG chains is mainly done by mass spectra. The m + 1 peak was observed at different points for different chains of PEG. The plain dendrimers having molecular weight 14,500 and 16 groups of different PEG chains have to be attached. Hence, the expected change

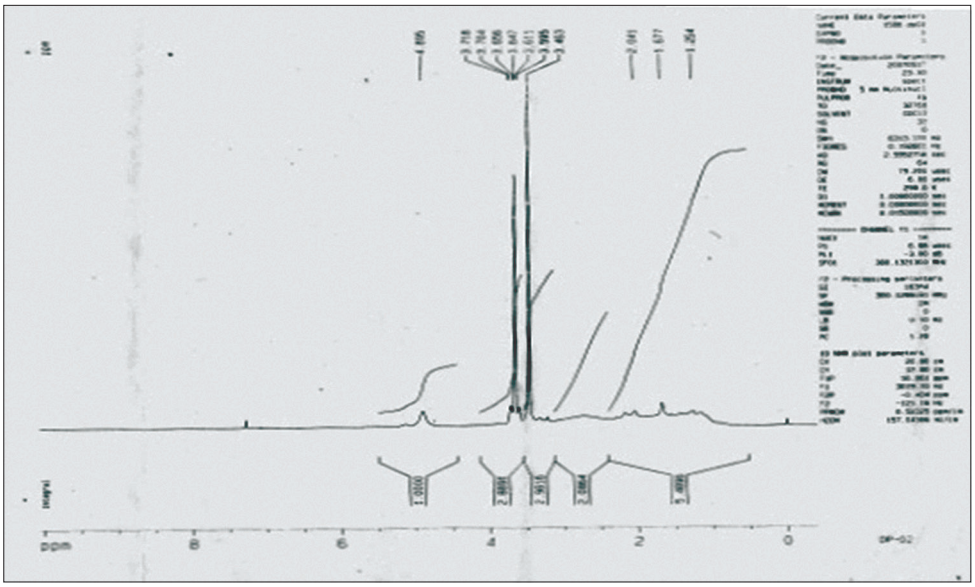

Figure 7: Nuclear magnetic resonance spectra of PEGylated dendrimers

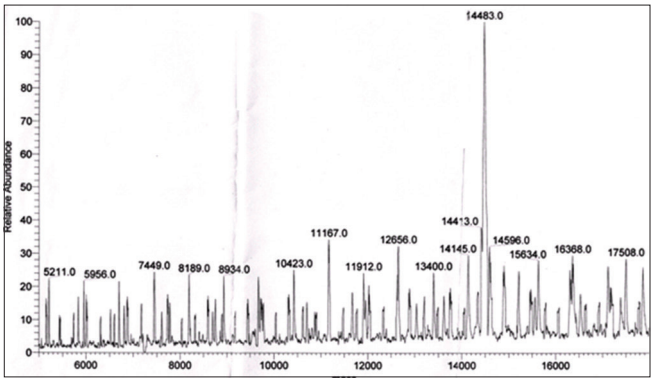

Figure 8: Mass spectra of 4.0 G polyamidoamine dendrimer

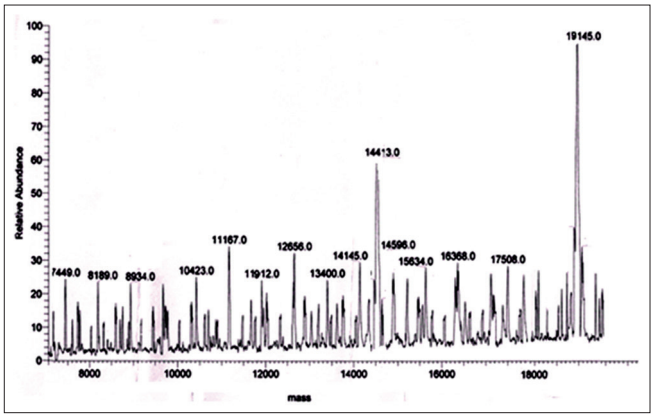

Figure 9: Mass spectra of 4.0 G polyamidoamine - polyethylene glycol (400) conjugate

in mass of dendrimers after PEGylation was compared with practical data. On comparing, it was found that PEG 400 and 600 shows value near their expected values, i.e., 19,150 and 19,780, respectively. This may due to that only PEG 400 and 600 attached at 16 chains of NH_2-terminated dendrimers. Whereas other PEG chains may show variable conjugation with dendrimers. This may also due to back folding of higher PEG chain such as 2000 and 6000 that stop the more attachment of PEG molecule with dendrimers and low-molecular PEG such as 200 and 300 may attach more than 16 chains of NH_2-terminated dendrimers. The mass spectra of 4.0 G and dendrimer - PEG (400) conjugate are given in Figures 8 and 9, respectively.

CONCLUSION

From the results of the present study, it can be concluded that PEGylation using PEG 400 and 600 gives a considerable level of attachment of PEG to dendrimers as compared to other PEG chains and also an easy, reproducible method and hence both PEG chain (400 and 600) can be used for optimized PEGylation of dendrimers.

REFERENCES

1. Abuchowski A, van Es T, Palczuk NC, Davis FF. Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. J Biol Chem 1977;252:3578-81.
2. Abuchowski A, McCoy JR, Palczuk NC, van Es T, Davis FF. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. J Biol Chem 1977;252:3582-6.
3. Kinstler OB, Brems DN, Lauren SL, Paige AG, Hamburger JB, Treuheit MJ. Characterization and stability of N-terminally PEGylated rhG-CSF. Pharm Res 1996;13:996-1002.
4. Wong SS. Chemistry of Protein Conjugation and Cross-linking. Boca Raton, FL: CRC Press; 1991. p. 13.
5. Francesco M, Mero A, Caboi F, Sergi M, Marongiu C,

Pasut G. Abstract of Papers, 32nd Annual Meeting and Exposition of the Controlled Release Society. Abstract 415. Miami, USA; 2005.
6. Robert J, Goodson L, Katare N. Site-Directed pegylation of recombinant interleukin-2 at its glycosylation site. Nature Biotechnology.1990;8:343-346.
7. Friman S, Egestad B, Sjövall J, Svanvik J. Hepatic excretion and metabolism of polyethylene glycols and mannitol in the cat. J Hepatol 1993;17:48-55.
8. Kawai F. Microbial degradation of polyethers. Appl Microbiol Biotechnol 2002;58:30-8.
9. Guiotto A, Canevari M, Pozzobon M, Moro S, Orsolini P, Veronese FM. Anchimeric assistance effect on regioselective hydrolysis of branched PEGs: A mechanistic investigation. Bioorg Med Chem 2004;12:5031-7.
10. Bailon P, Palleroni A, Schaffer CA, Spence CL, Fung WJ, Porter JE, *et al.* Rational design of a potent, long-lasting form of interferon: A 40 kDa branched polyethylene glycol-conjugated interferon alpha-2a for the treatment of hepatitis C. Bioconjug Chem 2001;12:195-202.
11. Hedden R, Bauer B, Smith A, Grohn F, Amis E. Templating of inorganic nanoparticles by PAMAM/PEG den-drimer—star polymers. Polmers 2002;43:5473-81.
12. Fen L, Cheng X, Xing C, Chun Y. Synthesis and investigation of PEG supported amino dendrimers. React Funct Polym 2006;66:952-6.
13. Heldt J, Durand N, Salmain M, Vessieres A, Jaouen G. Preparation and characterization of poly(amidoamine) dendrimers functionalized with a rhenium carbonyl complex and PEG as new IR probes for carbonyl metallo immunoassay. J Organomet Chem 2004;689:4775-82.
14. Liu M, Kono K, Fréchet JM. Water-soluble dendritic unimolecular micelles: Their potential as drug delivery agents. J Control Release 2000;65:121-31.

Source of Support: Nil. **Conflict of Interest:** None declared.