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A new approach for PEGylation of dendrimers

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ABSTRACT

Dendrimers have emerged as one of the most interesting themes for researchers as a result of unique functional architecture and macromolecular characteristics. The reported methods of PEGylation are very time consuming and required multisteps for synthesis. In present work we have synthesized PEGylated polyamidoamine (PAMAM) dendrimers using epichlorohydrin as a linker. The PEGylated dendrimers were evaluated for color reaction UV, IR and NMR studies and compared with standard data.

PEGylation defines the modification of a protein, peptide or non-peptide molecule by the linking of one or more polyethylene glycol (PEG) chains. This polymer is nontoxic, non-immunogenic, non-antigenic, and highly soluble in water and FDA approved. The 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] 'PEGylation,' the covalent coupling of polyethylene glycol (PEG) chains to drugs, has been the trailblazing innovation of the last few years. Important pioneering work in this field was performed by Abuchowski et al. (1977)[2] laying the cornerstone for the commercial success of this technology. PEGylation increases the hydrodynamic radius of a biopharmaceutical and shields its surface towards 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 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 polyethylene glycol 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 last 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, in order to assure a reproducible high quality.
- Availability of one single terminal reactive group for the coupling reaction, in order 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, poly dispersity of polyethylene glycol 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 by 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 in order to provide highly reproducible products maintaining maximum activity. In the majority of cases, high molecular weight

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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 by different authors, where the synthesis of PEGylated dendrimers and importance of PEGylation were discussed. The PEGylated dendrimer were found to be better candidate for drug delivery and due to long circulatory chain, it remains in blood and also solubility of chemical entity was found to be better in PEGylated dendrimers. Hedden et al. (2002)[11] prepared PAMAM/PEG conjugation by the reaction of *O*-[2 (vinyl sulfonyl) ethyl]-α-methyl-polyethylene glycol 5000 (Fluka), hereafter called 'PEG-VS', with PAMAM dendrimers. Fen et al. (2006)[12] have synthesized PEG-supported dendrimers with high-loading capacity, excellent solubility and thermal stability by using poly (ethylene glycol) (PEG) as soluble polymeric support, 2,4,6-trichloro-1,3,5-triazine (TCT) as dendrons and ethylenediamine as linkers. Heldt et al. (2004)[13] used the PEG grafted units of dendrimer to improve aqueous solubility. First, 4-fluoro-3-nitrobenzoic acid was coupled to the hydroxyl end group of monomethoxy-polyethylene glycols (mPEGs) in the presence of a catalytic amount of 4-dimethylamino-pyridine to yield esters. Liu et al. (2000)[14] explore the potential of dendritic unimolecular micelles in drug delivery systems.

Yang et al. (2004)[15] used monomethoxy-polyethylene glycols ($mPEG_s$, MW = 2000), tetrahydrofuran (THF), 4-nitrophenyl chloroformate and triethylamine for PEGylation of dendrimers. Akala and Pan (2004)[16] compared the effect of solvents for activation of PEG for PEGylation of dendrimers; they found that acetonitrile gives best result. Okuda et al. (2006)[17] studied and evaluated the physicochemical properties of PEGylated KG6 derivatives after the radio labeling. In order to prevent steric hindrance by PEG chains on the surface of the dendrimer, the DTPA-labeled KG6 was used for PEGylation.[17] Reported all above methods are time consuming and requires various costly chemicals and also extent of PEGylation is not adequate, hence in present work we tried PEGylation of PAMAM dendrimer using epichlorhydrin as a linker. The main drawback of dendrimers is, due to the presence of $-NH_2$ group at the surface, which causes hemolytic toxicity and it is toxic for the blood cells also hence in present work, we tried a new method for PEGylation of dendrimers to overcome this problem.

Synthesis of PAMAM (polyamidoamine) dendrimer was performed by divergent method. Construction of an EDA core PAMAM dendrimers consist 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 polyethylene glycol (PEGylation) was done using epichlorohydrin as a cross-linking agent. Epichlorhydrin used was 1.8 mM (1.8 g) and PEG used was also 1.8 mM (10.8 g), that is, in 1:1 ratio. One hundred milligram (6.3 μM) of lyophilized 4.0 G PAMAM dendrimer was dissolved in methanol. Sixteen molar times of poly ethylene glycol (PEG-600) was mixed with epichlorohydrin in separate container and stirred vigorously for 2 h and incubated for 36 h at room temperature (rt) 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 (Scheme. 1).

Identification of dendrimers was done by 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 was analyzed by UV–vis 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 λ_{max} values of plain and PEGylated system were observed.

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

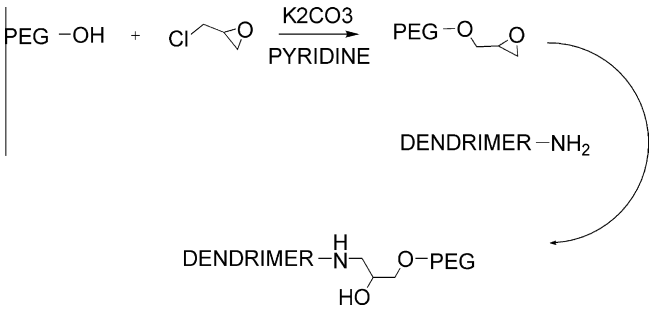
The sample was analyzed by NMR spectroscopy. The 4.0 G and PEGylated dendrimers were solubilized in D_2O using methanol as co-solvent and analyzed at 300 MHz various shifts in the peaks were observed, which were interpreted for different groups present in PEGylated system.

Further identification of simple and PEGylated dendrimers was carried out by using mass spectra. In case of reaction with copper sulfate the plain dendrimers gives violet color because of surface $-NH_2$ group, whereas after PEG attachment, it shows deep blue color due to absence of surface $-NH_2$ group.

The dendrimers were subjected to UV–vis spectrophotometer (Shimadzu-1700) for scanning where the changes in λ_{max} values were analyzed. The change in λ_{max} values from 283 to 353 nm was observed, which indicated the change in structure of dendrimers.[18]

The formed 4.0 G and PEGylated system were subjected to IR spectroscopy analysis by FTIR-470 Plus (Jasco, Japan). The IR peaks[18] confirmed the progress of PEGylation on dendrimers. The important peaks in IR spectrum of 4.0 G dendrimers was of N–H stretch of primary amine at 3310.21 cm^{-1}, N–H stretch of anti symmetric substituted primary amine at 3021.87 cm^{-1} and C–H stretch at 2947.66 cm^{-1}. In IR spectrum of PEGylated dendrimer, peak of C–O at 1100 cm^{-1} 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 anti symmetric peaks at 3021.87 cm^{-1} 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 PEGylation of dendrimer was further confirmed by NMR spectra. 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 co-solvent and analyzed at 300 MHz. Important shifts in NMR spectrum of 4.0 G dendrimers was 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



Scheme 1. PEGylation of dendrimers using epichlorohydrin as linking agent.

Table 1
IR interpretation of 4.0 G dendrimers

S.No.	Wave no. (peak) (cm^{-1})	Interpretation
1	3310.21	N–H stretch of primary amine
2	3022.87	N–H stretch anti symmetric primary amine
3	2947.66	C–H stretch
4	1668.12	C=O stretch of carbonyl group
5	1511.92 1417.42	N–H bending of N-substituted amine
6	1215.90	C–C bending

Table 2
IR interpretation of PEGylated dendrimers

S.No.	Wave no. (peak) (cm^{-1})	Interpretation
1	3434.6	N–H stretch of primary amine
2	3022.87	Carbonyl symmetric and anti symmetric peaks
3	2399.98	Carboxylic acid C=O and O–H stretch unconjugated
4	1473.25	CH–NH–C(=O) amide bending
5	1211.08	Ester unconjugated C=O and C–O stretching
6	1103.08	C–O stretch ether linkage strong and sharp
7	767.53	Aromatic C–H bending

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

S.No.	δ values range (ppm)	Interpretation
1	2.401, 2.425	Carbonyl (CH_2C=O)
2	2.539, 2.921	Amide –NH
3	3.164, 3.434	$-CH_2NH_2$ terminal group
4	4.822	–OH, methanolic due to residual solvent

and 4.82 for the –OH methanolic group. The NMR spectra[18] and shifts of PEGylated dendrimer as compared to simple dendrimers 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.

In the mass spectrum[18] of plain dendrimer, the m+1 peak was observed at 14652.36 whereas the calculated mass number of 4 G PAMAM was found to be at 14354.43. In MALDI TOF, one or two molecules of matrix may cause interference in the spectra of compound, the matrix used for mass spectra (MS) was 2,5-dihydroxy benzoic acid, which is soluble in $CHCl_3$, and may be responsible for this difference between observed and calculated values of mass number of 4 G PAMAMA dendrimer.

The plain dendrimers having molecular weight 14,500 and 16 groups of PEG-600 were attached in PEGylation. The PEGylated system showed the peak at 24067.28 and molecular weight was found to be 24,100. Hence there is expected change in mass of dendrimers after PEGylation, which indicated PEGylation was done and only 16 groups were attached.

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

S.No.	δ values range (ppm)	Interpretation
1	1.165–1.677	R_2CH_2 (secondary)
2	2.041–2.274	($-CH_2C$=O) carbonyl
3	3.583–3.880	Ether linkage
4	3.224–3.463	$-CH_2-NH_2$ (remaining free amines)
5	4.895, 5.127	Amide (–C=O–NH)

From the results of present study, it could be concluded that PEGylation is easily possible using epichlorohydrin as a linker gives considerable level of attachment of PEG to dendrimers and also a easy, reproducible and inexpensive method, which requires less time and proves to be a superior technique for PEGylation of dendrimers.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.100.

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