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ORIGINAL ARTICLE

Different approaches for determination of the attachment degree of polyethylene glycols to poly(anhydride) nanoparticles

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Abstract

Purpose: The aim of the study was to find an appropriate method for determination of the attachment degree of polyethylene glycol (PEG) to poly(anhydride) nanoparticles. *Methods*: The nanoparticles were modified with hydroxy-functionalized or with amino-functionalized PEGs. Three methods for their determination were applied and examined: in particular colorimetry, nuclear magnetic resonance (^{1}H-NMR), and elemental analysis (EA). *Results*: The attachment degrees determined by ^{1}H-NMR and colorimetry were similar. The associated amounts of PEGs estimated on the basis of EA differed significantly than those determined by colorimetry and ^{1}H-NMR. *Conclusion*: The colorimetric determination was considered fast and simple technique, but ^{1}H-NMR contributed to more precise results.

Key words: *Colorimetry; elemental analysis; nuclear magnetic resonance; pegylated nanoparticles; polyethylene glycol*

Introduction

Nanoparticles have been widely investigated as drug carriers for various routes of application such as oral, ocular, and parenteral. Independent on the type of administration, rapid nanoparticle elimination frequently occurred. The elimination mechanism includes phagocytosis (parenteral application), mucous turnover (after oral application), or nasolacrimal drainage (ocular administration). Various properties of nanoparticles such as their size, charge, and hydration may influence the nanoparticle residence time. In this view, the formation of a coating layer around nanoparticles is a promising approach intended for the achievement of suitable surface modification. Many studies have demonstrated that the surface modification with suitable polymers (e.g., polyethylene glycol (PEG), poloxamers, and poloxamines) enhanced the nanoparticle resistance against phagocyte uptake after parenteral application[1–3]. Regarding oral, nasal, and ocular administration, greater capacity of the modified nanoparticles for interaction with a certain mucosa was observed[4]. PEG and its derivatives have been considered more effective and the most commonly used among various coating agents. The surface modification with PEG, frequently denoted as pegylation, could solve many of the specific problems accompanying the various routes of nanoparticle administration. For instance, the radioactivity recovered in the blood 1 hour after the nasal administration of pegylated PLA nanoparticles was found to be 10-fold higher than that observed for nonpegylated PLA particles, demonstrating higher penetration of the pegylated particles through nasal mucosa[5].

The pegylation of the nanoparticles has been achieved by either covalent grafting or simple adsorbing of PEG chains bearing different functional groups like hydroxyl or amino groups. The layer thickness, surface chain density, and chain conformation are

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very important factors, determining in a high-degree nanoparticle behavior when in contact with mucin, various cells, or opsonins[3,6,7]. For instance, the low surface coating could lead to appearance of unprotected areas on the nanoparticle surface where opsonins could freely adhere. On the contrary, the high-surface coverage could hinder the mobility of PEG chains and, consequently, decrease the steric stabilization properties of the PEG layer[8].

Taking into account these considerations, the precise evaluation of both conformation state and amount of the efficiently attached PEG seemed very important. The aim of this study was to examine and compare the three methods for determination of the degree of PEG attachment to poly(anhydride) nanoparticles.

Materials and methods

Materials

Polyethylene glycol 2000, monomethyl ether of PEG 2000 (mPEG), *O,O′*-bis-(2-aminoethyl) polyethylene glycol 2000 (DAE-PEG), and *O,O′*-bis-(2-aminopropyl)-polypropylene glycol-polyethylene glycol-polypropylene glycol 2000 (DAP-PEG) were supplied by Fluka (Buchs, Switzerland). Poly(methyl vinyl ether-co-maleic anhydride) (PVM/MA) (Gantrez AN 119, Mw of 200 kDa) was a gift from ISP (Barcelona, Spain). Micro BCA Protein Assay Reagent Kit was obtained from Pierce (Rockford, IL, USA).

Preparation of pegylated PVM/MA nanoparticles

PVM/MA (100 mg) and different amounts (5, 10, or 25 mg) of PEG (PEG, mPEG, DAE-PEG, or DAP-PEG) were dissolved and stirred in acetone (5 mL) for 1 hour. After their incubation, 10 mL of a hydroalcoholic mixture (1:1, v/v) was added to the organic phase. The solvents were eliminated under reduced pressure (Buchi R-144, Postfach, Switzerland) and the freshly formed nanoparticles were centrifuged (2 times) at 52,600 × *g* for 20 minutes (Sigma 3K30, Osterode am Harz, Germany). Finally, the nanoparticle dispersion was lyophilized (Genesis 12-EL, Virtis, Gardiner, NY, USA) using sucrose as cryoprotector (5%, w/v).

The nanoparticle size and the zeta-potential were determined by photon correlation spectroscopy and electrophoretic laser Doppler anemometry using a Zetamaster analyzer (Malvern Instruments, Worcestershire, UK). The samples were diluted with 0.05 M phosphate-buffered saline (pH 7.4) and measured at 25°C at a scattering angle of 90°.

Determination of PEG attachment

Colorimetric method

The colorimetric assay was based on the measurement of nonassociated PEG in the supernatants after centrifugation of the freshly formed nanoparticles. The association degree was determined by the difference between the nonassociated and the total amount of the PEG used for preparation. Thus, 15 μL of iodine solution (10 mg/mL of iodine and 20 mg/mL of potassium iodine) is added to 1 mL of the supernatant obtained after nanoparticle purification. The absorbance of the complex was observed at 540 nm (Labsystems iEMS Reader MF, Vantaa, Finland). The calculations were made using a standard curve of PEG prepared under the same conditions (2–10 μg/mL, $r = 0.9994$) or mPEG (2–10 μg/mL, $r = 0.9995$).

In the case of DAE-PEG and DAP-PEG, the colorimetric method was based on the determination of their amino groups by Micro BCA Protein Assay Reagent Kit (Pierce). Equal volumes (150 μL) of the supernatants and the reagent were mixed in test plates and the samples were incubated at 37°C. The absorbance of the samples was determined after 2 hours at wavelength of 570 nm (Labsystems iEMS Reader MF). The calculations were made using standard curves of DAE-PEG and DAP-PEG prepared under the same conditions at concentration range of 2–10 mg/mL (for DAE-PEG $r = 0.9958$ and for DAP-PEG $r = 0.9993$, respectively).

Nuclear magnetic resonance

The amounts of the PEG attached to the nanoparticles were also determined by nuclear magnetic resonance (^{1}H-NMR) method (Brucer Avance 400, Rheinstetten, Germany). Thus, exactly weighted amounts of pegylated nanoparticles (5 mg) were dissolved in deuterated DMSO (0.5 mL) and the spectra were obtained at $n_s = 6400$ and 12800 (for DAE-PEG-NP and DAP-PEG-NP). ^{1}H-NMR spectra of free PEGs were performed using the same ratio and experimental conditions. The quantity of PEG attached to nanoparticles was calculated by the ratio of peak areas of the protons of ethylene units (3.51 ppm) detected in the spectra of pegylated particles to the free PEG.

Elemental analysis

The calculation of PEG association was made on the basis of the different hydrogen amounts in free PEGs and in pegylated nanoparticles obtained by elemental analysis (EA) (Leco CHN-900, St. Joseph, MI, USA).

Statistical analysis

One-way analyses of variance at α-level of 0.05 were performed to compare the associated amounts (μg/mg nanoparticles) of PEG (PEG, mPEG, DAE-PEG, or DAP-PEG) determined by the three tested methods—colorimetry, NMR, and EA using Origin 7.0 SR0 software (OriginLab Corporation, Northampton, MA, USA). The null hypothesis test is the hypothesis of equal means, H_0: $\mu_T = \mu_R$ (i.e., the population means are not significantly different), where μ_T and μ_R represent the expected mean difference of the test and reference formulations, respectively. The alternate hypothesis therefore is H_1: $\mu_T \neq \mu_R$ (i.e., the population means are significantly different).

Results and discussion

Pegylation technology has been widely applied taking into account the advantages of the resulting modified nanoparticle carriers compared with conventional particulate systems. To achieve the desired specific properties of the pegylated particles, it is of high importance to control the association degree of PEG. In this study, three different methods for determination of pegylation degree, in particular, colorimetry, ^{1}H-NMR, and EA were performed and the results were compared.

Pegylated nanoparticles based on PVM/MA were obtained by simple solvent displacement method. Pegylated nanoparticles were formulated by participation of hydroxy-functionalized PEGs (PEG and mPEG) or amino-functionalized PEGs (DAP-PEG and DAE-PEG). The pegylation occurred as a result of nucleophile reaction between functional groups of PEGs and anhydride residues of the copolymer. The pegylation achieved with both hydroxy-functional PEGs (PEG and mPEG) showed that the initial concentration did not influence significantly final association degree (Figures 1 and 2). On the contrary, the increase in the initial concentration of amino-functionalized DAE-PEG and DAP-PEG resulted in higher association (Figures 3 and 4). This fact was explained with the higher affinity of acid anhydride residues of PVM/MA copolymer to the amino groups compared to hydroxyl groups[9].

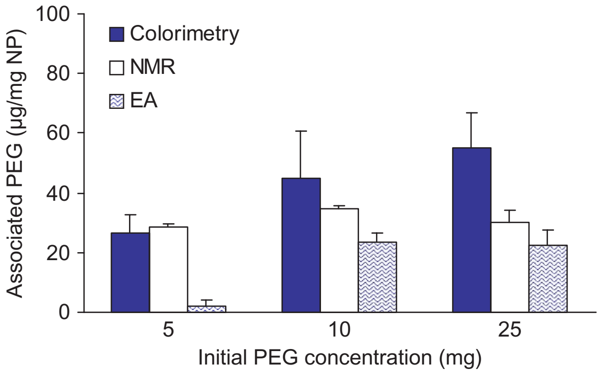

Figure 1. Determination of the association degree of PEG to poly(anhydride) nanoparticles. Mean ± SD ($n = 3$).

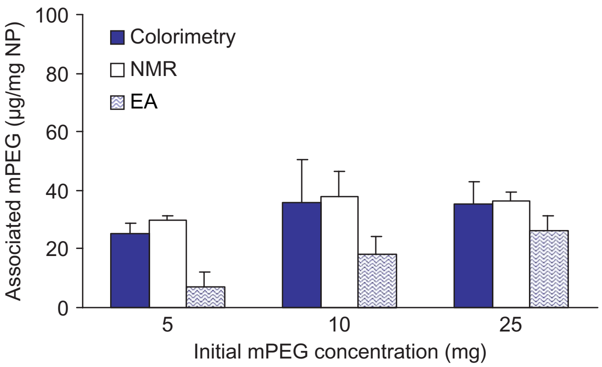

Figure 2. Determination of the association degree of mPEG to poly(anhydride) nanoparticles. Mean ± SD ($n = 3$).

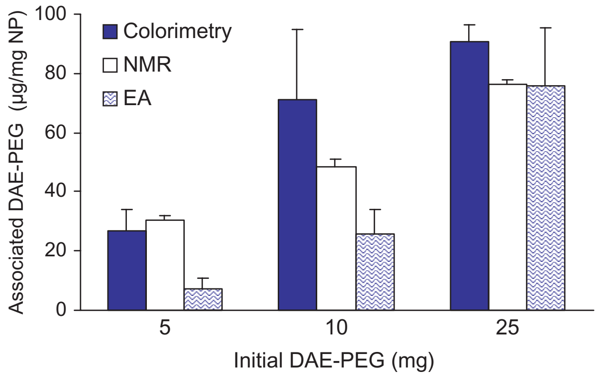

Figure 3. Determination of the association degree of DAE-PEG to poly(anhydride) nanoparticles. Mean ± SD ($n = 3$).

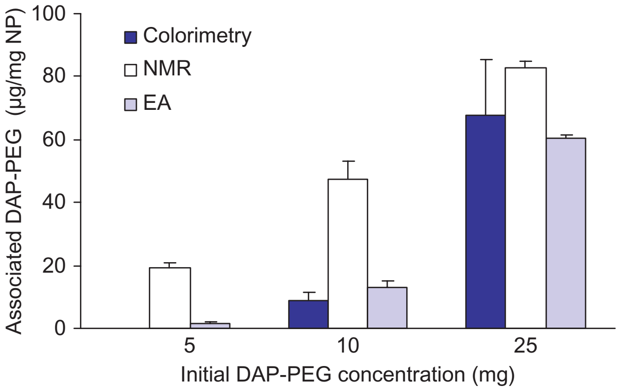

Figure 4. Determination of the association degree of DAP-PEG to poly(anhydride) nanoparticles. Mean ± SD ($n = 3$).

The main physicochemical properties of the pegylated nanoparticles are presented in Table 1. As shown, all types of the pegylated particles demonstrated lower

Table 1. Physicochemical properties of the nanoparticles modified with different types of polyethylene glycols (initial concentration of 10 mg).

Nanoparticles	Size (nm)	Polydispersity	Zeta-potential (mV)
PEG-NP	317 ± 5	0.218	−7.8 ± 0.5
mPEG-NP	254 ± 9	0.128	−19.7 ± 7.4
DAE-PEG-NP	387 ± 23	0.296	−11.9 ± 3.5
DAP-PEG-NP	347 ± 7	0.089	−4.1 ± 1.7
NP	289 ± 11	0.101	−58.8 ± 4.5

Data express the mean ± SD (n = 3). NP, nonmodified PVM/MA nanoparticles.

surface charge compared to that of the nonmodified particles. This observation was considered as indication for the surface attachment of PEG chains. The statement is in accordance with another study, reporting that the negative charge of poly(lactic acid) particles was shielded when they were modified with PEG[2]. The presence of PEG chains on the nanoparticle surface may shift the shear plane of the diffusion layer to a greater distance from the nanoparticles, leading to a decrease in the absolute value of zeta-potential.

The colorimetric determination of PEGs is based on the complex formation with a participation of iodine[10]. Interestingly, the colorimetric technique was even adapted for the defining of the surface conformation of mPEG chains[11]. The colorimetric determination could be made after nanoparticle hydrolysis or in a supernatant obtained after purification of freshly formed nanoparticles. In this study, the second technique was applied, because of its simplicity. Colorimetric determination of the standards (PEG and mPEG) showed good linearity in a certain concentration range (2–10 µg/mL). However, the determination of the attachment degrees of PEGs was characterized with relatively large deviations (Figures 1 and 2). The factors that influenced the measurement were associated with the properties of the complex. First, the complex tended to precipitate at higher concentrations of PEG and mPEG (higher than 10 µg/mL), which presumed appropriate dilutions of the samples. Second, the color intensity of the complex changed with time, which required fixing of the procedure time. The indirect determination by measurement of the unassociated fractions of PEGs also diminished the accuracy of the results.

Colorimetric determination of DAE-PEG and DAP-PEG was based on another mechanism, in particular the functional amino groups of DAE-PEG and DAP-PEG were determined through reaction with Micro BCA protein reagent. Determination of the attachment degree of both amino-PEGs was characterized with relatively small deviations (Figures 3 and 4). However, the limitation of this approach appears when nanoparticles modified with amino-PEG are loaded with another molecule possessing amino group. In our experiments, the amino-pegylated nanoparticles (DAP-PEG-NP) were loaded with model molecule as ovalbumin. In this case, the colorimetric determination gave the total amount of the nonassociated amino groups of DAP-PEG and ovalbumin.

Because of these limitations, other methods have been tested for evaluation of PEG association degree. In particular, ^{1}H-NMR and EA were carried out. The advantage of both methods is the direct determination of PEGs associated to the formulated nanoparticles. The measurement of the specific signal of ethylene units (3.51 ppm) by ^{1}H-NMR contributed to the characterization of pegylated particles. According to literature, NMR has been performed to estimate the copolymer composition, particularly for the grafted copolymers[12,13], or to hypothesize the surface conformation of PEG chains[7]. In the latter study, the surface conformation of PEG chains ('brush' or 'loop') was determined by following the quantitative changes of the protons of OH groups (or NH_2 groups) after nanoparticle pegylation. Here, the quantitative analysis was based on the data obtained for the intensity of the typical peak of ethylene units in the spectra of PEG standards and nanoparticle samples, respectively. The results illustrated values for PEG association close to those found by colorimetry (Table 2). Thus, independent of the different mechanisms of both determinations, nonassociated PEG measured by colorimetry or associated PEG found by NMR, the final values were in accordance. In contrast, the associated amounts of PEGs estimated on the basis of the data of EA differed significantly (Table 2). The calculations were made taking the values for hydrogen percent in PEG or mPEG as standard and deducting its percent found for the nonpegylated particles. Hence, one of the reasons for the different results could be due to the indirect mechanism of calculation. However, even in the case of amino-pegylated nanoparticles, where calculations were performed on the basis of nitrogen percent, the values were different compared to those computed by colorimetry or NMR spectroscopy (Figures 3 and 4). Thus, it seemed that EA was less appropriate for the determination of PEG association.

Table 2. Statistical analysis of the data obtained by the three methods: colorimetry, nuclear magnetic resonance (^{1}H-NMR), and elemental analysis (EA).

Compared amounts	F-ratio (degree of freedom)	P	F_{critical} at $\alpha = 0.05$	Decision at $\alpha = 0.05$
Colorimetry/ NMR	0.246 (1.67)	0.621	3.98	Means are not significantly different
Colorimetry/ EA	7.810 (1.53)	0.007	4.02	Means are significantly different
NMR/EA	8.312(1.56)	0.006	4.01	Means are significantly different

Taking into account that the data obtained from NMR spectra could contribute additionally to determination of the way of PEG association, NMR was considered more appropriate method providing quantitative and qualitative characterization of pegylated nanoparticles.

Colorimetric assay was considered as fast and simple evaluation of the attachment degree of hydroxy- or amino-functionalized PEG. ^{1}H-NMR contributed to more precise results independently free or bound were the functional groups of PEGs.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

References

1. Stolnik S, Davies MC, Illum L, Davis SS, Boustta M, Vert M. (1994). The preparation of sub-200 nm colloidal particles from poly(malic acid-co-benzoyl malate) copolymers and their surface modification with poloxamer and poloxamine surfactants. J Control Release, 30:57–67.
2. Gref R, Domb A, Quellec P, Blunk T, Muller RH, Verbavatz JM, et al. (1995). The controlled intravenous delivery of drugs using PEG-coated sterically stabilized nanospheres. Adv Drug Deliv Rev, 16:215–33.
3. Owens D, Peppas N. (2006). Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. Int J Pharm, 307:93–102.
4. Yoncheva K, Gomez S, Campanero MA, Gamazo C, Irache JM. (2005). Bioadhesive properties of pegylated nanoparticles. Expert Opin Drug Deliv, 2:205–18.
5. Tobio M, Gref R, Sanchez A, Langer R, Alonso MJ. (1998). Stealth PLA-PEG nanoparticles as protein carriers for nasal administration. Pharm Res, 15:270–5.
6. Yoncheva K, Lizarraga E, Irache JM. (2005). Pegylated nanoparticles based on poly(methyl vinyl ether-co-maleic anhydride): Preparation and evaluation of their bioadhesive properties. Eur J Pharm Sci, 24:411–9.
7. Yoncheva K, Guembe L, Campanero MA, Irache JM. (2007). Evaluation of bioadhesive potential and intestinal transport of pegylated poly(anhydride) nanoparticles. Int J Pharm, 334:156–65.
8. Storm G, Belliot SO, Daemen T, Lasic DD. (1995). Surface modification of nanoparticles to oppose uptake by the mononuclear phagocyte system. Adv Drug Deliv Rev, 17:31–48.
9. Pompe T, Zschoche S, Herold N, Salchert K, Gouzy MF, Sperling C, et al. (2003). Maleic anhydride copolymers—a versatile platform for molecular biosurface engineering. Biomacromolecules, 4:1072–9.
10. Sims GEC, Snape TJ. (1980). A method for the estimation of polyethylene glycol in plasma protein fractions. Anal Biochem, 107:60–3.
11. Bazile D, Prud'homme C, Bassoullet M, Marlard M, Spenlehauer G, Veillard M. (1995). Stealth Me.PEG-PLA nanoparticles avoid uptake by the mononuclear phagocytes system. J Pharm Sci, 84:493–8.
12. Olivier JC, Huertas R, Lee HJ, Calon F, Pardridge WM. (2002). Synthesis of pegylated immunonanoparticles. Pharm Res, 19:1137–43.
13. Dong Y, Feng SS. (2004). Methoxy poly(ethylene glycol)-poly(lactide) (MPEG-PLA) nanoparticles for controlled delivery of anticancer drugs. Biomaterials, 25:2843–9.