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New versatile approach for analysis of PEG content in conjugates and complexes with biomacromolecules based on FTIR spectroscopy.

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ABSTRACT

Here we report a new approach based on FTIR-spectroscopy for determining the degree of PEGylation in biomolecules. We show that the PEG C—O—C peak (at 1089 cm^{-1}) is the main analytically valuable band in IR spectra of PEG-containing systems: it is narrow and highly intense, it is well distinguished from absorption bands of other principal functional groups of proteins and other biopolymers (carbonyl, amide, hydroxyl etc), and therefore is easily identified in the IR spectra. The proposed method is therefore "reagent-free" and allows for fast and accurate determination of the PEGylation degree of biomolecules as well as the structural characteristics of bioconjugates from a single FTIR spectrum. The measurement is not dependent on PEG polymerization degree or branching and can be applied in a wide pH range, making it a convenient replacement of laborious and unreliable methods. The developed approach was successfully used to study the range of PEG-containing covalent conjugates with chitosan and non-covalent complexes with anionic liposomes. The composition of PEG-chitosan conjugates as well as their storage stability was determined by the method based on FTIR-spectroscopy. In the case of non-covalent complexes, not only PEG content, but also the binding constants of PEG-containing ligands to the liposome membrane were evaluated with this approach. The results obtained by the FTIR method were confirmed by DLS and zeta-potential experiments where the formation of electrostatic complex was monitored by the increase in the particle radius (from 80 nm to 105 nm) and zeta-potential neutralization (from −20 mV to −12 mV). Direct comparison of the results of FTIR approach with that of TNBS or OPA titration methods shows very good agreement between the measurements, with the FTIR method showing much lower deviation.

1. Introduction

Biopharmaceutical preparations, including recombinant proteins and enzymes, monoclonal antibodies, cytokines, hormones, polysaccharides, and others, constitute the most promising class of therapeutic agents. A number of preparations have already proved to be valuable drugs against several pathologies [1]. Their benefits, however, are not always fully realized due to side effects such as immunogenicity [2], as well as due to the limited half-life in blood stream.

The main approach that has been put into practice to solve this problem is conjugation with PEG molecules [1,3]. PEGylation is effective in extending a drug's half-life by decreasing kidney clearance, and shields sensitive sites at the biopolymer surface, such as antigenic epitopes and enzymatically degradable sequences. Thus, PEGylation has become a well-established technology used to ameliorate the pharmacokinetic properties of biopharmaceuticals [4]. Presently, several PEGylated proteins and enzyme preparations, namely PEG-L-asparaginase (Oncaspar), PEG-interferons (PEG-Intron and PEGASYS) and PEG-EPO (Mircera) as well as PEGylated liposomal drugs (Stealth-liposomes) (Doxil, Caelix) [5] are approved by the FDA as therapeutics. Furthermore, other PEGylated preparations, either low molecular weight drugs Pegamotecan (Enzon Pharmaceuticals, Inc.), peptides or proteins, such as PEG-granulocyte colony stimulating factor (PEG-filgrastim) [6], PEG-L-methioninase [7], PEG-arginine deiminase [8], PEG-arginase [9], PEG-uricase [10] etc. are currently in clinical studies.

To control the PEGylation degree in biomolecules one might use NMR-analysis, HPLC or MALDI-TOF MS methods [11–14]. Alternatively, for PEGylated proteins and aminopolysaccharides, methods based on titration of non-reacted amino groups of

Abbreviations: FTIR, fourier transformed infrared spectroscopy; TNBS, 2,4,6-trinitrobenzenesulfonic acid; CL, cardiolipin; DPPC, dipalmitoylphospatidylcholine; ATR, attenuated total reflection.

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biomolecules with orthophtalic aldehyde [15], TNBS [15,16] or ninhydrin reagents [17] are usually applied.

A common characteristic in this wide variety of protocols, is that they are time demanding, laborious and in many cases inaccurate due to background chemical reactions. A great challenge in bioanalytical chemistry is to determine PEGylation degree in high-molecular weight compounds, such as polysaccharides. The reaction rate in such systems depends on many factors; including the conformational state of a biopolymer in a specific system, and non-equilibrium colloid formation, both of which can result in an underestimation of PEGylation degree [18]. Free amino group titration methods also require the process pH to be in a certain range (9, 0 or higher), which is not always acceptable, as some aminopolysaccharides are insoluble in such conditions.

In the following study, we propose a new versatile approach for analyzing PEGylated conjugate composition based on FTIR spectroscopy. FTIR is robust and highly informative method for studying the structure and composition of biomolecules [19–22]. The important advantage of the proposed approach over existing methods is the possibility to analyze samples in solution, in suspension, as well as in other heterogeneous and microheterogeneous systems such as polymeric micelles, liposomes, hydrogels, and others. It is demonstrated here that the proposed method of degree of PEGylation analysis based on FTIR is highly specific, highly sensitive and rapid, thus, it could be used in routine characterization of PEG conjugates of different nature and composition.

2. Experimental

2.1. Materials

Samples of chitosan with a molecular weight (MW) of 15 and 90 kDa and deacylation degree of 12–15% were acquired from Bioprogress (Russia), the activated derivative of polyethylene glycol, monomethoxypolyethyleneglycolyl-*N*-hydroxysuccinimidyl-succinate (mPEG-suc-NHS) with a molecular weight 5 kDa was kindly provided by N.S. Melik–Nubarov (Faculty of Chemistry, Lomonosov MSU, Moscow), mPEG-OH MW 0, 4, 5, 20 kDa were obtained from Loba Feinchemie (Austria). Ethanol (96%), dimethylformamide, dimethylsulfoxide, other solvents, and TNBS were obtained from Sigma (USA). Cardiolipin disodium salt in chloroform (25 mg/ml) and dipalmitoylphosphatidylcholine in chloroform (25 mg/ml) were both from Avanti Polar Lipids (USA).

2.2. Synthesis of PEG-chitosan copolymers

Synthesis of chitosan-PEG copolymers was conducted using chitosan (MW 15 and 90 kDa) and mPEG-suc-NHS (MW 5 kDa) by the previously described procedure [20]. Briefly, chitosan was dissolved in 3% acetic acid, pH 4,0. After obtaining a clear solution its pH was adjusted to 6,5 with 5 mM sodium phosphate buffer. The resulting solution was mixed with a 5-35-fold molar excess of mPEG-suc-NHS in DMSO (per mole of deacylated amino groups of chitosan) added drop wise under stirring. During the reaction pH of the solution was adjusted to 7, 5 with sodium phosphate buffer. The reaction was carried out for 1–4 h and the target product was purified by dialysis against 5 mM sodium phosphate buffer, pH 7, 0.

Purification was carried out by HPLC, in a Knauer Smatline chromatography system (Germany) with BioFox 17 SEC agarose column, volume 15 ml. The eluent was phosphate buffered saline (15 mM phosphate, pH 7, 5, 150 mM NaCl); the elution rate was 0,5 ml/min, 25 °C. The purity was additionally verified by FTIR and UV spectroscopy.

2.3. Determination of the degree of modification of the copolymers by TNBS assay

The degree of modification of the copolymer was determined by a method based on the reaction between the free amino groups and TNBS [16]. Kinetic curves of the copolymer-TNBS adduct formation were measured at a wavelength of 420 nm. Degree of modification was calculated in percent through concentration of titrated amino groups in chitosan and copolymers. The concentration of amino groups in unmodified chitosan was considered as 100%. Concentration of colored product was determined via UV–vis-spectroscopy, using a UltroSpec 2100 spectrophotometer, Amersham Biosciences, thermostat Lauda E100.

2.4. Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared (FTIR) spectra were recorded using a Bruker Tensor 27 spectrometer equipped with a liquid nitrogen cooled MCT (mercury cadmium telluride) detector. Samples were placed in a thermostated cell BioATR-II with ZnSe ATR (attenuated total reflection) element (Bruker, Germany). The FTIR spectrometer was purged with a constant flow of dry air. FTIR spectra were acquired from 900 to 4000 cm^{-1} with 1 cm^{-1} spectral resolution. For each spectrum, 100 scans were accumulated at 20 kHz scanning speed and averaged. All spectra were registered in aqueous buffer in deionized water at 22 °C, pH was varied. Spectral data were processed using the Bruker software system Opus 7, 5 (Bruker, Germany), which includes linear blank subtraction, straight-line baseline correction and atmospere compensation. If necessary, seven- or nine-point Savitsky–Golay smoothing was used to remove white noise. Peaks were identified by standard picking-peak procedure [23,24].

2.5. Determination of the PEGylation degree of copolymers by FTIR spectroscopy

This method is based on analysis of intensity of PEG C—O—C absorption band at 1089 cm^{-1} in the FTIR spectrum. This band is narrow and highly intense; it is well distinguished from absorption bands of other principal functional groups of biopolymers including amide I and amide II in protein IR spectra; CH_2 valence oscillation bands at 2930–2850 cm^{-1} region, carbonyl band at 1750–1700 cm^{-1} and asymmetric valence oscillation band of phosphate at 1210–1270 cm^{-1} in a lipid spectra.

Modification degree was calculated from the intensity of the absorption band of C—O—C in the copolymer spectra with a calibration curve. The calibration curves associated the intensity of the absorption band at 1089 cm^{-1} with the concentration of PEG chain. These were constructed with samples of mPEG-OH of different MW. All spectra were registered for polymers solutions in 5 mM sodium phosphate buffer, pH 7,0; 22 °C.

2.6. Liposomes-copolymer complex preparation

Liposomes contained of DPPC and cardiolipin 80:20 (5 mg/ml), ionic strength of solution 0,05 M, obtained by sonication method [25] and copolymer PEG-chitosan 90 kDa DM 25%, were mixed in a base-molar ratio from 1:0, 25 to 1:10. The final total volume was 1 ml, with a final lipid concentration of 3 mg/ml, and the final concentration of polymer was varied.