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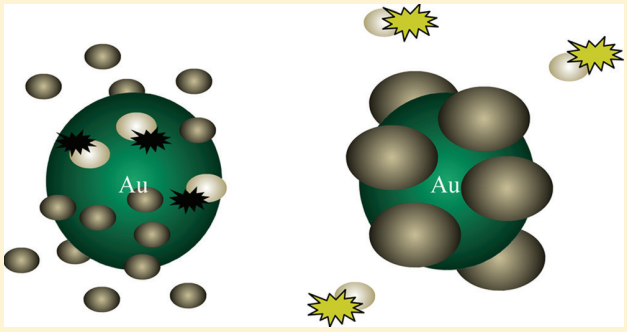
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Method for Determination of Polyethylene Glycol Molecular Weight

Sari Pihlasalo,* Pekka Hänninen, and Harri Härmä

Department of Cell Biology and Anatomy and Medicity Research Laboratory, Institute of Biomedicine, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland

ABSTRACT: A method utilizing competitive adsorption between polyethylene glycols (PEGs) and labeled protein to nanoparticles was developed for the determination of PEG molecular weight (MW) in a microtiter plate format. Two mix-and-measure systems, time-resolved luminescence resonance energy transfer (TR-LRET) with donor europium(III) polystyrene nanoparticles and acceptor-labeled protein and quenching with quencher gold nanoparticles and fluorescently labeled protein were compared for their performance. MW is estimated from the PEG MW dependent changes in the competitive adsorption properties, which are presented as the luminescence signal vs PEG mass concentration. The curves obtained with the TR-LRET system overlapped for PEGs larger than 400 g/mol providing no information on MW. Distinctly different curves were obtained with the quenching system enabling the assessment of PEG MW within a broad dynamic range. The data was processed with and without prior knowledge of the PEG concentration to measure PEGs over a MW range from 62 to 35 000 g/mol. The demonstration of the measurement independent of the PEG concentration suggests that the estimation of MW is possible with quenching nanoparticle system for neutrally charged and relatively hydrophilic polymeric molecules widening the applicability of the simple and cost-effective nanoparticle-based methods.



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Molecular weight (MW) is a key physical property for a polymeric molecule, and thus, simple and reliable methods are required for its determination at wide MW range. Size exclusion chromatography (SEC) and light scattering are widely used in industry for determination of polymer MWs. Other commercially available methods include electrophoresis, NMR, the measurement of viscosity, mass spectrometry, and ultracentrifugation.[1−14] However, most of the methods are not suitable for routine analysis needed in industry nor for high throughput screening. The methods can be time-consuming,[3,4] have limitations for low or high MW range,[15] and might require analyte concentrations as high as few grams per liter,[12] expensive equipment, and expertise.[7−9] Thus, there is a need to develop cost-effective and simple methods for fast routine analysis purposes.

Neutral organic polymers, such as poly(vinyl alcohol), polyvinylpyrrolidone, polyethylene glycol (PEG), poly(ethylene oxide), and dextran, are widely used and produced, e.g., in medicine, biomedical laboratories, and by various industries such as paper, textile, pharmaceutical, food, and cosmetic industry. The determination of PEG MW is important, as MW affect its properties, such as viscosity, melting point, solubility, degradation, and hygroscopicity.[16] Low MW PEGs are rapidly removed from human body making them suitable for pharmaceutical and food products.[17,18] Instead, the increased MW causes reduced kidney excretion and prolonged circulation of the drug in the blood.[18] PEGs have also different benefits to personal care and cosmetic products depending on their MW.[19] PEG MW is also optimized for the performance in different applications. PEG 6000 is effective in virus purification, whereas lower MW decreases the effectiveness in concentration of viruses.[20] Moreover, the PEG MW affects the biological or enzymatic activity of PEG−protein conjugates.[21]

Determination of neutral or charged polymers may need different protocols or there might be other limitations, if for instance the charge of the analytes and calibrators differs. Electrostatic interactions affect the properties of the charged polymers, and thus the determination of MW depends on the presence of charge, especially for methods, such as measurement of viscosity and diffusion, which utilize a correlation between MW and size.[22] In SEC, intermolecular interactions and adsorption to the column packing or the electrostatic interaction with the packing might lead to wrong estimation of MW.[23] In vapor phase osmometry, the counterions are also detected, and thus MW of the charged polymers is underestimated. In gel electrophoresis, the migration and the MW determination of neutral polymers is enabled with the binding of the sodium dodecyl sulfate. However, the binding to the neutral polymers can be relatively weak, identical charge-to-mass ratios are not achieved, and the resolution of the gel is

Received: December 18, 2014
Accepted: March 18, 2015
Published: March 18, 2015

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DOI: 10.1021/acs.analchem.5b00736
Anal. Chem. 2015, 87, 3918−3922

low.[24] The method may fail also with low MW polymers due to the low binding of sodium dodecyl sulfate to small polymers.[24]

Previously, we have developed the nanoparticle-based methods utilizing time-resolved luminescence resonance energy transfer (TR-LRET) and quenching for the quantification of proteins,[25−27] eukaryotic cells,[28] and detergents.[29,30] Recently, we demonstrated the novel application of the nanoparticle-based method utilizing TR-LRET for the determination of MW for polyethylenimines and polyamino acids.[31] The method utilized the size-dependent competitive adsorption between differently sized polymers and labeled protein to nanoparticles. These molecules have a total positive charge at the assay pH and polyamino acids contain hydrophobic sites giving optimal attractive electrostatic and hydrophobic interactions for adsorption to carboxylate-modified polystyrene particles. In this paper, we widened the nanoparticle-based application to hydrophilic PEGs having zero total charge. MWs for PEGs could be estimated with quenching nanoparticle system. The determination is based on the size-dependent adsorption to the nanoparticles (Figure 1). In the absence of analyte PEG, the

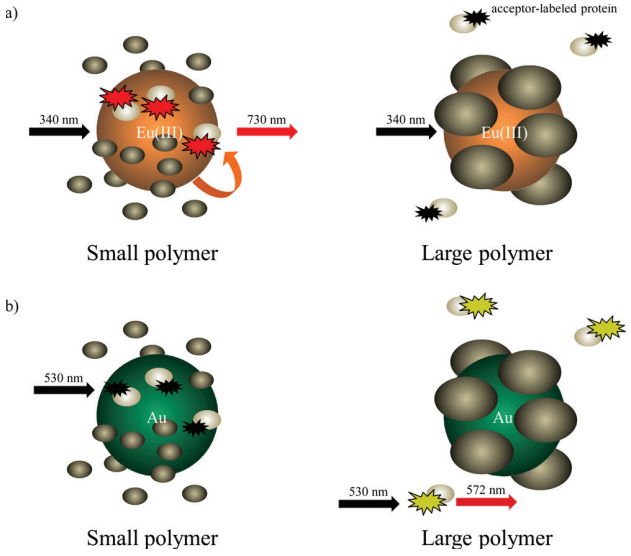


Figure 1. Principle for determination of polymer MW. Nanoparticle-based TR-LRET (a) and quenching (b) systems utilize the competitive adsorption between labeled protein and analyte polymer molecules. At constant analyte mass concentration, the increase in polymer MW reduces the adsorption of labeled protein, which is observed as a change in the signal. (a) In the TR-LRET system, an acceptor-labeled protein is adsorbed to the donor europium(III) polystyrene nanoparticles. Due to the close proximity of the donor−acceptor pair, luminescence resonance energy is transferred. The increase in MW of the polymer results in the decrease in the TR-LRET signal. (b) In the quenching system, a labeled protein is adsorbed to the quencher gold nanoparticles and the luminescence is quenched. The increase in MW of the polymer results in the increase in the luminescence.

labeled protein adsorbs to the gold nanoparticles and the luminescence is quenched. PEGs competed with labeled protein in the adsorption to the quencher gold nanoparticles leading to the increase in signal and the degree of the competition was related to MW. MW is assessed from the relationships between the luminescence signal and PEG mass concentration. The method was tested for PEG polymers with MW from 62 (monomer) to 35 000 g/mol. The assessment of PEG MW was enabled without prior knowledge of the PEG concentration, which was not achieved in our previous work.[31]

EXPERIMENTAL SECTION

Materials. γ-Globulins from bovine blood (γG), albumin from bovine serum (BSA), and polyethylene glycols (PEG 200, PEG 400, PEG 1500, PEG 3000, PEG 6000, and PEG 35000) were from Sigma-Aldrich Co. (St. Louis, MO). Ethylene glycol was from J. T. Baker (Deventer, The Netherlands). Carboxylate modified europium(III) polystyrene nanoparticles 92 nm in diameter were purchased from Seradyn Inc. (Indianapolis, IN) and colloidal gold particles 20 nm in diameter (having ∼100% monodispersity according to the manufacturer) from British Biocell International (Cardiff, U.K.). Alexa Fluor 680 carboxylic acid, succinimidyl ester was obtained from Molecular Probes (Eugene, OR). Dipyrrylmethene-BF_2 530 (BF530) dye was from Arctic Diagnostics Oy (Turku, Finland). NAP-5 gel filtration columns were ordered from GE Healthcare (Uppsala, Sweden). All the reagents used to prepare buffer solutions were obtained from Sigma-Aldrich Co. (St. Louis, MO). High purity Milli-Q water was used to prepare all aqueous solutions.

Methods. *Labeling of γ-Globulin with Alexa Fluor 680 and Bovine Serum Albumin with BF530.* Alexa Fluor 680 carboxylic acid, succinimidyl ester (Alexa) was conjugated to γ-globulin (γG), as recommended by the manufacturer. Dipyrrylmethene-BF_2 530 (BF530) dye was conjugated to albumin from bovine serum (BSA) as described previously for mouse monoclonal IgG anti-hAFP.[32]

Molecular Weight or Concentration Determination of Polymers. The MW or concentration determinations using nanoparticle application were performed in microtitration wells. In the TR-LRET system, 70 μL of the sample polyethylene glycol solution in 5 mM glycine buffer pH 3.0 and 10 μL of the europium(III) nanoparticles 92 nm in diameter in water were mixed. γG-Alexa was added in 10 μL of water. The final concentrations of europium(III) nanoparticles and γG-Alexa were 0.36 and 53 pM, respectively. In the quenching system, 70 μL of the sample polyethylene glycol solution in 5 mM glycine buffer pH 3.0 and 20 μL of the gold nanoparticles 20 nm in diameter in water were mixed. BSA-BF530 was added in 20 μL of 1.0 mM phosphate buffer, pH 7.4, containing 0.10 mM potassium chloride. The final concentrations of gold nanoparticles and BSA-BF530 were 64 and 890 pM, respectively. The size of europium(III) and gold nanoparticles was chosen according to our previous work.[25,27] The utilization of small gold nanoparticles (20 nm) is economical, as the lower amount of gold (mass) is required in the assay compared to larger nanoparticles. Luminescence emission intensities were measured with the Victor^2 multilabel counter (Wallac, PerkinElmer Life and Analytical Sciences, Turku, Finland). For the TR-LRET system, a 340 nm excitation and a 730 nm emission wavelength and a 50 μs window and a 75 μs delay time were used. For the quenching system, the emission was measured at 572 nm after the excitation at 530 nm.

RESULTS AND DISCUSSION

A simple mix-and-measure nanoparticle-based method was developed for the estimation of PEG MW. In this paper, the nanoparticle method is extended to the MW estimation of PEGs contrary to polyethylenimines and polyamino acids measurements published earlier.[31] As polyethylenimines and polyamino acids are positively charged at the assay pH of 3 and

DOI: 10.1021/acs.analchem.5b00736
Anal. Chem. 2015, 87, 3918−3922
ITM Exhibit 1024, Page 2 of 5

polyamino acids contain hydrophobic sites, the adsorption is efficient due to the attractive electrostatic and hydrophobic interactions to carboxylate-modified polystyrene nanoparticles. Here, we demonstrate that hydrophilic uncharged polymers such as PEGs having low affinity to the nanoparticles can also be measured on nanoparticles of different surface properties. PEGs are commercially available in a wide size range and their chemical structure is similar within the size series enabling the undistorted information on MW. We tested two nanoparticle-based systems utilizing TR-LRET or luminescence quenching to obtain the size related information for PEGs (Figure 1). The method exploits the adsorption competition between the labeled protein and the polymer to the europium(III) polystyrene nanoparticles or quencher gold nanoparticles. The estimation of MW is based on the size-dependent adsorption of the polymer, as a small polymer occupies the particle surface less efficiently than a large polymer. A large polymer may also cross-link several nanoparticles, as a single polymer can attach to many particles.[33] The cross-linking could be beneficial for the method, as a large polymer would cover larger total nanoparticle surface area than without cross-linking and the cross-linking would widen the dynamic range.

In the TR-LRET assay, γ-globulin labeled with acceptor-dye Alexa Fluor 680 (γG-Alexa) adsorbed to the donor europium-(III) polystyrene nanoparticles and the luminescence resonance energy was transferred from the donor to the acceptor. The donor was excited at 340 nm and the sensitized emission of the acceptor was measured at 730 nm. The TR-LRET signal was decreased, as PEGs prevented the adsorption of γG-Alexa. In the quenching assay, albumin from bovine serum labeled with dipyrrylmethene-BF_2 530 (BSA-BF530) adsorbed to quencher gold nanoparticles in the absence of the analyte and the luminescence of the BF530 dye was quenched (Figure 1b). The adsorption of BSA-BF530 was prevented by PEG polymers resulting in an increase of the luminescent intensity. The BF530 dye was excited at 530 nm and the emission was measured at 572 nm. The luminescence spectra of europium(III) nanoparticles, γG-Alexa, and BSA-BF530 and the absorption spectrum of gold nanoparticles have been published earlier by us.[28]

The europium(III) polystyrene nanoparticles have carboxylic groups giving a total negative surface charge ($pK_a(-SO_4H) < -3$, $pK_a(-SO_3H) = 1.9$, and $pK_a(COOH) = 4.7$) at the assay pH of 3. Similarly, the total surface charge is negative for colloidal gold nanoparticles due to the citrate groups (pK_a values: 3.2, 4.8, and 6.4) used in the synthesis. The negative total charge is also supported by ζ-potential measurements performed in literature studies.[34−37] These ionic groups provide ionic and hydrophilic character for the surface. However, both nanoparticles contain also neutral sites, reduced gold at oxidation state 0 for gold and chains of polystyrene for polystyrene nanoparticles giving the hydrophobic character for the surface. The assay performance on these nanoparticle surfaces depends on the strength of the interactions of PEGs or labeled protein and on the degree of exchange of PEG with labeled protein.

The response of both assay systems was tested for the competitive adsorption between labeled protein and PEG with different MWs and at varying PEG concentration (Figure 2). The curves for PEGs in Figure 2a were obtained by fitting the data to the modified Hill function with a constant offset.[38] The average coefficient of variation for the replicates was 9% and 6% for quenching and TR-LRET assay, respectively. For the TR-LRET system, the sensitized signal decreased at PEG concentrations above 10 g/L for all tested MWs between 400 and 35 000 g/mol and the dose−response curves nearly overlapped (Figure 2a). This decrease may be related to the steep increase in the viscosity of PEG solutions at concentrations above 200 g/L and the simultaneous decrease in diffusion preventing the adsorption of γG-Alexa.[39] As the adsorption of differently sized PEGs to the nanoparticle surface was essentially identical, the overlap of the calibration curves attained for PEGs between 400 and 35 000 g/mol enables the measurement of mass concentration independent of MW. Only monomeric ethylene glycol gave an opposite result, as increase in the signal was observed at concentrations above 3 g/L. This may be due to the change in the adsorbed layer of γG-Alexa in the presence of ethylene glycol and lower increase of viscosity compared to polyethylene glycols. Ethylene glycol is known to change the structure of γG.[40−42] Thus, more γG-Alexa may be adsorbed or the distance of Alexa dye from the europium(III) polystyrene nanoparticle surface may be decreased due to the changes in the α-helix and β-sheet structure. PEGs with different MWs can not be distinguished with the TR-LRET system, although size-independent mass concentration can be measured (Figure 2a).

As the size-related measurement failed with the TR-LRET system, we investigated the performance of the quencher gold nanoparticle system[25] for the MW determination of ethylene glycol and six corresponding polymers. The shape of the response curves were nearly identical to different PEGs, but no

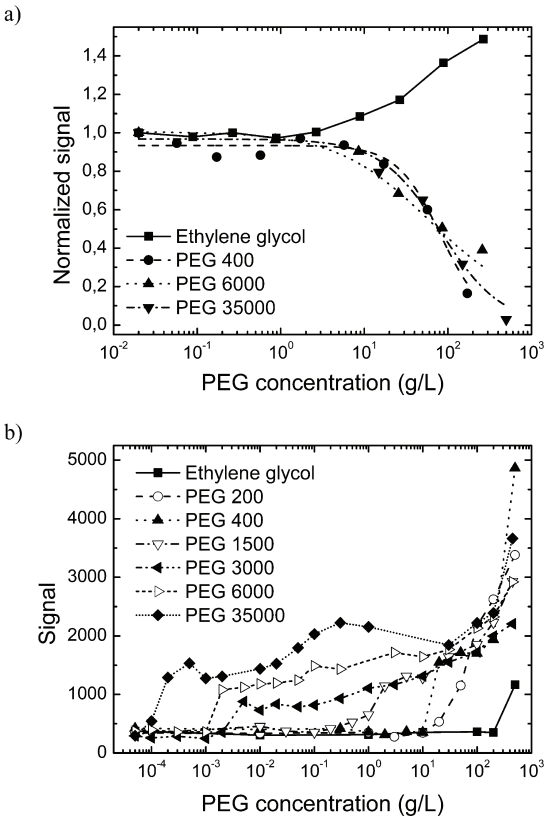


Figure 2. Response curves for polyethylene glycols measured with TR-LRET (a) and quenching (b) systems. The initial increase of the signal is moved to higher concentration, as the PEG MW is decreased. The data measured with TR-LRET system for PEGs was fitted to the modified Hill function with a constant offset.[38]

DOI: 10.1021/acs.analchem.5b00736
Anal. Chem. 2015, 87, 3918−3922

ITM Exhibit 1024, Page 3 of 5

obvious sigmoidal curve shape was measured for the quenching system (Figure 2b). The luminescence signal is increased, as the PEG concentration is increased. However, after the initial adsorption, a clear plateau is found for large-MW PEGs. As the PEG MW was reduced, the plateau became less distinct, until no plateau was identified for PEG 200 and monomer ethylene glycol. The multiphase adsorption to silica with several plateaus has been reported for PEG 400 in literature and interpreted as conformational changes of polymeric PEG chain.[43] As the PEG concentrations increased further, a clear increase in signal was observed at the PEG concentration above 100 g/L, which may be derived from the increase in viscosity similar to the observation in the TR-LRET system. PEGs have been shown to form fluorescent micelles at high concentrations with absorption at wavelengths below 350 nm and emission maximum at approximately 380 nm.[44] The emission at wavelengths higher than 500 nm is low. Thus, we tested the effect of PEG micelles to the fluorescence signal of the developed assay. This was confirmed to be insignificant at high concentration of PEG 400 and PEG 6000 in the assay buffer in the absence of gold nanoparticles and BSA-BF530 (data not shown).

The sensitivity of the quencher gold particle system for PEGs was improved by 5 orders of magnitude from the TR-LRET system. Unfortunately, the unorthodox curve shapes did not allow quantification of PEGs. However, the onset of the luminescence signal increase at varying specific concentration of differently sized PEGs indicates that MW of an unknown PEG sample could be estimated. We correlated the concentration at the luminescence signal level of 700 cts to the PEG MW (Figure 3a). This calibration requires a prior knowledge of analyte mass concentration and allowed the determination of the largest polymer PEG 35000 clearly below 1 mg/L and the smallest PEG 200 below the 100 g/L concentration. The entire MW range of PEGs from ethylene glycol to 35 000 g/mol could be measured. We also processed the data by presenting the range of plateau as a function of PEG MW (Figure 3b). The range of plateau was assessed as the ratio of PEG concentrations at the onset and end of plateaus. The end value was determined at the signal value two times the plateau signal value and the onset signal value was 700 cts. The plateau signal value was calculated as an average of values showing maximally 15% deviation from the plateau. For ethylene glycol and PEG 200 with no identified plateau, the concentration was assessed at signal value of 2500. The PEG MW could be assessed equally well from both calibrations. However, the data processing from the range of plateau requires no prior knowledge of PEG concentration. The curves in Figure 3 were obtained by fitting the data (PEG concentration vs MW or range of plateau vs MW) to the modified Hill function with a constant offset.[38]

The response of the quenching system was observed at different concentrations for PEGs with different MWs. Conversely, no size-dependent response was observed for TR-LRET system. The difference between the systems may be related to the different surface properties of the nanoparticle materials. Both surfaces contain hydrophilic and negatively charged groups and less hydrophilic neutral parts. However, gold is regarded as clearly more hydrophilic than polystyrene,[45] which potentially affects to the adsorption properties of PEGs with both hydrophilic and hydrophobic characteristics. The method utilizes the competitive adsorption and thus, the labeled protein may partly replace the adsorbed PEG depending on its size. However, the exact mechanisms of the competitive adsorption and the differences between the systems remain unclear.

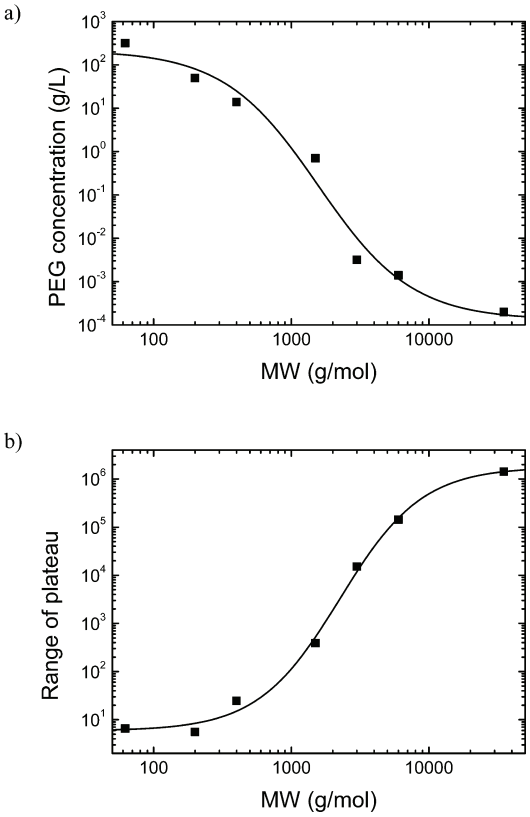


Figure 3. Polyethylene or ethylene glycol concentration at the signal level of 700 (a) or the range of plateau (b) as a function of MW for quenching system. The data was fitted to the modified Hill function with a constant offset.[38]

CONCLUSIONS

We have demonstrated the applicability of the quenching nanoparticle system for the determination of PEG MW in a high throughput format. The PEG MW was successfully measured over a range from 62 to 35 000 g/mol. The method is fast to perform in the microtiter plate format with standard luminescence plate readers, which is in contrast to the existing methods, such as mass spectrometry, analytical ultracentrifugation, and chromatography, requiring expensive instrumentation and expertise. Furthermore, we showed that the determination of PEG MW can be performed without prior knowledge of analyte concentration. This study with PEG suggests that the method is suitable also for neutral polymers, and thus the applicability shown by us earlier for charged compounds, polyethylenimines and polyamino acids, is widened. Thus, this paper indicates that the method is of potential value in research and industry for a wide range of polymers with different physical properties.

AUTHOR INFORMATION

Corresponding Author

*S. Pihlasalo. E-mail: sari.pihlasalo@utu.fi. Tel.: +358 2 333 7692.

DOI: 10.1021/acs.analchem.5b00736
Anal. Chem. 2015, 87, 3918−3922

ITM Exhibit 1024, Page 4 of 5

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the funding from the Academy of Finland (grant 258617) and the Graduate School of Chemical Sensors and Microanalytical Systems.

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ITM Exhibit 1024, Page 5 of 5