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Characterization of cationic polymers by asymmetric flow field-flow fractionation and multi-angle light scattering—A comparison with traditional techniques

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ABSTRACT

In the field of nanomedicine, cationic polymers are the subject of intensive research and represent promising carriers for genetic material. The detailed characterization of these carriers is essential since the efficiency of gene delivery strongly depends on the properties of the used polymer. Common characterization methods such as size exclusion chromatography (SEC) or mass spectrometry (MS) suffer from problems, *e.g.* missing standards, or even failed for cationic polymers. As an alternative, asymmetrical flow field-flow fractionation (AF4) was investigated. Additionally, analytical ultracentrifugation (AUC) and ^{1}H NMR spectroscopy, as well-established techniques, were applied to evaluate the results obtained by AF4. In this study, different polymers of molar masses between 10 and 120 kg mol^{-1} with varying amine functionalities in the side chain or in the polymer backbone were investigated. To this end, some of the most successful gene delivery agents, namely linear poly(ethylene imine) (LPEI) (only secondary amines in the backbone), branched poly(ethylene imine) (B-PEI) (secondary and tertiary amino groups in the backbone, primary amine end groups), and poly(L-lysine) (amide backbone and primary amine side chains), were characterized. Moreover, poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA), poly(2-(amino)ethyl methacrylate) (PAEMA), and poly(2-(*tert*-butylamino)ethyl methacrylate) (P*tBAEMA*) as polymers with primary, secondary, and tertiary amines in the side chain, have been investigated. Reliable results were obtained for all investigated polymers by AF4. In addition, important factors for all methods were evaluated, *e.g.* the influence of different elution buffers and AF4 membranes. Besides this, the correct determination of the partial specific volume and the suppression of the polyelectrolyte effect are the most critical issues for AUC investigations.

1. Introduction

Polyelectrolytes, in particular cationic polymers, are a highly promising class of compounds in biological, pharmaceutical, and medical research. They represent promising carriers for genetic material like DNA or RNA into cells [1–3]. The efficiency of gene delivery strongly depends on different parameters, such as the molar mass and architecture of the used polymer, since they influence the cytotoxicity, the cellular uptake, and transfection efficiency, or in the case of siRNA the protein knockdown. To investigate these structure–property relationships, a detailed molecular characterization of the polymers with respect to their physico-chemical properties is essential. In particular, key parameters such as molar mass, radius, architecture, intermolecular interactions, and conformation strongly influence the resulting macroscopic properties. For the determination of the molar mass, a large range of techniques are available in modern analytical and bioanalytical chemistry. Unfortunately, common methods like size exclusion chromatography (SEC) or mass spectrometry (MS) suffer problems or failed for polyelectrolytes, in particular for cationic ones [4,5]. While results from MS (MALDI–TOF MS or ESI–TOF MS) are difficult to achieve and the interpretation becomes more complex due to the probable multiply charged species in the polymer chain [6], SEC results should be regarded carefully, due to strong interactions of the polyelectrolytes with the column material and the lack of suitable standards for most of the cationic polymers [7]. Here, the development of modern stationary phases and the coupling of a multi-angle light scattering (MALS)

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detector to SEC can circumvent some of these limitations [8]. Other methods like viscosimetry or techniques based on colligative phenomena are applicable, but suffer the drawback that the constants in the Kuhn–Mark–Houwink–Sakurada equation are not available for most of these polymers, moreover, the determination of the degree of protonation of the polymer in water and the degree of dissociation are problematic. As a consequence, in solution the amount of species having counterions is not known. Further, important methods for characterization are NMR spectroscopy, static light scattering (SLS), and analytical ultracentrifugation (AUC). However, just average values and no or limited information about the polydispersity index (PDI) of the sample can be obtained. Having knowledge of the PDI is important from a synthetical and applicational point of view, particularly when structure–property relationships are investigated.

Due to intrinsic limitations described for the other analytical methods, asymmetric flow field-flow fractionation (AF4) coupled to a UV/RI and a MALS detector was investigated in this study as an alternative characterization method for cationic polyelectrolytes. AF4 was firstly introduced in 1966 by J. Calvin Giddings. It is an emerging technique and nowadays widely applied for colloids, *e.g.* nanoparticles or proteins [9]. Although preferred for the analysis of high molar mass samples, only rarely studies were performed using synthetic macromolecules, in particular polyelectrolytes of lower molar mass [10–13]. With AF4, the polymers are separated in a trapezoidal channel without any porous packing material according to their diffusion coefficient [14]. The separation of the sample is achieved by application of a cross-flow perpendicular to the direction of the sample flow through a semipermeable membrane with a defined molar mass cut-off (MWCO). A detailed description and theoretical consideration for the calculation of the diffusion coefficient based on the retention time was given by Wahlund and Giddings [15]. In comparison to classic chromatography techniques such as HPLC or SEC, AF4 contains no stationary phase, which reduces disturbing interactions and adsorption effects in the most cases. Moreover, the flow is less tortuous for the sample, due to the decreased shear forces in an empty channel. This is advantageous for sensitive biological samples [16]. Nowadays, in most cases, a MALS detector is used for the analysis after the fractionation process [17]. The calculation of molar mass or radius of gyration is based on the same principle as classic static light scattering. A common way to treat the data uses the well-known Zimm-plot. In contrast to classical SLS, the second virial coefficient A_2 can be neglected due to the high dilution during the fractionation process.

In contrast to AF4, analytical ultracentrifugation (AUC) and ^{1}H NMR spectroscopy are well-established techniques, which are used for many years for the characterization of biological and synthetic macromolecules [18–20]. It should be noted that both methods yield different molar mass averages. While ^{1}H NMR spectroscopy gives the number average molar mass (M_n), in AUC the sedimentation diffusion average molar mass (M_{sD}) is obtained from sedimentation velocity experiments and the Svedberg equation (1). These methods can be used for the comparison of the results and to show the potentials and possible limitations of AF4 with regard to the characterization of (cationic) polymers.

In this study, cationic polymers of different molar masses with varying amine functionalities in the side chain or the polymer backbone (Fig. 1) were investigated for the first time by AF4. As the most successful gene delivery agents, a tailor-made linear, and commercially available linear and branched poly(ethylene imine)s (L-PEI, B-PEI) were characterized [21]. Moreover, poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA), poly(2-(amino)ethyl methacrylate) (PAEMA), and poly(2-(*tert*-butylamino)ethyl methacrylate) (P*tBAEMA*) as polymers with primary, secondary, and tertiary amines in the side chain were studied. Additionally, two samples of different molar masses of commercially available poly(L-lysine) (PLL), a prominent polyamino acid in gene delivery research [22], are analyzed by AF4. As AF4-MALS is typically not applied to low molar mass ($M < 100\,kg\,mol^{-1}$) polymers, this study focuses on the evaluation of AF4 as a potential alternative for characterization of these cationic polyelectrolytes. Therefore, the results obtained from the synthesized methacrylate based cationic polymers are compared to well-established methods like ^{1}H NMR spectroscopy, SEC and AUC. Beside the determination of the molar masses and the polydispersity index values, different types of membranes and eluents were evaluated to identify optimal conditions for the analysis. This should also reveal potential interactions with the membrane and show how far it affects the retention behavior and the obtained results. PDMAEMA was studied in more detail by AF4 to gain deeper insight into the conformation as well as the influence of ionic strength and pH value on the retention behavior. This study shows that AF4 allows fast and reliable characterization of cationic polymers. Moreover, the limitations concerning molar mass limits and membrane interactions for different classes of cationic polymers are discussed in detail.

2. Experimental

2.1. Materials

Poly(L-lysine) (PLL) and branched poly(ethylene imine) (B-PEI$_{com}$) were purchased from Sigma Aldrich (Steinhausen, Germany). Linear poly(ethylene imine) (L-PEI$_{com}$) was purchased from Polysciences (Eppelheim, Germany). Methyl tosylate and 2-ethyl-2-oxazoline (EtOx) were purchased from Acros Organics (Geel, Belgium), distilled to dryness over barium oxide (BaO), and stored under argon. A second linear poly(ethylene imine) (L-PEI$_{600}$) was synthesized by acidic hydrolysis of poly(2-ethyl-2-oxazoline) (PEtOx) in a microwave synthesizer (Biotage) as described recently (see supporting info SI-I) [6].

2-(Dimethylamino)ethyl methacrylate (DMAEMA), 2-aminoethyl methacrylate hydrochloride (AEMA) and 2-(*tert*-butylamino)ethyl methacrylate (*tBAEMA*) were purchased from Sigma–Aldrich and purified by stirring in the presence of inhibitor-remover for hydroquinone or hydroquinone monomethyl ether (Aldrich) for 30 min prior to use. The initiators 4,4′-azo*bis*(4-cyanopentanoic acid) (ACVA), 1,1′-azo*bis*(cyclohexane carbonitrile) and 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid as well as 4-cyano-4-[(dodecylsulfanylthiocarbonyl) sulfanyl] pentanoic acid RAFT agents were purchased from

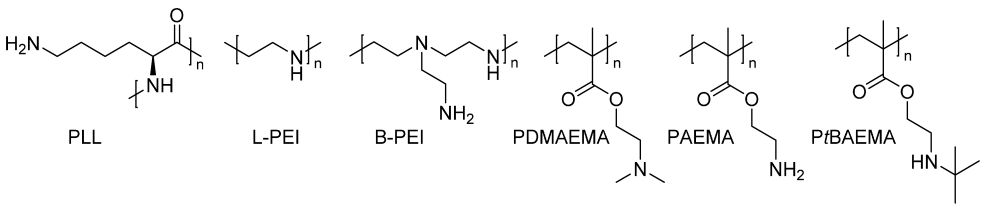

Fig. 1. Schematic representation of the structure of the polymers used in this study.