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Asymmetrical flow field-flow fractionation for human serum albumin based nanoparticle characterisation and a deeper insight into particle formation processes

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ABSTRACT

Nanoparticles used as drug delivery systems are of growing interest in the pharmaceutical field. Understanding the behaviour and effects of nanosystems in the human body is dependent on comprehensive characterisation of the systems especially with regard to size and size distribution. Asymmetrical flow field-flow fractionation (AF4) is a promising method for this challenge as this technique enables chromatographic separation of particles and solute molecules according to their respective size.

Within this study AF4 was used for the characterisation of human serum albumin (HSA) based nanoparticles. In a first part, the most important aspects of method development like the choice of cross flow rate, focusing and the increase of sample concentration via outlet stream splitting on the sample separation were evaluated. Sample fractionation was controlled by inline-coupling of a dynamic light scattering detector (DLS, Zetasizer) and was confirmed by DLS batch mode measurements. In a second part the applicability of field-flow fractionation for characterisation of the HSA particle formation process by a desolvation method was evaluated. A time dependent particle formation was observed which was controlled by the amount of desolvating agent. Furthermore, field-flow fractionation in combination with in-line dynamic light scattering was used to monitor the increase of particle diameter during PEGylation of the resulting HSA nanoparticles. The separation of nanoparticles from dissolved polyethylene glycol (PEG) could successfully be used for determination of the particles' PEGylation degree.

1. Introduction

Specific uptake of therapeutics in diseased cells (drug targeting) for increasing activity while on the other hand reducing toxicity is a benefit of nanoscaled delivery systems. With the discovery of biodegradable materials such as albumin, poly(lactic-co-glycolic acid) (PLGA), polyalkylcyanoacrylate (PACA) just to name few, a rapid trend to nanosized systems could be observed especially in the field of oncology. Besides liposomes solid particles such as Abraxane®, albumin bound paclitaxel, were established for the effective treatment of metastatic breast cancer [1]. In addition to the choice of the starting material, particle size and surface morphology are the most important factors for the behaviour of nanoparticles (NP) in the human body. Regarding starting material for NP preparation biodegradable polymers are of major interest. Besides PLGA human serum albumin (HSA) is a common excipient for preparation of NP. Based on HSA most often NP are prepared by desolvation techniques [2–4]. During the desolvation process the resulting particle size can be influenced either by the pH of the protein solution or the choice of desolvating agent [5]. After intravenous application of nanoparticulate suspensions, the particles are rapidly opsonised by plasma proteins and enriched in the mononuclear phagocyte system (MPS: liver, spleen, lung, and bone marrow) [6]. MPS accumulation can be controlled by appropriate choice of particle size and surface structure.

According to Kreuter in pharmaceutics NP are defined as colloidal polymer particles with a size below 1 μm [7]. With regard to dimension it is necessary to control the size of NP during production since particles, which are larger than 200 nm are rapidly eliminated from the blood stream by macrophages after opsonisation. In contrast smaller particles with a narrow size distribution are characterised by a prolonged circulation time [8].

On the other hand the grafting of hydrophilic polymers such as polyethylene glycol (PEG) to particle surfaces is a well accepted modification technique for increasing the plasma circulation time.

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PEG modification prevents opsonisation and as a consequence uptake by the mononuclear phagocyte system (MPS). Among others Gref et al. established PLGA-based PEG-coated nanospheres and observed, that liver uptake could be decreased from 66% within 5 min (uncoated PLGA-nanospheres) to less than 30% uptake of 20 kDa PEG coated particles within 2 h [9]. Schädlich et al. showed, that NP formed of diblock copolymer PEG-polylactic acid were circulating in the blood vessels over 6 h after intravenous application, while non-PEGylated PLA NP showed half-life times of just a few minutes [10]. Besides the modification of NP PEGylation is also used for the modification of biopharmaceuticals such as protein-based drugs (i.e. Oncaspar®) [11]. For that reason there is a need for characterisation methods of PEGylated products in the nanometer size range.

The major aspects of nanoparticle characterisation cover particle size and polydispersity. Particle size analysis is commonly performed by one of the following methods or a combination thereof: microscopy, in particular scanning electron microscopy (SEM), is often applied as it represents an absolute sizing method. Additional information about particle shape and morphology can be obtained while measuring the size of an individual particle. However, for statistical reasons the minimum number of particles to be measured has to be at least 10,000 depending on the accepted sampling error of the particle size distribution. Therefore SEM is time consuming [12]. On the other hand methods like laser diffraction (LD) and static light scattering (SLS) can obtain results of a particle sample within few minutes, though the particle size is strongly depending on the used analysis model and correct parameters [13,14]. Among these optical techniques dynamic light scattering (DLS) like photon correlation spectroscopy (PCS) is nowadays the method of choice because of its fast and easy performance [15]. In PCS the particle diameter and polydispersity index (PDI) can be easily obtained by a measurement of the light intensity scattered by dispersed particles. As particle size is related to the diffusion coefficient via the Stokes–Einstein equation and the diffusion coefficient determines the Brownian motion of dispersed particles, an autocorrelation function between the scattered light and the delay time can be calculated. Using this method, most laboratories could achieve valid results [16]. However, the resolution of DLS is rather low and larger particles are overestimated in bimodal nanosized populations [17] and the result is depending on the average value used [18].

For a better resolution, fractionation methods such as analytical ultracentrifugation [19], hydrodynamic chromatography, size exclusion chromatography, and particularly field-flow fractionation (FFF) can be used previous to sizing [20]. Field-flow fractionation comprises a versatile group of elution techniques, which follow all the same underlying principle: like other chromatographic separation methods FFF uses a flow for carrying the analyte to the detectors. But in contrast to HPLC separation takes place in a thin, ribbonlike channel without any stationary phase. Due to a channel height between 100 μm and 500 μm [21], a parabolic flow profile is created, where flow velocity increases from the channel walls to the centre. Perpendicular to this flow, a second field is applied, which induces a force that accumulates the particles at the channel wall. Contrary to this force, particles diffuse back to the centre of the channel until an equilibrium distribution according to their diffusion coefficient is achieved [22]. The samples form zones with exponential concentration profiles and leave the channel in the order of small to big ones. This elution mode is called Brownian, as it describes the nature of the analyte concentration profile [23].

If this field perpendicular to the laminar flow throughout the channel is created by a flow, the so-called cross flow, this method is called flow field-flow fractionation, which is an universal method and can be applied for samples in a size range between 1 nm and 50 μm [24]. The highest flow velocity is in the centre of the channel and is most sluggish at the channel walls. In asymmetrical flow field-flow fractionation (AF4) the upper channel wall is impermeable to the carrier flow. Drawing one part of the channel flow through the lower wall creates the cross flow and forces the eluate to accumulate there [25]. Opposite to this, the analyte diffuses back to the channel centre to equilibrate concentration until a stationary layer is formed. As the cross flow is equal for all solute materials, the mean layer thickness of the analytes only varies with the diffusion coefficient. So, separation is caused by the differences in solute diffusion coefficients [26].

The major advantage of AF4 in comparison to classical chromatographic methods such as HPLC is that nanoparticle samples can be analysed without any pretreatment such as i.e. centrifugation for particle separation. AF4 was used the first time to analyse the desolvation process during the preparation of HSA NP. A deeper insight into the time and solvent dependent formation of HSA NP was achieved. Furthermore the suitability of AF4 for characterisation of particle PEGylation as well as determination of the PEGylation degree was investigated. AF4 is separating particles continuously and non-destructively so that differences in particle structure due to PEGylation could be recorded. Especially in combination with DLS detection the influence of PEGylation on particle size could easily be determined.

2. Materials and methods

2.1. Reagents

HSA (fraction V, purity 96–99%) and glutaraldehyde 8% solution were obtained from Sigma–Aldrich (Steinheim, Germany). Ethanol was obtained from Merck (Darmstadt, Germany). Methanol and salts used for the preparation of the mobile phase ($NaH_2PO_4 \times 1\ H_2O$, $Na_2HPO_4 \times 2\ H_2O$, and NaCl) were purchased from Roth (Karlsruhe, Germany). All PEG succinimidyl active esters were purchased from Rapp Polymere (Tübingen, Germany): mPEG-5,000-NHS, mPEG-10,000-NHS, mPEG-20,000-NHS, Fmoc-PEG-5,000-NHS. All chemicals were of analytical grade and used as received. The water used in this work was deionised and purified using a PURELAB flex water system distributed by ELGA LabWater (Celle, Germany).

2.2. Instruments

Formation of HSA NP was performed in glass beakers (diameter 27.5 mm; height 57.0 mm) with Teflon coated stirring bars (diameter 6.0 mm; length 19.7 mm), placed onto a Variomag Multipoint magnetic stirrer (Thermo Scientific, Langenselbold, Germany). Desolvation agent was added by a Reglo tubing pump (Ismatec, Wertheim–Mondfeld, Germany).

For particle purification an Eppendorf Centrifuge 5424 (Eppendorf, Hamburg, Germany) and a Sonorex ultrasonic bath (Bandelin electronic, Berlin, Germany) were used. Afterwards, the particles were analysed regarding diameter and polydispersity index (PDI) using a Zetasizer Nano S (Malvern Instruments Ltd, Worcestershire, UK).

Incubation procedures during particle PEGylation were performed in an Eppendorf Thermomixer comfort (Eppendorf, Hamburg, Germany). The PEGylation degree of HSA NP via Fmoc-PEG fluorescence was measured using a Synergy Mx multi-mode microplate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany).

For nanoparticle analysis by asymmetrical flow field-flow fractionation (AF4) an AF 2000 MT-system was used, consisting of an AF 2000 module box, channel oven (PN 4020), PN 5300