

Stabilizer-Induced Viscosity Alteration Biases Nanoparticle Sizing via Dynamic Light Scattering

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The size dependent features of colloids at the nanometer scale have been issues of increasingly intensive research. In order to be able to correctly relate characteristics to certain size-populations, accurate and reliable particle sizing by dynamic light scattering (DLS) is a main prerequisite. So far, the complexity of the systems due to the presence of surfactants, proteins, and so forth in the nanoparticle suspensions has not been accounted for. In this work, practically relevant quantities of the frequently used PEO-PPO triblock copolymer surfactant Pluronic F-68 were studied for their effect on the size determination of nanoparticles by DLS. Induced changes in the tenside-content of the nanosphere suspension were monitored using a photometric assay and were correlated to the respective variances in mean particle size. These measurements showed that alterations in the range from 0.005 to 2% of Pluronic content are associated with shifts in diameter of 200 nm-particles by as much as 65 nm. The considerable changes that were found have been attributed to the surfactant-concentration-dependent fluctuations in the viscosity of the nanoparticle suspension, which affect the dimensions of colloids calculated according to the Stokes–Einstein relation.

Introduction

The diversity of the exceptional features of nanoparticles is manifold, ranging from the photostability of semiconductor quantum dots^{1,2} to the passive accumulation of nanoparticulate drug delivery vehicles in tumor sites due to the enhanced permeability and retention (EPR) effect.^{2,3} These characteristics have in common that they cannot be generalized for submicrometer colloids but rather are dependent on the material properties and, very importantly, the actual size of the particles. In the case of quantum dots, this is strikingly illustrated by the precise interdependence between the particle's diameter and the wavelength at which fluorescence is emitted. However, not only the physical properties of nanoparticles but also their interaction with cells, subsequent intracellular trafficking, and therefore possible toxicity have been increasingly discussed issues.^{4–7} It is assumed that these characteristics are governed in a similarly grave manner by the dimensions of the colloids. The first evidence of such size dependence has been given by studies on the cellular and tissue uptake of nano- versus microparticles in the 1990s. It was shown that the uptake efficiency of 100 nm colloids is 15–250-fold higher as compared with that of 1–10 μm particles in a rat in-situ intestinal loop model.⁷ Similar tendencies toward the enhanced uptake of smaller particles were reported by Jani et al. using radiolabeled 50 nm–3 μm polystyrene microspheres in a rat in-vivo study.⁸

Although it has been known for several years that distinct size differences are inflicted with differential cellular uptake, account has been given lately that even very small alterations in size influence the cell association and intracellular processing of nanoparticles.^{9,10} Lai et al., for example, recently showed that small polymeric nanoparticles (<25 nm) but not larger ones (>42 nm) enter cells via a nondegradative pathway.¹⁰

Beyond the impact on the uptake of nanoparticles, the physical size also determines the biodistribution behavior of the colloids. In case of the EPR effect, this is illustrated by the accumulation of particles in the range from 70–200 nm because only such are suited to pass the fenestrated endothelium of tumor blood vessels.¹¹

These issues make clear that an accurate determination and knowledge of the physical dimensions of the particles are crucial prerequisites not only for toxicity studies but also for the rational design of nanoparticulate drug-delivery devices.

Currently, the method of choice for sizing nanoparticles in suspension is dynamic light scattering (DLS). If performed under standardized conditions, results obtained with this technique are rather comparable and reproducible. However, nanoparticle suspensions in practice represent complex and changing systems, which in most cases contain not only the dispersed colloids but also varying amounts of stabilizers, salts, proteins, and so forth. These substances not only physicochemically interact with the particles but also alter the properties of the dispersant. In the production process of single- or double-emulsion solvent evaporation techniques, for example, large amounts of surfactant serve the purpose of avoiding coalescence during the solidification of the nanodroplets. In the course of further treatments and modifications of the particles, however, the tenside content of the suspension and thus the viscosity vary considerably. Although such variations are expected to occur frequently and with a

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practical impact, to the best of our knowledge, the importance of the factor viscosity on DLS measurements has not been accounted for.

In the present contribution, the existence and dimensions of such effects were investigated using the poly(ethylene glycol)-poly(propylene glycol) (PEO-PPO) triblock copolymer Pluronic F-68 and PLGA nanospheres as a model system. Pluronics, which are also referred to as poloxamers because of their surface activity and a wide range of tuneable hydrophilicity–lipophilicity balance, have been extensively used in the production and coating of colloids made from materials such as polystyrene,^{12–14} poly(lactic acid) (PLA),^{15–16} poly(D,L-lactic-co-glycolic) acid (PLGA),^{17–21} poly(β -malic acid-co-benzyl malate),²² chitosan,²³ and solid lipid nanoparticles (SLN).²⁴

Experimental Section

Materials. Resomer RG503H (PLGA, 50:50 lactide/glycolide, inherent viscosity 0.32–0.44 dL/g, acid number > 3 mg of KOH/g) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Pluronic F-68, ammonium thiocyanate, and cobalt(II) nitrate hexahydrate were obtained from Sigma (Vienna, Austria). All other chemicals used were of analytical purity.

Preparation of PLGA Nanospheres. PLGA nanospheres were prepared by a water-in-oil-in-water solvent-evaporation technique.²⁵ Briefly, 400 μ L of distilled water was emulsified with a solution containing 400 mg of PLGA in 2 g of ethyl acetate by sonication for 60 s (sonifier, Bandelin electronic UW 70/HD 70; tip, MS 72/D, Berlin, Germany). Following the addition of 6 mL of a 10% aqueous solution of Pluronic F-68, the emulsion was sonicated again for 50 s, yielding the (w/o)/w emulsion that was poured into 100 mL of a 1% aqueous solution of Pluronic F-68. After mechanical stirring (600 rpm) for 1 h at room temperature, the residual ethyl acetate was removed under reduced pressure. The resulting nanosphere suspension was filtered (1 μ m pore size) to eliminate aggregates.

Particle Size Analysis and Processing of the Nanospheres. The mean particle size and distribution were determined by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Instruments Ltd, U.K.). All measurements were carried out in triplicate at 20 °C after 5 min of equilibration time.

The removal of Pluronic was practically achieved by diafiltration. For this, 20 mL of a PLGA nanosphere suspension was repeatedly washed with 40 mL of distilled water on a tangential flow filtration system based on an ultrafiltration membrane (Vivaflow 50; 100 000 MWCO PES, Sartorius Vivascience GmbH) which was operated by a peristaltic pump (MV-CA 8, Ismatec, Glatbrugg) at a system pressure of 2.5 bar.

Quantification of the PEO-PPO Block Copolymer. Pluronic F-68 was quantified using a photometric assay relying on the complexation of a purple cobalt thiocyanate reagent with the

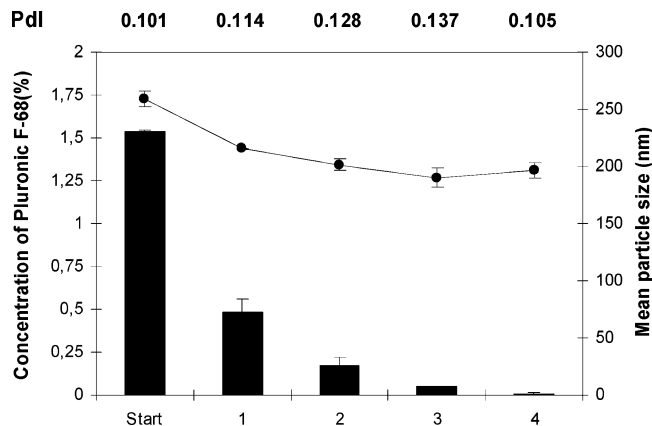


Figure 1. Polydispersity index (PdI) and mean particle size of PLGA nanoparticles determined by dynamic light scattering (—●—) as well as the respective content of Pluronic F-68 in the suspension (solid bars) in course of purification by diafiltration.

copolymer.²⁶ The cobalt thiocyanate reagent used for the photometric quantification was prepared by dissolving 20 g of ammonium thiocyanate and 3 g of cobalt(II) nitrate·6H₂O in 100 mL of distilled water. Ethanol (80 μ L, 96%), ethyl acetate (200 μ L), cobalt thiocyanate reagent (100 μ L), and the sample (50, 150, or 250 μ L, depending on the concentration range) were mixed in a 2 mL Eppendorf cup. Following centrifugation of the precipitated complex at 10 400 rpm for 1 min (Centrifuge 5804R, Eppendorf), the supernatant was removed. After the tube walls and pellet were washed with ethyl acetate, the precipitate was dissolved in 2 or 0.5 mL of acetone, and the absorbance at 328 nm was determined using a U-3000 UV/vis spectrophotometer (Hitachi).

Viscosity Measurements. The dynamic viscosities of aqueous solutions of Pluronic were determined according to run-time measurements on a microviscometer (AMVn, Anton Paar GmbH, Graz) at 20 °C using calibrated capillary/ball sets at an angle of inclination of 60°. The densities of the samples that are required to calculate the viscosities were determined with an oscillating U-tube densitometer (Anton Paar GmbH, Graz). All measurements were performed in triplicate with water as a reference, and the dynamic viscosity η was calculated according to eq 1 with K as the calibration constant, t as the run time of the ball, ρ_B as the density of the ball, and ρ_S as the density of the sample.

$$\eta = Kt(\rho_B - \rho_S) \quad (1)$$

Results and Discussion

To investigate concentration-dependent effects of Pluronic on the particle sizing of nanospheres, a method for the quantification of the tenside is required. This was provided by a cobalt thiocyanate assay described in the literature.²⁶ Modifications of the sample size as well as the acetone volume for dissolution of the reagent/Pluronic complex lowered the detection limit of the assay and thereby allowed the direct determination of the tenside in aqueous solutions in the range from 2.5 to 0.005%.

When the Pluronic content of a PLGA nanoparticle suspension was analyzed in the course of diafiltration with distilled water, a rapid decrease in the concentration was observed, reaching the detection limit of the photometric assay after four washing steps (Figure 1).

Starting with a PLGA nanosphere suspension containing 1.54% Pluronic, $96.7 \pm 0.35\%$ was removed during the first three washings, and 99.74% of the tenside was removed after one more purification step. Thus, a considerable amount of Pluronic contained in nanoparticle suspensions prepared according to the

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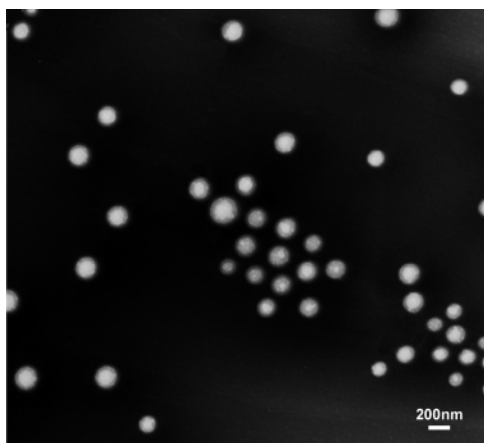


Figure 2. TEM image of PLGA nanoparticles suspended in a 1.54% aqueous solution of Pluronic, negative stained with uranyl acetate.

double-emulsion solvent-evaporation technique described above can be removed in a controllable manner by diafiltration with distilled water. In parallel, possible shifts in the particle size distribution were monitored by sizing the colloids via dynamic light scattering after each washing step. Commonly used purification methods such as (ultra)centrifugation impede the complete redispersibility of the pellet into single nanospheres as a result of the high rotation rates necessary for sufficient sedimentation. In contrast, tangential flow filtration allows to study the implication of washed-out surfactant in an unaffected manner.

These particle size measurements showed, concurrent with the previously discussed decrease in Pluronic content, that the sizes were notably lowered from 259 ± 7 to 196 ± 6 nm within the first four purification steps (Figure 1). During the next three washings, the suspension remained rather stable as controlled by DLS, and the mean particle size was not reduced below a threshold of about 195 nm.

These monitored size differences might partially be due to the desorption of surfactant molecules from the particle surface in the later stages of the washing procedure. However, according to Baker and Berg, size alterations in the hydrodynamic diameter of the colloids due to the adlayer of Pluronic F-68 solely amount to 6 nm.¹⁴

Furthermore, the seeming drop in particle size by 63 ± 6 nm cannot be attributed to a gradual degradation of the nanospheres because otherwise the decrease would have progressed in the course of further washing steps. It is also known from molecular weight loss studies of microparticles made from PLGA with the same lactide/glycolide ratio of 50:50 that the polymer decomposes rather slowly as illustrated by a half-life (50% molecular weight loss) of 15 days.²⁷

Thus, the apparent decrease in the mean particle size during the first four washing steps might be attributed to the lowering of the Pluronic concentration (Figure 1). To narrow the observed effect down to an interference of varied Pluronic levels with DLS, the physical dimensions of the particles suspended in a 1.54% aqueous solution of Pluronic were analyzed by an independent imaging method (Figure 2). Indeed, as assessed by transmission electron microscopy (TEM; Zeiss EM 902) measurements, the mean particle size of the colloids is only about 200 nm compared with 259 ± 7 nm determined via the light scattering technique.

To determine if the apparent decrease in size during diafiltration is reversible, 0.5, 1, 1.5, and 2% tenside were added to 1 mL

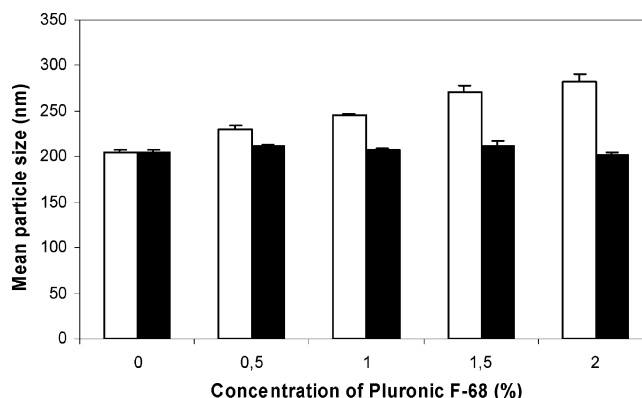


Figure 3. Mean particle size of PLGA nanoparticles dispersed in aqueous solutions of Pluronic F-68. Viscosity of water as dispersant property (open bars, DLS instrument's standard parameter). Results recalculated with the respectively determined dynamic viscosities (solid bars).

Table 1. Dynamic Viscosities of Aqueous Solutions of Pluronic F-68 Determined at 20 °C

dispersant	dynamic viscosity at 20 °C (mPa s)
water (reference)	1.0100 ± 0.002
0.5% aqueous solution of Pluronic F-68	1.0765 ± 0.021
1% aqueous solution of Pluronic F-68	1.1595 ± 0.009
1.5% aqueous solution of Pluronic F-68	1.2578 ± 0.0001
2% aqueous solution of Pluronic F-68	1.3772 ± 0.003

aliquots of a purified nanoparticle suspension with a Pluronic content of $<0.005\%$. As illustrated in Figure 3 (open bars), this results in a stepwise increase in the determined mean particle size with rising Pluronic content.

The cause of these reversibly controllable size shifts was considered to be linked to particle size calculation by the DLS instrument. Using this technique, the random movement of colloids is monitored according to fluctuations in the intensity of the scattered light. From this, a decaying exponential correlation function is derived, and it contains the translational diffusion coefficient D . According to the Stokes–Einstein relation (eq 2), D is inversely proportional to the dynamic viscosity η and the radius of an idealized sphere r with k as the Boltzmann constant and T as the absolute temperature. Thus, if the temperature is held constant, the diffusion coefficient for a sphere with the given radius r will be solely dependent on the dynamic viscosity of the suspension.

$$D = \frac{kT}{6\pi\eta r} \quad (2)$$

Experimentally, the influence of Pluronic on this variable was confirmed by a determination of the dynamic viscosities of 0.5, 1, 1.5, and 2% aqueous solutions of tenside. While viscosity measurements implicate shear stress of the sample, dynamic light scattering experiments are performed under static conditions. If the fluid exhibits Newtonian behavior, however, the viscosity can be extrapolated to that of a system under no shear stress and thus can be applied for this purpose. Because of the low polymer content used in the experiments, it can be assumed that the determined viscosity of the fluid is rather shear-independent. Measurements illustrated in Table 1 show that increasing concentrations of the surfactant lead to incremental increases in the dynamic viscosity of the solution.

The effect of this increased viscosity on the size calculation by the DLS instrument, as compared to that of the standard dispersant water, was assessed by a re-evaluation of the previous

results from suspensions with different Pluronic content using the respectively adjusted viscosities. Consequently, the sizes of all samples were determined to be approximately 205 nm (Figure 3, solid bars), which corresponds to that of the control sample with less than 0.005% Pluronic as a reference.

Furthermore, preliminary results from viscosity measurements of PVA, another frequently used stabilizing agent for the production of polymeric nanoparticles,^{20,28,29} imply a similar effect on nanoparticle sizing as observed for Pluronic. The viscosities of 1 and 2% aqueous solutions of practically used PVA ($M_w \approx 67\,000$) were determined to be 1.735 ± 0.002 and 2.922 ± 0.012 mPa s, respectively. When extrapolating these viscosities to sizing results of nanospheres with a mean diameter of 200 nm, apparent increases to 425 nm (1% PVA) and 687 nm (2% PVA) can be expected.

Summary

In the present work, it has been demonstrated that the results obtained from nanoparticle sizing by dynamic light scattering are prone to influences by tensides contained in the suspension. Practically relevant concentration variances between 0.005 and 2% PEO-PPO surfactants in a PLGA nanoparticle suspension are associated with significant alterations of the determined mean particle size. This effect has been attributed to the Pluronic-

concentration-dependent fluctuations in the dynamic viscosity of the nanoparticle suspension, which directly affect particle sizing by the dynamic light scattering technique. Because of the general cause of this implication, it can be deduced that the presence of any substance that might alter the suspension's properties needs to be considered.

To receive reliable results, the respective dynamic viscosities of the samples that can be determined either directly by viscometry or indirectly by quantification of the Pluronic concentration have to be supplied for the size calculation process. This will provide more accurate information on the actual size of the colloids, which is of special importance for studies on size-dependent properties of nanoparticles.

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