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Research paper

Protein–nanoparticle interactions evaluation by immunomethods: Surfactants can disturb quantitative determinations

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ABSTRACT

The adsorption of proteins on nanoparticle surface is one of the first events that occur when nanoparticles enter in the blood stream, which influences nanoparticles lifetime and further biodistribution. Albumin, which is the most abundant protein in serum and which has been deeply characterized, is an interesting model protein to investigate nanoparticle–protein interactions. Therefore, the interaction of nanoparticles with serum albumin has been widely studied. Immunomethods were suggested for the investigation of adsorption isotherms because of their ease to quantify the non-adsorbed bovine serum albumin without the need of applying separation methods that could modify the balance between the adsorbed and non-adsorbed proteins. The present work revealed that this method should be applied with caution. Artifacts in the determination of free protein can be generated by the presence of surfactants such as polysorbate 80, widely used in the pharmaceutical and biomedical field, that are needed to preserve the stability of nanoparticle dispersions. It was shown that the presence of traces of polysorbate 80 in the dispersion leads to an overestimation of the amount of bovine serum albumin remaining free in the dispersion medium when determined by both radial immunodiffusion and rocket immunoelectrophoresis. However, traces of poloxamer 188 did not result in clear perturbed migrations. These methods are not appropriate to perform adsorption isotherms of proteins on nanoparticle dispersions containing traces of remaining free surfactant. They should only be applied on dispersions that are free of surfactant that is not associated with nanoparticles.

1. Introduction

Nanoparticle interactions with blood proteins are key factors when nanoparticles are intended for intravenous administration, since they will influence the nanoparticle lifetime in the blood as well as their biodistribution [1–4]. Among blood proteins, serum albumin has been widely studied, because it is one of the most prevalent proteins in the blood (around 4%). Due to its high concentration, serum albumin is one of the first adsorbed proteins onto nanoparticle surface when nanoparticles enter the bloodstream [4–7]. Diverse methodologies have been used in previous works for the study of albumin adsorption onto nanoparticle surface [4,8–11]. Immunomethods (rocket immunoelectrophoresis and radial immunodiffusion) represent appropriate methodologies for these studies, since they were proved to give reproducible, feasible and easily interpretable results, with the possibility of obtaining quantitative data [4,11–13]. In addition, they can be performed using little equipment. Another interesting advantage is that they can be applied directly on samples without the need to separate the nanoparticle dispersion for the analysis, which could modify the balance between adsorbed/non-adsorbed protein. Therefore, they have been used to study adsorption isotherms of serum albumin on nanoparticles in the previous studies [4,11]. The nanoparticles considered in these previous reports were made of poly(isobutyl-cyanoacrylate) (PIBCA) – dextran copolymers. The dispersions were purified to remove all traces of remaining free polymers after the synthesis. In the present work, the methods were applied to study the adsorption isotherm of bovine serum

Abbreviations: BSA, bovine serum albumin; CMC, micellar critic concentration; DLS, dynamic light scattering; P, poloxamer 188; PBS, phosphate buffered saline; PIBCA, poly(isobutyl-cyanoacrylate); PIC, phase inversion composition method; PLGA, poly-(lactic-co-glycolic acid); RRPE, redox radical emulsion polymerization method; T, polysorbate 80.

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albumin (BSA) on poly-(lactic-co-glycolic) acid (PLGA) nanoparticles [14]. The dispersions contained remaining traces of polysorbate 80 (an ethoxylated nonionic surfactant), which was required to maintain the stability of the nanoparticles, hence to prevent their aggregation. As the application of the immunomethods to the determination of adsorption isotherms generated inconsistent results, the aim of the present work was to investigate the role of polysorbate 80 in the discrepancy of the method comparing data obtained by the two immunomethods (rocket immunoelectrophoresis and radial immunodiffusion) from BSA solutions of know concentrations, BSA solutions at the same concentration but containing polysorbate 80 and BSA solutions at the same concentration in which the nanoparticles were added. In addition, PIBCA – dextran nanoparticles were used for comparison purposes, as a model suitable for immunomethods, as well as PIBCA – chitosan nanoparticles coated with the nonionic triblock copolymer poloxamer 188 [15] were also used to test the adequacy of immunomethods in the presence of another surfactant.

2. Results

The measurement of the interaction of the bovine serum albumin (BSA) with nanoparticles was performed using rocket immunoelectrophoresis and radial immunodiffusion methods. Samples were incubated with different concentrations of BSA, in 10 mM PBS, at pH 7.4. Samples tested included the following:

(a) Solutions of BSA as the standard calibration curve (BK).
(b) Aqueous solutions of BSA with polysorbate 80 (T) at a concentration of 3 wt% (T_3), since that was the surfactant concentration used for the preparation of the template nano-emulsions of NP-A (PLGA nanoparticles).
(c) Aqueous solutions of BSA with polysorbate 80 at a concentration of 0.3 wt% ($T_{0.3}$). This concentration corresponded to a dilution of 1/10 of the solution used to prepare NP-A. This dilution was applied arbitrary to take into account the surfactant loss during the purification of the nanoparticles by dialysis.
(d) Aqueous solutions of BSA with polysorbate 80 at a concentration of 0.001 wt% ($T_{0.001}$), to include a solution of the surfactant at a concentration below the critical micellar concentration (CMC).
(e) Aqueous solutions of BSA with poloxamer 188 (P), at a concentration of 3 wt% (P_3), to compare with another surfactant.
(f) Aqueous solutions of BSA with poloxamer 188, at a concentration of 0.3 wt% ($P_{0.3}$), to compare with the other surfactant.
(g) Aqueous solutions of BSA with poloxamer 188, at a concentration of 0.03 wt% ($P_{0.03}$), to study a surfactant concentration below the CMC (CMC of poloxamer 188 = 0.0334 wt% at 25 °C, Amicogen).
(h) PLGA nanoparticles, containing traces of polysorbate 80 (NP-A).
(i) PIBCA nanoparticles coated with poloxamer 188 and chitosan (NP-B).
(j) PIBCA – dextran nanoparticles (NP-C).

Rocket immunoelectrophoresis consists in performing the migration of proteins, in this case BSA, in a gel containing specific antibody. While the protein migrates in the gel because of the electric field, they find antibody and from antigen–antibody complexes that are precipitating in the gel with a maximum precipitation for a 1/1 antigen–antibody ratio, forming a line of precipitation which occurs as a peak in the case of rocket immunoelectrophoresis. The height of the peak is proportional to the concentration of the protein that serves as antigen in the gel. Rocket immunoelectrophoresis performed with standard solutions of BSA gave a linear response between the height of the peak formed by the precipitation line and the BSA concentration deposited in the well consistently with what was expected. Then, the experiment was repeated with the different samples containing different concentrations of surfactants or nanoparticles. A relationship between the height of peak and the BSA concentration was found. However, when comparing the results with those obtained with the standard BSA solution of the calibration curve, results obtained with samples containing polysorbate appeared inconsistent. With polysorbate solutions at a concentration higher than the CMC, the migration peaks due to the precipitation of BSA with its antibody in the gel were higher than those found with the standard solution of BSA at the same concentration (Fig. 1a). Lower peaks were observed when the concentration of polysorbate was below the CMC (Fig. 1c). Experiments considering NP-A showed a much further increase in the height of peaks appearing on the gel compared with those of the standard solution of BSA at the same concentration. This suggested that free polysorbate 80 remained in the nanoparticle dispersion at a higher concentration than the CMC.

The other surfactant tested in this study, poloxamer 188 (P), did not produce variation of the height of the peak of BSA compared with corresponding standard solutions (Fig. 1b and c). So did the NP-B, which were produced using this surfactant as the height of the peak of BSA was not disturbed by poloxamer 188 and it remained unchanged in the presence of NP-B. It could be suggested that all the BSA introduced in the NP-B dispersions remained free in the dispersion and that BSA was not adsorbed on these nanoparticles.

NP-C were used in this study as control nanoparticles known to adsorb BSA. Consistently with the previously published results [4], the height of the peaks of BSA was reduced compared to those given by the standard solutions of BSA at the same concentration, confirming that these nanoparticles adsorbed BSA and that the method can be applied if no molecules disturbing the analysis are present in the dispersing media.

The differences noted from direct measurements of the peaks appearing on the rocket immunoelectrophoresis were quantified using BSA calibration curves. BSA concentrations plotted against the migration height (and the surface ring for radial immunodiffusion) showed a linear relationship (Fig. 2).

After normalization of the quantified results, taking ratios given by the height of the peak of the sample over that of the standard solution of BSA of the same concentration, higher differences between the BSA standard solution and some tested samples were highlighted (Fig. 3).

Concerning radial immunodiffusion experiments, BSA is left to diffuse in a gel containing the antibody where it precipitates when the diameter of the circle allows optimal precipitation of the antigen–antibody complexes at a 1/1 ratio. Both, the visual study of the gels and the normalization of the surfaces of the circles were performed, as for the rocket immunoelectrophoresis. In this case, the higher the protein concentration, the larger the precipitation rings. An example of the resulting rings is presented in Fig. 4. The same samples than those for rocket immunoelectrophoresis were used, although in this case, only BSA concentrations of 100 and 200 μg/mL were tested, since lower concentrations studied did not result in reliable results (results not shown). The results agreed well with those of the rocket immunoelectrophoresis (Fig. 1). As expected, all tested samples showed a linear relationship between the BSA concentration and the ring surface area. Nevertheless, as it happened for rocket immunoelectrophoresis, the immunoprecipitating rings for those aqueous solutions containing polysorbate 80 at concentrations higher than the CMC were much larger than