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Protein adsorption and complement activation for di-block copolymer nanoparticles

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ABSTRACT

Four types of nanoparticles with core-diffuse shell structures have been synthesized through self-assembly of PICBA-Dextran block copolymers. These nanoparticles are designed to carry pharmaceutically active molecules into the human body through injection into the blood stream. In this work, we have determined how the characteristics of the diffuse shell influence the adsorption of three types of proteins: Bovine Serum Albumin (BSA), fibrinogen, and a protein from the complement system that triggers recognition and elimination by macrophages. We have determined the structural characteristics of the diffuse shells using Nuclear Magnetic Resonance (NMR), Small Angle Neutron Scattering (SANS) and Quasi-Elastic Light Scattering (QELS). We have measured the adsorption of Bovine Serum Albumin (BSA) through Immuno-diffusion methods, and found that it adsorbed in substantial amounts even when the distance between dextran chains at the core-diffuse shell interface is quite short. We have observed the aggregation of the nanoparticles induced by fibrinogen, and found that it was prevented when the density of dextran chains protruding from the core surface was sufficiently high. Finally we have measured the activation of the complement system by the nanoparticles, and found that it was also limited by the surface density of dextran chains that protrude from the core and by their mesh size within the diffuse shell.

1. Introduction

Colloidal particles are often used to carry molecules that are pharmaceutically active from the point of administration to a target site in the body. The requirements for such a particle are threefold (a) it should carry a sufficient number of active molecules; (b) it should avoid the body's mechanisms for recognition of foreign particles; (c) it should be able to deliver the active molecules to the target. In order to perform these functions, most colloidal carriers rely on a *core-diffuse shell* structure. Typically, the core is made of a hydrophobic polymer that dissolves or traps the active molecules. The diffuse shell (also called corona) is made of hydrophilic polymers that "hide" this hydrophobic core [1].

Colloidal carriers that are injected into the blood stream find themselves in a concentrated protein solution. The most abundant protein, serum albumin, is present in blood at a concentration of 4%. Consequently, the first event that takes place when a particle is injected in the blood is the adsorption of proteins, mainly serum albumin. Subsequently, other proteins may either displace the bound serum albumins or bind to the absorbed serum albumin layers [2–4]. Recognition and elimination of these foreign particles by the body defense mechanisms including macrophages may then occur through the absorption of specific proteins, e.g. the C3 protein of the complement system [5]. Upon binding, the C3 protein will change conformation, expose a reactive site, and release a signaling molecule that triggers the chain of bio-chemical events called the complement activation cascade [6]. This will lead to the elimination of the particles by the macrophages [7].

There are indications that some chemical characteristics of the diffuse shell have an effect on complement activation. Indeed a chain length effect has been found for diffuse shells made of PEG or polysaccharides [8,9]. Also, an effect of the grafting density of hydrophilic chains on the particle surfaces has been found in the case of PEG chains [8]. The nature of the hydrophilic chains may also be important. These effects may be caused by interactions of C3 with albumins that are already adsorbed [10]. Indeed, since the absorption of the C3 complement protein takes place after that of serum albumin, it will be influenced by the amount of bound serum

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albumins, their configurations at the surface, and their accessibility. Thus, in order to design the diffuse shell for avoiding complement activation, we need to know more about the adsorption of albumin.

Recently we have synthesized a collection of nanometric-sized drug carriers through self-assembly of block copolymers. As a hydrophobic block we chose poly(isobutylcyanoacrylate) (PIBCA), which is a bioerodible and bioeliminable polymer that has already been used to deliver in vivo a number of drug molecules [11,12]. As a hydrophilic block, we chose dextran, which is a polysaccharide known to restrict protein adsorption [13–15]. The self-assembly of these copolymers does yield particles with a core-diffuse shell structure that is determined by the respective lengths of the hydrophobic and hydrophilic blocks [9,16]. By changing the conditions of polymerization, we have produced two types of particles that differ only by the core size and by the density and extension of the diffuse shell. Remarkably, these two types have very different effects on complement activation. This opens the possibility of finding how the characteristics of the diffuse shell control protein adsorption and complement activation.

In this paper we report characterizations of these polymer particles by Small Angle Neutron Scattering (SANS), measurements of the adsorption of the abundant blood proteins, serum albumin, through rocket-immunoelectrophoresis [17] and radial immunodiffusion [18], measurements of fibrinogen-induced aggregation through SANS, and measurements of the activation of the complement system, through 2D immunoelectrophoresis [9,19]. We attempt to answer the following questions:

(a) Does the diffuse shell limit protein adsorption, what is the location of the adsorbed proteins, and what is their configuration?
(b) What is the relation between the number of hydrophilic chains that protrude out of the core and the number of proteins that can be adsorbed?
(c) Is there a relation between the number or the configuration of adsorbed proteins and the activation of the complement system?

2. Materials and methods

2.1. Preparation of nanoparticles

PIBCA nanoparticles were prepared by redox radical emulsion polymerization (RREP) according to the method of Bertholon et al. [9,16]. The polymerization initiated on dextran produces bloc-copolymers which self-assemble into nanoparticles with a *core-diffuse shell* structure.

Two dispersions were prepared named A and B, differing by the amount of initiator (dextran-cerium). Nanoparticles A were obtained in 8 mL of nitric acid 0.2 M containing 0.137 g of dextran (molar mass 69300 Da). The solution was placed under vigorous magnetic agitation at 40 °C and purged with nitrogen for 10 min. Then, 2 mL of a solution of cerium ammonium nitrate (0.08 M in nitric acid 0.2 M) and 0.5 mL of isobutylcyanoacrylate (IBCA) were added successively and the polymerization was allowed to proceed for 1 h at 40 °C. For nanoparticles B, the volume of nitric acid 0.2 M was 9.3 mL, the amount of dextran was 0.0502 g and the volume of cerium ammonium nitrate was 0.7 mL. All other conditions were the same.

Immediately after polymerization, the nanoparticle dispersions were cooled down to room temperature using an ice bath. They were purified by dialysis (Spectra/Por® membrane 100 000 g/mol molecular weight cut off) against 1 L of water 4 times to remove unreacted reagents and to raise the pH to a neutral value. Further purification included a first centrifugation at 720-× *g* for 10 min followed by a second centrifugation at 17,000-× *g* for 15 min to remove any aggregates. After the second centrifugation, the lower part of the supernatant (340 μl) was collected as fractions A_{middle} and B_{middle} respectively and the upper part of the supernatant (600 μl) as fractions A_{top} and B_{top} respectively. The volume fractions of the dispersions were around 1%.

2.2. Particle sizes and structures

2.2.1. Quasi-elastic light scattering (QELS)

The hydrodynamic radii of the nanoparticles were measured through QELS using a Zetasizer ZS90 instrument (Malvern, France). Measurements were performed at 25 °C at a scattering angle of 90°. The intensity correlation function was fitted through the instrument software to yield a volume-weighted distribution of particle sizes. From this distribution we calculated the total volume and total surface area of the nanoparticles, and the radius that describes the corresponding hydrodynamic volume/surface ratio. This radius is identical to the z-averaged radius of the particles defined by Eq. (1).

$$R_z = \sum N_i R_i^3 \Big/ \sum N_i R_i^2 \quad (1)$$

Dispersions were diluted in Milli® Q water filtered over a 0.22 μm membrane prior to the measurement.

2.2.2. Scanning electron microscopy (SEM)

Images of freeze-dried nanoparticle dispersions were obtained using a scanning electron microscope LEO 1530 (LEICA) equipped with a Gemini column. Prior to observation, freeze-dried samples were mounted on supports and coated with a 2 nm Pt/Pd layer using a Cressington sputter coater 208HR apparatus (Cressington, France) operating under argon atmosphere. The sizes of more than 300 individual nanoparticles were measured to determine the mean radius and the parameters of the size distribution.

2.2.3. Small Angle Neutron Scattering (SANS)

Scattering patterns from liquid nanoparticle dispersions were obtained using the instrument D11 at the ILL. The experiments yielded scattering patterns consisting of intensities as a function of scattering vector $\boldsymbol{q}$. Since the dispersions were isotropic, the scattering patterns were averaged over all directions of $\boldsymbol{q}$, yielding spectra of intensity I vs. magnitude q of the scattering vector, which is related to wavelength λ and scattering angle θ by $q = (4\pi/\lambda)\sin(\theta/2)$. The range of q values was 6×10^{-3} to 9×10^{-2} nm^{-1}. In this range, the interference patterns probe variations of the density of scattering length $\boldsymbol{\rho}(r)$ with a resolution of 10 nm. Scattering is then produced by the difference between the scattering density of a particle, $\boldsymbol{\rho}(r)$, and that of the solvent, $\boldsymbol{\rho}_s$. For the characterization of the particles, the dispersions were made in H_2O, as the difference of scattering density between the particles and H_2O was sufficient to produce strong scattering [20]. Spectra were fitted with a calculated curve for a lognormal distribution of spheres. The z-averaged value (R_z) was calculated using Eq. (1). Other experiments were made with dispersions made in H_2O-D_2O mixtures that matched the scattering density of the PIBCA-dextran nanoparticles, in order to enhance the contrast of the adsorbed proteins [20].

2.3. Composition of the nanoparticles

The monomer compositions of the PIBCA-dextran copolymers were determined through (^{1}H) NMR spectroscopy. The copolymers were dissolved at 40 °C in dimethyl-d_6-sulfoxide (DMSO, d_6) (Carlo Erba) at the concentration of 20 mg/mL. Spectra were then recorded on a 300 MHz Brucker instrument (Brucker, France). The composition was deduced from the spectra by calculating the ratio between the integral of dextran peaks (4.3–5 ppm) and that of the CH peak of PIBCA (1.8–2.0 ppm) [21]. The molecular weight of the PIBCA part of the copolymer was calculated from the composition in dextran and PIBCA deduced from the NMR spectra and the known molecular weight of dextran (69 300 g/mol), assuming that the copolymer was composed of one chain of PIBCA and one chain of dextran as shown by Bertholon et al. [16].

2.4. BSA adsorption through immunomethods

Nanoparticles at a volume fraction of 0.5% were incubated with different concentrations of BSA in 10 mM phosphate buffer for 3 h at 37 °C. After incubation, the amount of non-adsorbed BSA was determined using immunochemical methods including immunoelectrophoresis [17] and radial immunodiffusion [18] as previously described [20].

In both cases, agarose gel plates (12 cm × 8.5 cm) were prepared on Gelbond® films for agarose gel (GE healthcare, BioScience, Sweden). The agarose solution (13 mL) at a concentration of 1% contained 0.3 mL of the bovine serum albumin antisera (Sigma, France) diluted at 1/5 in a saline phosphate buffer (10 mM, NaCl 140 mM, KCl 25 mM, pH 7.5). For the radial immunodiffusion technique, the gel was prepared in the saline phosphate buffer (10 mM, NaCl 140 mM, KCl 25 mM, pH 7.5) whereas tricine buffer (lactate calcium 1 mM, Tris 63 mM, Tricine 27 mM, pH 8.6) was used to prepare gels for the immunoelectrophoresis method. Wells holding 5 μL samples were formed using a 2 mm diameter punch on the gel plates. After deposition of the samples in the wells (5 μL) including standard solutions of BSA and nanoparticles incubated with the different concentrations in BSA, gels were placed in a humid chamber and let to diffuse in a quiet place for 48 h at room temperature in the case of the radial immunodiffusion technique. The conditions for the immunoelectrophoresis were 18 h, 230 V, 12 mA (Electrophoresis power supply EPS 600, Amersham, Pharmacia Biotech, Orsay, France) using Tricine buffer as the running buffer for the electrophoresis performed in a Pharmacia LKB Multiphor II apparatus (Amersham, Pharmacia Biotech, Orsay, France). At the end of both types of experiments, the gels were dried using Watmann® filter papers and stained with coomassie blue to reveal of the formation of BSA-antibody immunoprecipitate. Each gel contained a set of 5–6 standard solutions of BSA of concentrations ranging from 20 to 900 μg/mL for internal calibration purpose. In the case of the radial