European Journal of Pharmaceutics and Biopharmaceutics 130 (2018) 48–58

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Characterization of nanomedicines' surface coverage using molecular probes and capillary electrophoresis

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ARTICLE INFO

Keywords:
Nanomedicine
Capillary electrophoresis
Characterization
Surface coverage
Molecular probes

ABSTRACT

A faithful characterization of nanomedicine (NM) is needed for a better understanding of their *in vivo* outcomes. Size and surface charge are studied with well-established methods. However, other relevant parameters for the understanding of NM behavior *in vivo* remain largely inaccessible. For instance, the reactive surface of nanomedicines, which are often grafted with macromolecules to decrease their recognition by the immune system, is excluded from a systematic characterization. Yet, it is known that a subtle modification of NMs' surface characteristics (grafting density, molecular architecture and conformation of macromolecules) is at the root of major changes in the presence of biological components. In this work, a method that investigates the steric hindrance properties of the NMs' surface coverage based on its capacity to exclude or allow adsorption of well-defined proteins was developed based on capillary electrophoresis. A series of proteins with different molecular weights (MW) were used as molecular probes to screen their adsorption behavior on nanoparticles bearing different molecular architectures at their surface. This novel strategy evaluating to some degree a functionality of NMs can bring additional information about their shell property and might allow for a better perception of their behavior in the presence of biological components. The developed method could discriminate nanoparticles with a high surface coverage excluding high MW proteins from nanoparticles with a low surface coverage that allowed high MW proteins to adsorb on their surface. The method has the potential for further standardization and automation for a routine use. It can be applied in quality control of NMs and to investigate interactions between proteins and NM in different situations.

1. Introduction

The number of nanoparticle-based medicinal products that have reached clinical development has increased over the last years [1–7]. This is a positive signal acknowledging major general advances of nanomaterials clinical translation. However, several challenges remain unanswered [2,8–10]. Among them, the next important step in nanomedicine (NM) development is expected to come from the comprehension of their interactions with biological components encountered *in vivo* [11]. On this purpose, preliminary efforts are needed to improve the physicochemical characterization of nanomaterials. Indeed, incomplete or inappropriate characterization of important NMs' parameters were identified as critical points in project failures by actors involved in the development of NMs [12]. The characterization has been pointed out as a hurdle that delays and still hampers the development of projects involving nanomaterials [2,11,13–19].

Nanomaterials composing NMs include a wide range of components and their intended use suits for many applications considering different routes of administration, hence facing various delivery and safety challenges [2,20]. The precise knowledge of NMs' characteristics is an important process in the understanding of their biological activity and safety. This is also needed for quality control analysis to ensure batch to batch consistency of NMs and evaluate nano-similarity in generic version of NMs that has appeared on the market since 2013 [18]. A therapeutic molecule can be characterized from its well-defined signature deduced from its NMR spectra. In contrast, NMs are complex structures resulting from the combination of multiple components. Their functional behavior including their biological activity and safety depends on many parameters that are defined by fine-tuned of chemical and physical traits for each application [18,21]. Characterization of these

Abbreviations: CE, capillary electrophoresis; CZE, capillary zone electrophoresis; DLS, dynamic light scattering; ID, immunodiffusion; IE, immunoelectrophoresis; NM, nanomedicine; NP, nanoparticle; PC, principal component; PCA, principal component analysis; PEO, polyethylene oxide; pI, isoelectric point; RC, repellent capacity; SDS, sodium dodecyl sulfate

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https://doi.org/10.1016/j.ejpb.2018.06.014
Received 11 May 2018; Received in revised form 12 June 2018; Accepted 12 June 2018

nanomaterials is complex and parameters to evaluate remains the subject of open discussions. They are often decided on a case by case basis from discussion between companies and health authorities' actors [9,22–25].

Today, properties such as size and surface charge are well controlled and validated to ensure batch to batch consistency. On their own, they are not sufficient to warrant NMs' functionality hence *in vivo* fate reproducibility [19,26,27]. A short list proposed by Crist and McNeil [18] from the National Characterization Laboratory of the National Institute of Health includes "the level of surface coverage required for optimal biological performance". The notion of surface coverage includes the density, length, architecture and/or conformation of macromolecules grafted on NM's surface. This parameter regulates the way nanomaterials interact with proteins that is a major phenomenon occurring *in vivo* in biological fluids containing proteins, with a tremendous impact on the functionality and the biological activity of nanomaterials [28–36]. The importance of the understanding of the surface coverage of NMs has also recently been highlighted in a work studying different macromolecular grafting by Bertrand et al [37]. This suggests that characterization methods of the nanomaterial surface coverage should reach the molecular level, degree of precision needed for the intended use. So far, such evaluations require the use of highly specialized methods that are not generally available [12]. To sum up, the characterization of surface properties has been identified to be not optimal. Regulatory agencies emphasized the need for new methods on this purpose [12,19,23,26,27,38].

To answer this yet unaddressed need, the purpose of our work was to develop a method evaluating the surface coverage of nanomedicines that can be implemented in a quality control analytical cascade. Straightforward characterization of the density, architecture and/or conformation of macromolecules grafted on NM's surface and of the thickness formed by the layer would be highly demanding and cannot be envisaged for a routine use. The proposed method was based on an indirect approach investigating repellent capacities of the NM surface coverage to the adsorption of a series of proteins having well-defined size characteristics. In this method, the proteins served as molecular probes to explore the steric exclusion capacity of the NM surface coverage assuming that a part of it is driven by steric hindrance effect due to the surface coverage property [39]. Proteins taken as molecular probes were chosen from several criteria including knowledge on their characteristics (molecular weight, size through their hydrodynamic diameter, conformation) and their availability as affordable marketed compounds at a high purity grade. The principle of the method was based on the evaluation of the adsorption of individual proteins and the establishment of a map of surface adsorption/exclusion assumed to reflect the surface coverage property of the tested NM. The choice of proteins to probe surface properties of NM was also consistent with their key role in mechanisms controlling the *in vivo* activity of these compounds [37,40,41]. The proposed method was designed to provide a simple model evaluating NM surface functionality that can be implemented in routine to be used in quality control of NMs. It is noteworthy that the most relevant method to explore the surface properties of NM would certainly consist on the determination of the protein corona that identifies and quantifies all proteins that adsorbed on NM surface after incubation in plasma. The profile of adsorbed proteins deduced from this analysis determines the so called "biological identity" of the nanomaterial [42]. It is very sensitive to the surface coverage properties of the nanomaterial and its determination raised interests to develop predictive models for the *in vivo* behavior of NMs including their cellular uptake, stability and biodistribution [41,43–45]. The method would provide a screenshot of what happens after NM's injection into the blood [41,42,46–48]. However, currently, methods allowing the establishment of the protein corona are based on the most advanced techniques applied in proteomic analysis using last generation of liquid chromatography - mass spectrometry (LC-MS) instruments. A high expertise is also needed to interpret the data [46,47,49]. The preparation of samples prior to the proteomic analysis is based on many manipulations that may introduce bias in the determination of the protein corona [50,51]. These possibly disturbing steps might be at the root of the major hurdles encountered for a clear correlation of predictive model between synthetic identity, biological identity formed after administration and outcome of NMs. Thus, it is unrealistic to use such a method at a large scale to characterize nanomaterials on a routine basis for now.

The proposed method was designed to include two steps that can be easily implemented into a high throughput and automated analytical method. They included an incubation of the NM with a series of individual protein and the evaluation of the non-adsorbed fraction of the protein using a general analytical method. A challenge was to choose an analytical method that could be applied straightforward on the sample without pretreatment. Capillary electrophoresis (CE) was selected consistently with its growing interest in studies dealing with protein-nanomaterial interactions [52–56]. Thanks to its adaptability to different purposes, it was assumed that it could be applied on samples without pretreatment. Another advantage is the low sample consumption. This paper describes the different steps of the development of the method and reports the evaluation of its potential to discriminate nanoparticles with low and high protein repellent capacity of the surface coverage using a series of 5 polymer nanoparticles designed with different densities, conformations and lengths of chains of dextran grafted on their surface [29,57–59].

2. Materials and experimental methods

2.1. Materials

Reagents and buffer components: Agarose, polyethylene oxide (PEO) (200,000 Da), ammonium hydroxide, hexadimetrine bromide (Polybrene®), tricine, tris Base (Sigma 7-9®), benzoic acid, sodium chloride and sodium dodecyl sulfate (SDS) were purchased from Sigma (Saint-Quentin-Fallavier, France). Hydrochloric acid 1 M and sodium hydroxide 1 M were supplied by Prolabo (Paris, France). Sodium dihydrogenophosphate monohydrate was obtained from Carlo Erba (Valde-Reuil, France). Calcium lactate, glacial acetic acid, coomassie brilliant blue R-250 were supplied by Thermo Fisher Scientific (Villebon-sur-Yvette, France). Gel-Fix™ for agarose (265 × 150 mm) was obtained from Serva Electrophoresis (Heidelberg, Germany). All chemicals were of reagent grade and used as purchased. Water used during the study was ultrapure water from a MilliQ® system (Merck, Fontenay-sous-bois, France).

Proteins and antibodies: Goat anti-human IgG, apo-transferrin from human plasma, human immunoglobulin G and transthyretin from human plasma were provided by Merck (Fontenay-sous-bois, France) with a purity > 95%. Aprotinin from bovine lung (purity = 80%), bovine serum albumin (purity > 96%), insulin B chain from bovine pancreas (puritiy > 80%), orosomucoïd from human plasma (purity > 99%), ovalbumin from chicken egg white (purity > 98%), thyroglobulin from bovine thyroid (purity > 90%), fibrinogen fraction I type 1 from human plasma (purity = 66%) and rabbit anti-bovine serum albumin were purchased from Sigma (Saint-Quentin-Fallavier, France).

2.2. Experimental methods

2.2.1. Nanoparticles synthesis and characterization

Model nanoparticles made of poly(isobutylcyanoacrylate) (PIBCA) and dextran were used in this work. Three types of nanoparticles (NPs) were prepared according to the radical redox emulsion polymerization method described by Bertholon et al. (R1, R2 and R3) [57]. Two types of NPs were prepared via anionic emulsion polymerization method (A1 and A2) [57–60]. Aqueous dispersions were obtained after purification by dialysis against water. NPs were then characterized in terms of