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Fluorescence labels may significantly affect the protein adsorption on hydrophilic nanomaterials

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ABSTRACT

Fluorescently labelled proteins are often used to study processes *in vitro, e.g.* the binding of proteins to cell surfaces or the adsorption of plasma proteins on drug nanocarriers. However, the fact that the fluorescent labelling may affect the protein properties is frequently neglected. On the example of a simple model system, we reiterate the importance of this issue by showing that even a single label may perturb interactions between hydrophilic starch-based nanocapsules and serum albumin and thus prevent binding.

1. Introduction

Different forms of nanomaterials/-particles are currently being investigated in terms of their toxicology and applicability in nanomedicine. While a careful physico-chemical characterization of such nanomaterials is necessary their interaction with biological components is of great importance [1]. When nanoparticles (NPs) come into contact with biological fluids such as blood plasma, proteins from the environment begin to associate with the foreign interface (NP surface), forming a new 'biological' interface. This NP-protein interaction is referred to as the formation of a 'protein corona' and is being investigated extensively using various experimental methods [2–13]. Many of these methods, *e.g.* dynamic light scattering (DLS) [14], isothermal titration calorimetry (ITC) [15,16], liquid chromatography-mass spectrometry (LC-MS)[4,17,18] or differential centrifugal sedimentation (DCS) [3,9] do not require any special modification of the studied nanoparticles or proteins. However, some of the most sensitive methods like microscale thermophoresis [19] or fluorescence correlation spectroscopy (FCS) [8,10–13,20], require fluorescent labelling of the studied nanoparticles or proteins for successfull measurements. Here, a critical point is the required sensitivity of the studies and thus the size of the nanomaterials under investigation determines the labelling utilized for measurement. If the protein corona is large enough compared to the bare NPs, its formation can be studied by DLS or FCS using fluorescent nanoparticles and non-labelled proteins. For example, Nienhaus et al. [12,13] and Mukhopadhyay et al., [6,7] used either quantum dots or gold NPs to study non-labelled protein adsorption with FCS. Due to the very small size of the studied NPs (~10 nm), both groups were able to detect the formation of a protein monolayer on the nanoparticle surface. However, if the studied NPs are larger (as is commonly the case when drug nanocarriers are considered [21]) or the adsorption of one or few proteins per NP has to be detected, greater sensitivity is needed and FCS with fluorescently labelled proteins must be employed [8].

The use of fluorescently labelled proteins is not only important for the FCS studies of the protein corona, but is also relevant when other *in vitro* an *in vivo* processes are investigated with modern experimental techniques such as confocal microscopy, fluorescence-activated cell sorting (FACS), fluorescence resonance energy transfer (FRET), microscale thermophoresis (MST), etc. However, while it is well known that the attachment of fluorescent labels may change the physico-chemical properties of the used proteins [22–24], and thus affect their interaction with other species [25–27], this effect is often neglected or fully ignored. In the present study we highlight the impact of fluorescently labelling proteins on experimental outcomes via a simple protein corona formation model system.

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2. Results and discussion

As a model nanomaterial we used nanocapsules (NCs) composed of hydroxyethyl starch (HES) [28]. Due to their hydrophilic nature the HES-NCs exhibit a relatively low protein affinity compared to other, more hydrophobic nanomaterials. We compared the impact of different fluorescent labels attached to bovine and human serum albumin (BSA/HSA) on the interaction of these proteins with HES-NCs and found that the labelling may have a strong impact, up to complete suppression of protein adsorption. In order to investigate the influence of the label's chemical structure, three different fluorescent dyes, namely Alexa Fluor® 488 (AF488), Bodipy® FL (BP) and Fluorescein (FITC) were chosen.

The dyes exhibit different characteristics like polarity and absorption/emission maxima (see Fig. 1). Their dipole moments were calculated using ChemBio 3D Ultra GAMESS Interface and are shown in Fig. 1 together with the chemical structures. Commercially available conjugates of those dyes with BSA were purchased, while a BP-HSA conjugate was synthesized and purified in our group (see Experimental Part, Supplementary information).

The labelling of HSA was performed to obtain a protein with only one fluorescence label attached, since all commercially available conjugates feature several dye molecules per protein, as confirmed by FCS (see Table 1). The BP-HSA conjugate was characterized with high performance liquid chromatography (HPLC) to ensure complete conversion of the HSA (see Figs. S1–S3). Using FCS, the number of dye molecules per protein was confirmed to be one based on the fluorescent brightness measurements (see SI for details). Hydrophilic HES-NCs with an R_h of 128 ± 13 nm and hydrophobic polystyrene nanoparticles (PS-NPs) with an R_h of 59 ± 6 nm (sizes determined by DLS, size distributions see Fig. S4) were synthesized according to previously published procedures [28,29].

Table 1
Isoelectric focusing data of labelled and unlabelled albumin.

Protein	Number of attached dye molecules per protein[a]	IEP range of bands	IEP mean value
BSA	–	5.3–5.7	5.5
HSA	–	5.4–5.8	5.6
AF488-BSA	5	4.4–4.6	4.5
FITC-BSA	5	4.9–5.3	5.1
BP-BSA	5	4.7–5.1	4.9
BP-HSA	1	5.4–5.8	5.6

[a] As given by the supplier and roughly confirmed with FCS.

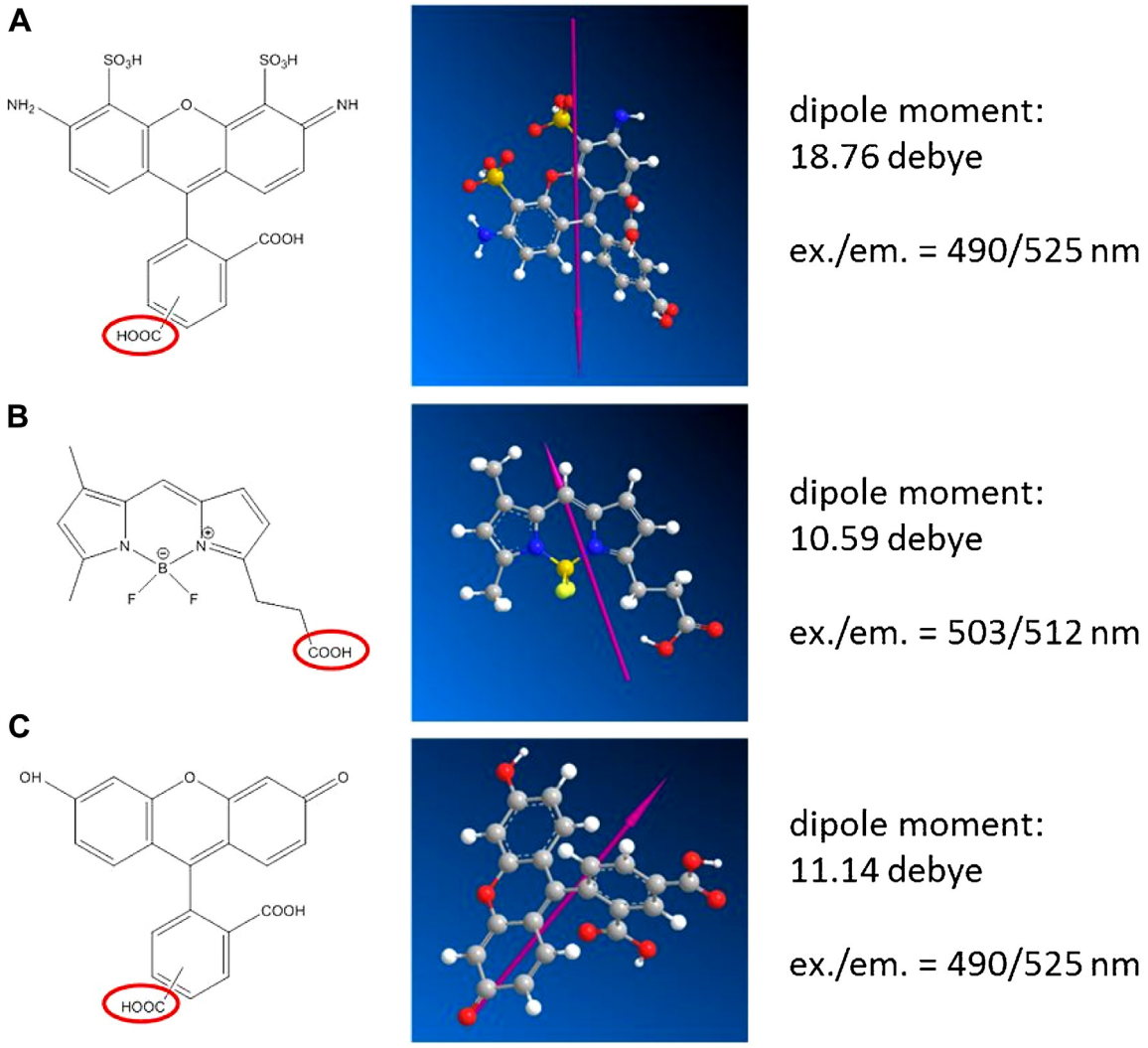

Fig. 1. (Left) Chemical structures of the fluorescent dyes used with a red circle indicating the carboxylic acid group coupled to the protein as amide bonds. For each structure the 3D orientation and dipole moment is displayed using a magenta arrow. The length of the arrow corresponds to the value of dipole moment. (right) Corresponding characteristics of used dyes: dipole moment calculated with ChemBio 3D Ultra GAMESS Interface and excitation/emission wavelengths as given by the supplier. A) Alexa Fluor® 488, B) BODIPY® FL, C) Fluorescein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)