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Electrokinetic elucidation of the interactions between persistent luminescent nanoprobes and the binary apolipoprotein-E/albumin protein system†

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The affinity between functional nanoparticles (NPs) and proteins could determine the efficacy of nanoprobes, nanosensors, nanocarriers, and many other devices for biomedical applications. Therefore, it is necessary to develop analytical strategies to accurately evaluate the magnitude of these protein corona interactions in physiological media. In this work, different electrokinetic strategies were implemented to accurately determine the interactions between PEGylated ZnGa1.995Cr0.005O4 persistent luminescent NPs (ZGO-PEG) and two important serum proteins: human serum albumin (HSA), the most abundant serum protein, and apolipoprotein-E (ApoE), associated with the active transport of NPs through the blood–brain barrier. Firstly, the injection of ZGO-PEG in a background electrolyte (BGE) containing individual proteins allowed an affinity study to separately characterize each NP–protein system. Then, the same procedure was applied in a buffer containing a mixture of the two proteins at different molar ratios. Finally, the NPs were pre-incubated with one protein and thereafter electrokinetically separated in a BGE containing the second protein. These analytical strategies revealed the mechanisms (comparative, cooperative or competitive systems) and the magnitude of their interactions, resulting in all cases in notably higher affinity and stability between ZGO-PEG and ApoE ($K_a = 1.96 \pm 0.25 \times 10^{10} \text{M}^{-1}$) compared to HSA ($K_a = 4.60 \pm 0.41 \times 10^6 \text{M}^{-1}$). For the first time, the inter-protein ApoE/HSA interactions with ZGO-PEG were also demonstrated, highlighting the formation of a ternary ZGO-PEG/ApoE/HSA nanocomplex. These results open the way for a deeper understanding of the protein corona formation, and the development of versatile optical imaging applications for ZGO-PEG and other systemically delivered nanoprobes ideally vectorized to the brain.

Introduction

In the current context of nanomedicine, one of the most representative challenges is to successfully target therapeutic NPs to cells or organs of interest.1 However, some constraints such as their detection by the immune system,2 degradation,3 bioaccumulation by organs,4 or the presence of physiological barriers limit their adequate targeting and function.5 For instance, the blood–brain barrier (BBB) is one of the most impermeable physiological barriers in an organism, and it enables the protection of the brain from the peripheral circulation and toxic substances but restricts the transport of many therapeutically relevant nanodrugs.6 In this context, some early studies have shown a clear correlation between apolipoprotein-E (ApoE) adsorption onto the NP surface and passage through the BBB, and different kinds of nanocarriers have been successfully used for the transport of drugs to the brain.7,8 However, once in vivo, the NPs interact with plasma proteins and other biomolecules, forming new complexes and dynamic entities in which an active exchange of proteins from solution to the NP surface occurs.1 Thereby, the rapid formation of a protein corona could critically affect the interactions of the NPs with living systems, and thereby, their distribution and therapeutic action.9

The use of effective imaging agents will help to clarify the precise mechanism for NP distribution and interactions in the body, and to diagnose diseases in earlier stages. As reported by our group, ZnGa1.995Cr0.005O4 persistent luminescent NPs

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(ZGO-NPs) represent a new generation of optical nanoprobes, whose persistent luminescence can be activated before administration on live systems, as well as in vivo through living tissues. This in situ re-activation in the therapeutic window results in the ability to make observations of the probe without any time constraints, opening new perspectives for a great variety of diagnosis applications. For instance, the ability of these PEGylated ZGO-NPs for in vivo passive tumor targeting without long-term toxic effects has been demonstrated. These advantages help to consider the adsorption of plasma proteins at the surfaces of these NPs, in order to program the final fate of ZGO-NPs or other functional NPs in the organism.

A few recent publications showed different ways by which capillary electrophoresis (CE) can be applied to obtain qualitative and quantitative information about the interactions between NPs and various types of proteins. Different approaches have been used for this purpose, principally the affinity CE mode (ACE), leading to the determination of binding constants and kinetics, stoichiometry and cooperativity. The application of the Hummel–Dreyer CE (HDCE) mode for evaluation of the interactions between ZGO-PEG NPs and bovine serum albumin (BSA) was also demonstrated previously by our group. Furthermore, capillary zone electrophoresis (CZE) has shown to be an important tool in the analysis of ApoE protein involvement in the translocation of nanocarriers through the BBB, and it has also been used as a complementary method to study the adsorption of specific peptides onto PEGylated nanoparticles.

In the present work, the affinity between ZGO-PEG NPs and a binary system of proteins (HSA and ApoE) was systematically evaluated through an integral set of tests including comparative, cooperative, and competitive models in a capillary electrophoresis system. The presented methodology allows the rapid and versatile determination of both stable and short-lived NP–protein nanocomplexes with fast or slow association/dissociation rates under physiologically relevant conditions. Since protein adsorption by NPs provides them new identity and properties, this strategy can enhance the development of novel biomedical applications of NPs, such as biosensors, optical imaging, and targeted therapy. Furthermore, the possibility of modifying the ZGO-PEG surface with ApoE by a simple and fast incubation step is discussed in this work, in order to envision the potential application of the ZGO-PEG/ApoE nanocomplex for the development of optical imaging agents to detect brain pathologies.

**Experimental**

The detailed experimental protocols used for the synthesis, functionalization, and characterization of ZGO-PEG are presented in the ESL.

**Analytical procedures for evaluation of interactions between nanoparticles and proteins by CE**

Initially, the proteins were individually analyzed by means of CZE. Injections of 1.44 μM HSA or ApoE suspensions were performed hydrodynamically by applying 20 mbar pressure at the capillary inlet for 10 s (10 nL). The background electrolyte (BGE) was 30 mM ACB (pH 7.4). The separation was performed at a voltage of 14 kV, and the signals were obtained using a UV-Vis detector (λ = 200 nm). The molar extinction coefficients of the proteins were determined at 200 nm under these experimental conditions according to the Lambert–Beer law, and considering the equation $A = εCL$ (where $A$ = absorbance, $ε$ = molar extinction coefficient, $C$ = molar concentration and $L$ is the light pathway). Viscosity and conductivity measurements of the BGE containing mixtures of proteins at different ratios were carried out according to a previously reported CE protocol.

To evaluate the interactions between ZGO-PEG and the proteins, three different CE approaches were used: (a) comparative interactions of NPs with individual HSA or ApoE proteins at different concentrations under the same analytical conditions, (b) cooperative interactions of NPs with a binary system of proteins mixed at different ratios, and (c) incubation of ZGO-PEG NPs with one of the proteins for 15 minutes, and thereafter the analysis of their competitive interactions in a separation medium containing the second protein, and vice versa.

The overall method used for the different experiments performed in this section consists of five principal steps: (i) equilibrium of the capillary, consisting of successive flushes with 0.1 M NaOH (2 min), H$_2$O (2 min) and ACB (2 min) at the corresponding ionic strength (15 or 30 mM); (ii) capillary pre-conditioning, in which the BGE was flushed for 3 minutes. For comparative or competitive interactions, the BGE consisted of HSA or ApoE at various concentrations dispersed in 30 mM ACB solutions. For cooperative interactions, the BGE was a mixture of HSA and ApoE at different ratios dispersed in 15 mM ACB solutions; (iii) sample injection, in which 0.2 mg mL$^{-1}$ ZGO-PEG or ZGO-PEG pre-incubated with one of the proteins was hydrodynamically injected (applying 20 mbar pressure at the capillary inlet for 10 s) for comparative or competitive interactions, respectively. In the case of cooperative interactions, ZGO-PEG samples were injected only for 5 s at the same pressure in a capillary filled with protein mixtures. An electropherogram of 0.001% DMF in the BGE was obtained after each sample analysis (4 replicates) to control the electroosmotic flow; (iv) electrokinetic separation, consisting of the application of a voltage ranging from 12 to 14 kV (see the precise value in the footnote of every figure). The signal registration was made with a UV-detector at λ = 200 nm; and (v) analysis of the interactions, based on two different approaches for numerical data interpretation. First, the Hummel–Dreyer method (HDCE) was applied in the cases in which no shifts in the electrophoretic mobility of the involved species (protein, NPs, and their complex) were detected upon protein concentration variation. There, the injection of ACB solutions under the same analytical conditions was used for external calibration, and the peak areas were monitored to obtain the portion of protein bound to NPs and the analytical parameters were derived. Affinity CE (ACE) was applied for the interpretation of the interactions in which considerable shifts in the
electrophoretic mobilities of the nanocomplex formed between ZGO-NPs and the proteins were detected after variation of the protein concentration.

A Model G7100A CE system from Agilent Technologies equipped with a UV–Vis absorbance detector was used for the analysis. Separations were carried out with a fused silica capillary [37 cm (effective length 28.5 cm) × 50 μm I.D., purchased from Polymicro Technologies (Phoenix, USA)] activated by successive flushes (925 mbar) with 1.0 M NaOH (15 min), 0.1 M NaOH (15 min) and H₂O (5 min), respectively.

Results and discussion

ZGO-NPs were synthesized, and afterward a three-step functionalization sequence was set up to obtain the PEG-modified NPs (Fig. 1A). The PEG chains are intended to reduce the NP interactions with various proteins and biological elements, to reduce their detection by the mononuclear phagocyte system,⁴⁴ and thereby, to improve their biodistribution and applications as an optical imaging tool. An average solid diameter of 30 nm with a semi-spherical shape was observed via TEM (Fig. 1B), while the success of PEGylation was confirmed by FTIR spectroscopy (Fig. 1C). The hydrodynamic diameter of the PEGylated NPs was 177.6 ± 7.6 nm in 30 mM ammonium carbonate (ACB) at pH 7.4, with a polydispersity index smaller than 0.1, demonstrating that monodisperse ZGO-NP formulations were obtained (Fig. 1D). The zeta potential was almost neutral, with a value of −1.7 ± 0.6 mV. This is attributed to the long chains of PEG (5 kDa) screening the charge of the NPs. A detailed description of the physicochemical characterization of these NPs in physiologically relevant media is presented in the ESI,† indicating a preserved colloidal stability of ZGO-PEG under all the range of conditions used in the present work.

Interactions between ZGO-PEG and individual proteins: affinity electrophoretic studies with HSA or ApoE suspensions

In a first step, the HSA and ApoE proteins were electrophoretically characterized before studying their interactions with ZGO-PEG so as to discard any possible interference (Fig. 2). The electrophoretic mobilities of HSA and ApoE in 30 mM ACB (pH 7.4) are both negative as obtained by means of CZE (−1.52 ± 0.12 × 10⁻⁴ and −1.65 ± 0.09 × 10⁻⁴ cm² V⁻¹ s⁻¹, respectively), which are in agreement with their isoelectric points (4.7 and 5.5, respectively). As evidenced in the electropherograms, the absorbance intensities of the proteins are however significantly different. According to the Lambert–Beer law, the molar extinction coefficients (ε) were determined at 200 nm using a CE apparatus under the experimental conditions (30 mM ACB, pH 7.4), resulting in ε = 1.48 × 10⁶ ± 0.03 cm⁻¹ M⁻¹ and ε = 1.17 × 10⁵ ± 0.02 cm⁻¹ M⁻¹ for HSA and ApoE, respectively. At this wavelength, the absorbance arises primarily from the peptide backbone, independently of the protein sequence.²⁵ HSA is notably bigger (M.W. 66 500 Da, 585 amino acids) than ApoE (M.W. 34 200 Da, 299 amino acids).²⁶ Thereby, the difference in the observed ε values could be related to the considerable variation in the size of the analyzed proteins.

![Fig. 1](image-url) (A) Schematic representation of the ZnGa₁.₉₉₅Cr₀.₀₀₅O₄ surface functionalization sequence. (B) TEM image of the ZGO-PEG NPs. The inset shows the corresponding histogram for size distribution. (C) FTIR-ATR spectra of the intermediary functionalized ZGO-NPs. (D) DLS profile of ZGO-PEG in 30 mM ACB (pH 7.4).
While HSA is easily observed, the analysis of ApoE is limited to concentrations higher than 1.44 µmol L$^{-1}$.

In a second step, the ZGO-PEG NPs were electrokinetically separated in 30 mM ACB (Fig. 3Ab and Bb). The low surface charge density of ZGO-PEG and thereafter the quite neutral electrophoretic mobility are due to the presence of the 5 kDa PEG layer. When the ZGO-NP–protein interactions are analyzed, at least two possible systems can be expected: (i) stable NP–protein complexes or (ii) short-lived complexes with fast association/dissociation rates. The choice of the adequate electrokinetic separation mode for evaluation of the NP–protein interactions is therefore crucial. In the present section, an “affinity” methodology was implemented, consisting of the electrokinetic separation of the ZGO-PEG NPs in a BGE containing individual proteins at different concentrations. This methodology resulted in two markedly different electrophoretic behaviors when HSA and ApoE were analyzed under the same analytical conditions (Fig. 3).

Considering the colloidal stability of the ZGO-PEG NPs, the variations in the electrophoretic profiles can be exclusively associated with the NP/protein or inter-protein interactions.

Furthermore, the DLS and LDE ($\zeta$-potential) experiments performed under similar conditions did not provide exploitable results. Indeed, the separation ability of CE is paramount to detect small changes in the magnitude of association parameters, and thereby, for the determination of interactions.

In the case of interactions with HSA (Fig. 3A), two peaks were evidenced when HSA was present in the BGE. A “negative peak” (vacancy peak) appears at a constant electrophoretic mobility corresponding to the one of HSA, and is due to a local depletion of HSA in the BGE originating from the ZGO-PEG/HSA complex formation. A “positive peak” at a nearly zero electrophoretic mobility corresponds to the ZGO-NPs or their nanocomplexes with HSA. A gradual increase in the vacancy peak area of HSA was evidenced upon the increase of its concentration in the BGE, while no significant shift in the electrophoretic mobility or variation in the shape of the “positive peak” was observed. This electrophoretic evolution of ZGO-PEG as a function of the HSA concentration (from 0 to 1.44 µM) in 30 mM ACB corresponds to the Hummel–Dreyer CE (HDCE) mode.

On the other hand, only one positive peak was evidenced with a gradual shift in the electrophoretic mobility upon the increase of the ApoE concentration in the BGE (Fig. 3B). The lower molar extinction coefficient of ApoE (one order of magnitude compared to HSA, as determined above) can explain the absence of the ApoE signal at the used concentrations. The variations in the electrophoretic mobility of the positive peak indicate the formation of a stable nanocomplex between ZGO-PEG and ApoE. Since ApoE is negatively charged under the experimental conditions, the interaction of ZGO-PEG with ApoE induces a gradual increase (in negative value) of the electrophoretic mobility. This evolution corresponds to the affinity CE (ACE) mode, being the first evidence of the stable NP–ApoE interaction.
The significant differences in the electrophoretic profiles obtained for the evaluation of the interactions between ZGO-PEG and HSA or ApoE could be explained in terms of interaction strengths and kinetics. The electrophoretic mobility shifts derived from the interactions with ApoE demonstrate a fast binding with ApoE, and a high stability of the ZGO-PEG/ApoE complex. The electrophoretic mobility shifts towards more negative values at higher protein concentrations are due to the increase of charged groups provided by the ApoE molecules at the surface of the PEGylated NPs. On the other hand, the absence of variations in the shape and electrophoretic mobility of ZGO-PEG upon interaction with HSA, and the presence of a vacancy peak of the protein, could indicate a faster association/dissociation kinetics compared to ApoE. The protein size could strongly impact the affinity with the NPs, since a smaller size can lead to better spatial distribution and thus stronger interactions. This CE methodology is then a convenient analytical tool for comparison of the interaction dynamics between ZGO-PEG and both HSA or ApoE proteins.

Since the electrophoretic profiles observed for ZGO-PEG interactions with both HSA and ApoE proteins present significant differences, two numerical approaches have been applied to determine the ZGO-PEG/protein binding constants and association parameters. A detailed description of the equations and models applied in this work is presented in the ESI.† For the ZGO-PEG/HSA nanocomplex in 30 mM ACB (pH 7.4), the model fitting parameters according to the HDCE mode lead to $K_a = 4.60 \pm 0.41 \times 10^6 \, \text{M}^{-1}$ and a number of binding sites $n = 4.35 \pm 0.24$. Otherwise, the ACE mode leads to $K_a = 1.96 \pm 0.25 \times 10^{10} \, \text{M}^{-1}$ and a slightly cooperative association ($n_H = 1.47 \pm 0.07$) for the ZGO-PEG/ApoE nanoconjugate. A stronger ZGO-PEG/ApoE binding compared to ZGO-PEG/HSA was evidenced by these calculations, resulting in a difference of four orders of magnitude. In general, proteins could have affinity with other substances through several interactions like H-bonds, pi-stacking, and electrostatic attractions, which could be responsible for the interactions observed in this work. The qualitative adsorption of ApoE onto other PEGylated NPs has also been verified by 2D-polyacrylamide gel electrophoresis (2D-PAGE).27,28 Thereby, PEGylation does not exclude the NPs to interact with circulating proteins, for instance those related to the immune system or mononuclear phagocyte system.29 This stronger affinity between ZGO-PEG and ApoE could be related to the high affinity for the lipid-binding region in the C-terminal domain (amino acids 244–272) present in this protein. This region appears to be linked to the primary ApoE functions, such as ligand binding.30 The smaller size of ApoE compared to HSA could also enhance the interactions by improving their spatial distribution and orientation at the NP surface.

**Interactions between ZGO-PEG and a mixture of the HSA and ApoE proteins**

In order to compare the ZGO-PEG/protein interactions in a binary system, the capillary was pre-conditioned with mixtures of HSA and ApoE at different ratios, and then ZGO-PEG was injected. Fig. 4 shows a full panorama of these interactions. The electropherograms derived from the injection of ZGO-PEG in capillaries pre-conditioned with pure ApoE or HSA are presented in Fig. 4A and B, respectively, for the direct comparison of the effects derived from the addition of a second protein in the system.

For evaluation of the cooperative interactions between ZGO-PEG and a binary system of proteins, the HSA and ApoE ratio was varied, with concentrations ranging from 0 to 0.72 μM (Fig. 4C and D). So as to facilitate the analysis of the results obtained from Fig. 4, a schematic representation of the phenomena observed in these systems is presented in Fig. 5.

In Fig. 4C and D, the superimposed dashed lines represent the injection of an ACB zone in the capillary pre-conditioned as indicated. These experiments demonstrate the inter-protein interactions between HSA and ApoE, evidenced by the formation of a positive broad peak (a plateau) with a mixture of the proteins with concentrations higher than 0.36 μM. As previously indicated in the literature, some peptides and more than thirty-five proteins can interact with HSA, including both high and low abundant proteins (e.g. apolipoproteins, angiotensinogen, ceruloplasmin, clusterin, hemoglobin (Hb), plasminogen, prothrombin, and transferrine).31,32

When the HSA concentration in the pre-conditioned capillary was fixed at 0.72 μM and the ApoE concentration was varied (Fig. 4C), a negative peak related to the HSA vacancy appears as previously explained, but it is affected by the concentration of ApoE. Since no significant variations in the area of the negative peaks were detected with low ApoE concentrations (ApoE ≤ 0.24 μM) compared to those observed for separations in a BGE with only 0.72 μM HSA, it is deduced that HSA was not initially removed from the ZGO-PEG surface. However, when the ApoE concentration was increased (ApoE ≥ 0.36 μM), the equilibrium was modified and the vacancy peak area slightly diminished, indicating an increase in the HSA concentration in the BGE, which could be due to lower HSA adsorption at the ZGO-PEG surface.

According to Fig. 4A and C, the electrophoretic mobility of the ZGO-PEG/protein nanocomplex was significantly decreased (in absolute value) in the presence of a fixed 0.72 μM HSA concentration with respect to experiments with only ApoE. A direct comparison of these values is presented in Fig. 6. As described before, higher ApoE concentrations induce a gradual increase in the electrophoretic mobility (in absolute value) of the ZGO-PEG/ApoE nanocomplex due to the negative charges provided by the protein. However, when a fixed concentration of HSA (0.72 μM) was added in the BGE, this electrophoretic mobility (in absolute value) increased more slowly, probably due to the presence of HSA as well on the NP surface. This indicates the formation of a ternary ZGO-PEG/HSA/ApoE nanocomplex with slow dissociation kinetics, which could be assisted by the initial HSA/ApoE interprotein association.

Furthermore, when the ApoE concentration in the mixture was ≥0.36 μM (Fig. 4C), a plateau appeared, connecting the ZGO-PEG/protein signal and the HSA vacancy peak, with a higher intensity compared to those experiments in which only
the BGE was injected (dashed lines). This observation is in accordance with the formation of a ternary ZGO-PEG/protein nanocomplex.

Fig. 5B schematizes the hypothetical complexes formed during this experiment: whereas the ZGO-PEG/HSA and ZGO-PEG/ApoE systems have fast and slow dissociation kinetics, respectively, the ternary ZGO-PEG/protein nanocomplex satisfies two conditions: (1) it has a slow dissociation kinetics and (2) it leads to the release of some HSA from the NP surface at a given ApoE concentration. At low ApoE concentrations,
this ternary complex could exist with both HSA and ApoE proteins at the ZGO-PEG surface, without affecting the vacancy peak area. Nevertheless, when increasing the ApoE concentration, some HSA could be released from the NP surface, which suggests the displacement of HSA by ApoE from the NP surface through competitive interactions, triggering variations in the vacancy peak area. Due to the fast dissociation kinetics of the ZGO-PEG/HSA interaction, a ternary nanocomplex in which HSA connects ZGO-PEG and ApoE is not expected. As a slow dissociation nanocomplex was formed, its structure should correspond to a ternary ZGO-PEG/ApoE/HSA nanocomplex in which ApoE acts as a bridge between ZGO-PEG and HSA.

In Fig. 4D, in which the ApoE concentration was fixed at 0.72 μM and the HSA concentration was varied, a peak associated with the ZGO-PEG/ApoE nanocomplex was present in all the electropherograms at 3.6 min (the same time observed for the ZGO-PEG/ApoE nanocomplex formed in a capillary containing only 0.72 μM ApoE). The migration time was kept constant when the HSA concentration was increased. However, a shoulder and then peak deformation were evidenced and a broad peak between the HSA vacancy and the nanocomplex peak was observed. These results provide further evidence of the ZGO-PEG/ApoE nanocomplex stability (slow dissociation kinetics), even in the presence of HSA, with the possible formation of the ternary ZGO-PEG/ApoE/HSA nanocomplex.

Globally, this set of experiments clearly demonstrates the stronger affinity of ZGO-PEG for ApoE compared to HSA, and the stability of the ZGO-PEG/ApoE nanocomplex, even in environments with higher HSA concentrations.

It further evidences the HSA/ApoE association that seems to lead to the formation of a more complex interaction system involving ZGO-PEG and the two proteins through cooperative interactions, in which the most stable complex is formed with ApoE acting as a bridge between ZGO-PEG and HSA.

**Competitive interactions between ZGO-PEG and a mixture of the two proteins: pre-incubation effects**

In order to better understand the interaction mechanisms between ZGO-PEG, ApoE, and HSA in a competitive system model, different pre-incubation approaches were compared in various BGE compositions (Fig. 7). The incubation of ZGO-PEG with proteins was performed at the most elevated concentration analyzed in this work (1.44 μM) in order to guar-

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![Fig. 5](A) Schematic representation of the interactions between ZGO-PEG and the binary system of HSA and ApoE proteins in a CE system. (B) Hypothetical forms of the ZGO-PEG/binary-protein interactions.

![Fig. 6](Effect of HSA (0.72 μM) on the electrophoretic mobility of the ZGO-PEG/ApoE nanocomplex formed in a capillary pre-conditioned with ApoE at different concentrations, according to Fig. 4C.)
actions in a competitive system were then analyzed. The electropherograms are identified according to the BGE composition (BGE) and the pre-incubated sample (S): (a) BGE = ACB, S = ZGO-PEG, (b) BGE = ACB + HSA, S = ZGO-PEG, (c) BGE = ACB + ApoE, S = ZGO-PEG, (d) BGE = ACB, S = ZGO-PEG/HSA, (e) BGE = ACB, S = ZGO-PEG/ApoE, (f) BGE = ACB + ApoE, S = ZGO-PEG/HSA, and (g) BGE = ACB + HSA, S = ZGO-PEG/ApoE. The protein concentration was 1.44 μM for all cases. The initial BGE was 30 mM ACB (pH 7.4). E = 12.0 kV.

Fig. 7 Set of representative electropherograms obtained at λ = 200 nm for analysis of the competitive interactions. The electropherograms are systematically evidenced through the application of the comparative, cooperative, and competitive methodologies. Firstly, the interaction of nanoparticles with individual proteins in the capillary and thereafter the comparison of the same migration time and area as those for free HSA (Fig. 7b), and a second superimposed peak appears at an intermediary migration time between ZGO-PEG and the ZGO-PEG/ApoE nanocomplex. This indicates a competitive interaction between HSA and ApoE, in which HSA is replaced by ApoE at the ZGO-PEG surface due to its stronger interaction, and the formation of a ternary ZGOPEG/ApoE/HSA complex.

For this purpose, ZGO-PEG was first incubated with HSA and then injected in a BGE containing ApoE, both of them at the same concentration (1.44 μM). In Fig. 7f, the peak of HSA is observed, with a smaller peak area compared to free HSA (Fig. S5†), while a second superimposed peak appears at an intermediary migration time between ZGO-PEG and the ZGO-PEG/ApoE nanocomplex. This indicates a competitive interaction between HSA and ApoE, in which HSA is replaced by ApoE at the ZGO-PEG surface due to its stronger interaction, and the formation of a ternary ZGOPEG/ApoE/HSA complex.

Finally, in Fig. 7g, the pre-incubated ZGO-PEG and ApoE are separated in a BGE containing HSA. A very slight negative peak corresponding to the HSA vacancy is observed. Furthermore, a broad peak (or a peak followed by a plateau) is also present at an electrophoretic mobility between the ones of ZGO-PEG in Fig. 7d and e. The broad peak is attributed to the ternary ZGO-PEG/ApoE/HSA nanocomplex, which does not seem very stable. This is additional evidence of the participation of HSA in the ternary nanocomplex. Accordingly, it is clearly demonstrated again that: (1) the ZGO-PEG/HSA complex is ruled by a fast association/dissociation kinetics, (2) ZGO-PEG/ApoE is a stable nanocomplex, even in the presence of competing HSA, (3) ApoE is able to displace HSA from the ZGO-PEG surface, and (4) an interaction between HSA and ApoE occurs, and a ternary bioconjugated nanocomplex ZGO-PEG/ApoE/HSA is formed under the analysis conditions. Considering the great stability of the ZGO-PEG/ApoE nanocomplex, even in the presence of higher concentrations of other proteins such as HSA, the pre-formation of an ApoE protein corona around ZGO-PEG by means of a simple 15 min pre-incubation step can be explored as a strategy for their translocation across the BBB, representing a potential tool for optical imaging in the brain region. The set of methodologies presented herein can be potentially adapted to study other interacting NPs or specific proteins. This work also opens perspectives for the evaluation of interactions between NPs and multiplex protein systems.

Conclusions
A systematic set of electrokinetic methodologies was implemented to deeply study the protein corona formation around PEGylated persistent luminescent NPs (ZGO-PEG). A binary system of proteins including HSA, the most abundant serum protein, and ApoE, associated with the active transport of NPs through the blood-brain barrier, was analyzed. The stronger affinity between ZGO-PEG and ApoE compared to HSA was systematically evidenced through the application of comparative, cooperative, and competitive methodologies. Firstly, the interaction of nanoparticles with individual proteins in the capillary and thereafter the comparison of the model fitting parameters indicated binding constants $K_a = 4.60 \pm 0.41 \times 10^6$ M$^{-1}$ and $K_s = 1.96 \pm 0.25 \times 10^{10}$ M$^{-1}$ for the ZGO-PEG/HSA and ZGO-PEG/ApoE nanoconjugates, respectively, in 30 mM ammonium carbonate buffer (pH 7.4). The
evaluation of the ZGO-PEG interactions with a mixture of these proteins at different ratios revealed three important parameters: the stronger interactions with ApoE, even in a system with a high HSA concentration, the presence of HSA–ApoE inter-protein interactions, and the cooperative interactions allowing the formation of a ternary ZGO-PEG/ApoE/HSA nano-complex. Finally, the competitive model showed dynamic NP–protein interactions in which ApoE displaces HSA from the ZGO-PEG surface. These CE characterization studies were found to be sensitive enough to detect small changes in the magnitude of association parameters. Even if the presence of only two proteins is far from the real protein composition in a physiological sample, at the current state of research in which the interactions between nanoparticles and individual proteins are normally considered, the analysis of interactions in a binary system of proteins represents a great advance in the simulation of physiologically relevant conditions. This methodology could allow a better understanding of the interactions between nanoparticles and proteins, improving the development of novel functional nanomaterials for different biomedical applications like diagnostic biosensors, targeted therapy, and optical imaging. Furthermore, the Apo-E adsorption onto ZGO-PEG induced by a fast incubation step could be explored as a simple modification strategy for its translocation across the blood–brain barrier, representing a potential tool for optical imaging in the brain region.

Author contributions

Conceptualization – ideas: GRG, FdO, and AV; formal analysis, investigation, and methodology: GRG, FdO, and AV; project administration: AV; resources: GRG, CR, NM, FdO, and AV; supervision: AV; validation: GRG, FdO, and AV; visualization: GRG; writing – original draft: GRG; writing, review and editing: GRG, FdO, CR, NM, and AV.

Conflicts of interest

The authors declare no competing interests.

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