



Serum-PEG and BSA-PEG hydrogels as advanced platforms for evaluating plasma protein binding

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ABSTRACT

The binding of bioactive compounds to proteins is critical for their availability and ADME/Tox profile. Specifically, binding to serum proteins affects both the distribution and elimination of drugs, while permeation through protein-enriched matrices, such as skin, is also influenced by protein interactions. Although several methods exist to evaluate ligand-protein binding, they often fail to replicate the high protein concentrations and molecular crowding conditions found *in vivo*. In this study, we investigate the use of protein-PEG hydrogels with low crosslinking density as 3D matrices to quantify ligand-protein affinity. Two types of hydrogels were developed: one using bovine serum albumin (BSA) and a more physiologically relevant one using serum. BSA was chosen as a model protein due to its similarity to human serum albumin.

The hydrogels were characterized for swelling, stability, mechanical properties, and porosity, and the structural integrity of BSA within the hydrogel was confirmed using circular dichroism, ¹H NMR and fluorescence spectroscopy. To assess protein functionality, we evaluated the binding affinity of various ligands, including a homologous series of fluorescent amphiphiles with different hydrophobicity (NBD-Cn, where n = 4, 6, and 8), two pesticides (malathion and chlorpyrifos), and six pharmaceutical drugs (acetaminophen, chlorpromazine, diclofenac, labetalol, salicylic acid, and verapamil).

Our results demonstrated that the structural and functional properties of BSA remained intact within the hydrogel, and the large mesh size allowed for rapid and selective ligand binding. A comparison of BSA and serum hydrogels confirmed the major role of serum albumin in ligand binding, while highlighting some differences between cationic and anionic ligands. Altogether, these hydrogels offer an effective and reliable 3D platform for the fast and accurate evaluation of plasma protein binding of drugs and other bioactive compounds.

1. Introduction

The binding of small molecules to proteins is a key property with

biological, pharmacological, and environmental implications, making it highly relevant across scientific fields such as agriculture, food chemistry, chemical biology, pharmaceutical sciences, and medicine [1–6].

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The evaluation of ligand binding to plasma proteins is of particular relevance in pharmacokinetics and ADME/Tox (absorption, distribution, metabolism, excretion, and toxicity) studies due to effects of sequestration in the bioactivity of the ligands, influencing their blood circulation time, stability, and effective permeability through biological barriers [3,7]. Serum albumin is the most abundant protein in the blood of vertebrates, and is present in relatively high concentrations in interstitial fluids [8]. Due to the presence of a large number of binding sites with distinct properties [9,10], binding to serum albumin is also a good model to evaluate non-specific binding to protein-enriched matrices [6, 11], allowing the evaluation of the retention and transport of drugs and general xenobiotics.

There are several methods that may be used to characterize the binding of small molecules (hereafter named as ligands) to proteins [12–17]. The gold standard is equilibrium dialysis (ED), with the physical separation between the ligand associated with the protein and the free ligand in solution, followed by quantification of the later. This method has however many disadvantages, notably the long equilibration time required and the adsorption of the ligand to the dialysis membrane. Those disadvantages are overcome by methods that do not require the physical separation between the bound and free ligand. However, in this case, the amount of ligand bound (or free) is not directly obtained. Instead, it is estimated from the observed variation in a property of the system, of the ligand or of the protein [14,18]. Some examples based on variations in systems properties include the use of isothermal titration calorimetry [16,19–28] and surface plasmon resonance [21,29–34]. While the ligand and/or protein property followed may be based on its absorption and/or fluorescence spectra [35,36], electrophoretic or thermophoretic signal [16,18,24] or by nuclear magnetic resonance spectroscopy [37,38]. The most common property followed is however protein fluorescence intensity due to its wide applicability, ligand binding being evaluated through the efficiency of fluorescence quenching [16,20–23,25–27,30,31,33]. A common drawback of these indirect methods is the assumption of a direct and simple relation between the observed property and the amount of ligand bound. If this relation is not well defined, large inaccuracies will be observed for the binding affinities estimated with those methods. This is particularly relevant for the method based on quenching of protein fluorescence due to ligand binding, where a proper analysis of the results would require knowledge of the quenching efficiency for each protein fluorophore and ligand bound to each binding site [39,40].

Methods that allow the quantification of the fraction of ligand bound (directly or from the quantification of the ligand free in solution), but without the need to use separation membranes or filters, would be the most appropriate. Protein hydrogels may be particularly relevant in this respect, provided that the protein maintains its native structure and that hydrogel aqueous content and porosity allows the free diffusion of the ligand and its accessibility to the protein binding sites.

Protein hydrogels have been extensively explored for biomedical applications [41–66], serum albumins being particularly promising due to their properties and high natural abundance. The simplest methods to prepare albumin-based hydrogels rely on albumin's tendency to form aggregates and organized structures, as is observed when bovine serum albumin (BSA) structure is modified by pH [42] or temperature [55]. These hydrogels are of interest in a large variety of biomedical applications, but cannot be used to characterize ligand-protein binding due to the non-native properties of the protein in the hydrogel. Other methods are based on the establishment of a covalent protein network due to crosslinking with polar agents [44,46–48,50–55,58,61,62,64]. As expected, the porosity and water content of the hydrogels are influenced by the length of the crosslinking agent [49,50,66], and by the concentration of protein and crosslinker [48,56]. The optimal porosity depends on the application, with more compact hydrogels being of interest in controlled drug delivery [45,53,55,60], as sensors for very small ligands [57], or as biocompatible and stable implants [52]. However, applications as sensors for molecules with moderate-to-large sizes, or to

evaluate ligand binding to the protein in the hydrogels, require a high porosity and a low degree of protein crosslinking. This is both to allow the free diffusion of the ligand within the hydrogel [53,54,67–69], and because the reduced conformational flexibility of the proteins in a highly reticulated hydrogel may lead to a decrease in ligand-protein affinity [51,52]. Attempts to increase the porosity and decrease the conformational constraints imposed on the proteins in the hydrogels, include the use of longer crosslinking agents, and the decrease in the number of reactive groups in the protein. This has been attempted in the case of BSA-PEG hydrogels, namely through the previous reaction of the protein with polyethylene glycol (PEG) activated in only one of its terminals to block some of the reactive amine groups in the protein [48]. PEGylation of the protein has also been shown to directly influence the protein structure and/or stability [44,61,62,70], although with little impact on ligand-binding affinity for modification at a single site in the protein [64].

In addition to the advantages of avoiding the separation membrane needed in equilibrium dialysis, the use of hydrogels to evaluate ligand-protein binding and enzymatic activity may in fact correspond to a better approach to mimic the conditions found *in vivo*. This is particularly relevant for proteins that are present at very high concentrations in biological fluids, thus corresponding to very crowded solutions. The concentration of serum albumin in the plasma varies between 20 and 50 mg/mL [9,71–76], corresponding to 300–750 μM and representing up to 50 % of the total serum proteins with the remaining being mostly globulins [71–73,75–79]. At this high protein concentration most ligand would be bound, even for moderate binding affinities (≥ 90 % ligand bound for $K_b \geq 10^3 \text{ M}^{-1}$ and considering a single binding site in the albumin). While *in vivo* binding to albumin must compete with many other binding agents present, decreasing the amount of ligand associated with albumin, when characterizing ligand binding using purified *in vitro* model systems, much lower protein concentrations must be used to allow the accurate estimation of the binding affinity. Large differences in protein concentrations may be a problem when the properties of the protein are influenced by its concentration. This is the case of serum albumin, which has been shown to form aggregates of different sizes depending on protein concentration, even at physiological conditions of pH, temperature and ionic strength [44,47,59,70,80,81].

In this work we explore the use of hydrogels prepared from whole serum and from serum albumin crosslinked with PEG, for application in the quantification of drug plasma protein binding in the blood. BSA and bovine serum were used, given the resemblance with human serum [16, 19,20,24,82–84] at a much lower cost. In contrast with previous works using BSA-PEG hydrogels as binding agents of bioactive molecules for their controlled release [45,46,53,55,60,64,65], PEG with a low activation degree was used in this work. Under these conditions, most of the PEG molecules are activated only at one of the two terminals, and only a small fraction is activated at both terminals. Therefore, the number of cross-linking nodes in the gel will be low, while the surface of the protein will be partially decorated with PEG, allowing for an efficient entrapment in the hydrogel. Some preliminary results on the use of these BSA-PEG hydrogels to evaluate ligand-BSA binding have been previously reported by us [85]. That work is now extended through a detailed characterization of the hydrogel structural properties, the inclusion of hydrogels prepared from whole serum, and their use as a 3D matrix to characterize ligand-protein binding for several bioactive molecules. The mechanical properties of the hydrogels were determined via compression and tensile mechanical tests, while hydrogel morphology was characterized by surface electron microscopy (SEM), and possible effects on the protein structure resulting from inclusion in the hydrogel were evaluated using fluorescence spectroscopy and circular dichroism (CD). The ability of the protein to bind ligands with distinct hydrophobicity was also evaluated. For this purpose, a homologous series of fluorescent amphiphiles was used, where the polar region is maintained (the fluorescent moiety NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl) and the non-polar region is varied with aliphatic chains of different lengths

(NBD-Cn with $n = 4, 6$ and 8). The results obtained show that the structure of BSA in the hydrogel is similar to that of the protein free in solution at a comparable concentration. It is also shown that the binding affinity obtained is very similar to that observed for BSA in solution [36], and that serum albumin is the major binding agent in serum hydrogels. The ability of the hydrogels to bind structurally diverse ligands was evaluated through the characterization of the binding affinity for several bioactive molecules, including two pesticides (chlorpyrifos and malathion) and six pharmaceutical drugs highly prescribed worldwide (diclofenac, salicylic acid, chlorpromazine, verapamil, labetalol, and acetaminophen). The results obtained show that BSA-PEG and serum-PEG hydrogels with a large mesh size are adequate 3D matrices for a straightforward, accurate and fast evaluation of drugs' plasma-protein binding.

2. Materials and methods

2.1. Materials

Bovine serum albumin was from Fisher Scientific (Lisboa, Portugal), bovine serum from Sigma-Aldrich (Algés, Portugal), polyethylene glycol 6000 (PEG) from Merck (Algés, Portugal), 4-nitrophenyl chloroformate (NPCF) 97 % purity from Acros Organics (Gell, Belgium), and 4-dimethylaminopyridine (DMAP) > 99 % purity from Fluka Analytical (Algés, Portugal). The fluorescent amphiphiles NBD-Cn were synthesized and purified following the procedure described in the literature [36]. Whenever commercially available, the ligands used were of analytical grade and used without further purification. Chlorpyrifos, labetalol, salicylic acid, and chlorpromazine hydrochloride were obtained from Sigma-Aldrich (Algés, Portugal); sodium diclofenac and acetaminophen (also known as paracetamol) were from Supelco (acquired to Sigma-Aldrich, Algés, Portugal); (+/-)verapamil hydrochloride was from ACROS (acquired to Thermo Fisher Scientific Inc., Waltham, USA); malathion was from Chem-Lab (Zedelgem, Belgium). The aqueous medium was saline phosphate buffer (PBS), prepared with 10 mM sodium phosphate, 150 mM sodium chloride, and 0.02 % (w/v) sodium azide, all from Sigma-Aldrich (Algés, Portugal), with the pH adjusted to 7.4 or 8.5 by addition of small aliquots of HCl or NaOH as required. Purified water with a resistance ≥ 18 M Ω -cm was used to prepare the solutions (first distilled and then passed through activated carbon filters and deionized by ion exchange cartridges in an equipment ARIOS UP, from Human, Seoul, Republic of Korea), and additional solvents, salts and buffers were of analytical grade.

2.2. Hydrogel preparation, stability and swelling

Hydrogels were prepared according to the method described by Fortier and co-authors [49,50]. Briefly, PEG was activated by reaction with NPCF (2 molar equivalents) in the presence of the activator DMAP (2 molar equivalents), all dissolved in dichloromethane which was previously incubated overnight with molecular sieves 3A to remove vestigial water. The mixture was allowed to react under agitation at room temperature (~ 23 °C) for 15 min to 2 h (depending on the intended activation degree). The product was first purified by precipitation with cold diethyl ether, resuspended in dichloromethane, and cleaned by water extraction. Traces of water in the organic phase were removed by filtration over sodium sulfate, and the product was again precipitated with cold diethyl ether and dried under vacuum. An aliquot of the white-to-yellowish powder obtained was dissolved in deuterated methanol and characterized by ^1H NMR (Bruker Avance III 400 spectrometer, Bruker, MA, USA). The degree of activation (dA) was calculated from the relative areas of the signals assigned to the aromatic protons from the covalently linked nitrophenyl group and that of PEG methylene groups, see Section S1 of the Supporting Information (SI) for the NMR spectra (Fig. S1) and further details.

In most of the studies performed in this work with BSA-PEG

hydrogels, 100 mg of BSA was dissolved in 1 mL PBS at pH 8.5, and mixed with 180 mg of PEG (dA = 0.5) dissolved in 1 mL of water. The high concentration of BSA and PEG contributes significantly to the volume of the solution, leading to a final BSA concentration of 46 mg/mL. Serum hydrogels were prepared by mixing 1 mL serum with 1 mL activated PEG (dA = 1) at the same conditions used for the BSA-PEG hydrogels. Aliquots of the mixture (typically 100 μL each) were immediately transferred to plastic dishes (to obtain thin disk-shaped hydrogels) or to a cone-shaped silicone rubber platform (to obtain cone-shaped hydrogels), and allowed to polymerize and dry at room temperature and atmosphere for about 24 h (see Fig. S2). The materials of the supports where the hydrogel was formed have been selected to allow an easy detachment of the dry gel units. The gel units had a strong yellow color due to the presence of significant amounts of 4-nitrophenylformate in the activated PEG sample, as observed in the ^1H NMR spectra (Fig. S1), and their further production due to the reaction of the activated PEG. The dry gel units were stable for at least several months when stored in the dark in a dry atmosphere. One day before used, the gel units were extensively washed in water at 4 °C, with the yellow contaminant (4-nitrophenylformate) being efficiently removed leading to translucent hydrogels (Fig. S2). The cleaned hydrogel units were allowed to dry (to obtain the dry mass in swelling experiments or for storage during longer periods), or were equilibrated with fresh PBS and stored at 4 °C until use. Before the binding assays, a final incubation of the hydrogel in PBS at 37 °C with orbital agitation for 2 h was performed to stabilize the hydrogels at the conditions used in the binding assays.

Hydrogel swelling was determined as the ratio between the mass increase in the fully hydrated gel and its dry weight, Equation (1). The dry gel units were incubated with the required solvent at 37 °C with orbital agitation, and at specified times excess solvent was removed (first by pipette aspiration and then by gentle adsorption into paper), and the hydrogel mass was quantified. The effective mass of the swollen hydrogel considered in Equation (1) was obtained by subtracting the contribution from the solvent components (salts and buffer).

$$\text{Swelling factor} = \frac{\text{mass of hydrated gel} - \text{mass of dry gel}}{\text{mass of dry gel}} \quad (1)$$

The amount of protein leached out from the BSA-PEG hydrogels was calculated from its fluorescence intensity ($\lambda_{\text{exc}}/\lambda_{\text{em}} = 268 \text{ nm}/380 \text{ nm}$) in 96 wells black plates from Fisher (Lisboa, Portugal) using the plate reader SpectraMax iD5 (Molecular Devices, Berkshire, UK). The selected wavelengths correspond to an optimal sensitivity, with a balance between the intensity of fluorescence emission from BSA and a small contribution from light scatter and autofluorescence from the plate (calibration curve in Fig. S3, with a detection limit of 0.1 $\mu\text{g}/\text{mL}$). This method could not be used in the first steps of hydrogel washing due to the presence of significant amounts of contaminants that absorb in the UV-vis (e.g. 4-nitrophenylformate). In those solutions, the amount of protein was quantified by precipitation of the protein with trichloroacetic acid followed by quantification with amido black [86], with a detection limit of 0.2 $\mu\text{g}/\text{mL}$. Some selected samples were first lyophilized and redissolved in a smaller volume of water to increase the sensitivity in the quantification of protein. For solutions in PBS, dissolution of the high amount of sample after lyophilization required a moderate volume of water. To avoid interference of the salts in the characterization of the protein in the sample and to further increase the final protein concentration, an additional desalting step was required (using Zeba spin desalting columns, 7k MWCO, from ThermoFisher Scientific). The desalted solution was lyophilized again and redissolved in a small volume of water.

Due to the complexity of the solutions, and difficulty in the establishment of an accurate calibration curve for protein quantification by fluorescence, the protein concentration in the washing solutions from the serum-PEG hydrogels was always characterized by protein precipitation and staining with amido black [86]. These samples were also characterized by SDS-PAGE to evaluate the profile of the proteins

leached from the hydrogel. A discontinuous system with 1.5 mm thick slab gels, consisting of a 12 % polyacrylamide running gel and a 4 % polyacrylamide stacking gel, was used with a running buffer composed of Tris-glycine and SDS. The procedure was performed using a Bio-Rad Mini Protean III electrophoresis apparatus. Samples were diluted 1:1 with a denaturing buffer solution containing 4 % SDS, 8 M urea, and 10 % β -mercaptoethanol, and heated at 95 °C for 10 min before being applied to the gel. A low molecular weight marker was loaded into the gel as a reference for protein size determination. Electrophoresis was carried out at 150 V for 1 h. Following electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 and imaged for further analysis.

2.3. Characterization of the structural properties of albumin in the BSA-PEG hydrogel using spectroscopic methodologies

2.3.1. Circular dichroism spectroscopy

Far-UV circular dichroism experiments were performed on an Olis DSM20 CD spectropolarimeter calibrated with D-10-camphorsulfonic acid. BSA-PEG hydrogels were polymerized *in situ* between two quartz surfaces, washed by immersion in water, and equilibrated with PBS at pH 7.4. Free BSA was dissolved in PBS at 1.0 mg/mL and inserted in a 0.1 mm pathlength quartz cuvette. Spectra were recorded between 200 nm and 260 nm with a step resolution of 1 nm, an integrated time of 5 s, using a bandwidth of 1.0 nm. The final spectrum was the average of five scans and was corrected by subtraction of the signal from the buffer. Results were expressed as mean residue ellipticities $[\Theta]_{MRW}$, where Θ_{obs} stands for the observed ellipticity (in millidegrees), MRW for the mean residue weight, c for concentration (mg/mL), and l for the path length (cm): $[\Theta]_{MRW} = (\Theta_{obs} \times 0.1MRW) / (l \times c)$. In the case of the hydrogel, the product of the pathlength and protein concentration was calculated from its absorption at 280 nm, assuming a molar extinction of BSA of $4.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and a BSA molar weight of 66.5 kDa [9]. Secondary structure was estimated from the far-UV CD spectra by making use of the BeStSel algorithm [87].

2.3.2. Size-exclusion chromatography (SEC)

Size-exclusion chromatography (SEC) experiments were performed on a Wyatt WTC-030S5 column (MW range from 5 to 1250 kDa) coupled to a Pharmacia P-500 pump (Pharmacia Biotech AB, Uppsala, Sweden) and a Gilson UV detector (Gilson, Inc., Middleton, WI, USA). The system was always allowed to equilibrate for at least 5 column volumes of phosphate saline buffer (PBS) at a flow rate of 0.5 mL/min, before sample injection (10, 100 or 300 μL , for BSA concentrations of 750, 75 and 5 μM , respectively). Apparent molecular weights were calculated by interpolation based on an elution volume *versus* log(molecular weight) calibration curve of six protein standards: thyroglobulin (669 kDa), β -amylase (200 kDa), BSA (66.5 kDa), carbonic anhydrase (29.0 kDa), cytochrome *c* (12.4 kDa), and aprotinin (6.5 kDa).

2.3.3. Photoluminescence spectroscopy

Intrinsic fluorescence emission spectra of BSA solutions and of BSA-PEG hydrogels were collected on a Horiba-Jobin-Ivon SPEX Fluorolog 3–22 spectrometer (excitation wavelengths at 280 nm and 375 nm, emission from 300 to 500 nm and from 400 to 600 nm, respectively). In solution, the emission spectra were collected in a 0.5 cm quartz cell using a right-angle geometry, except for the more concentrated samples, i.e., with a BSA concentration of 750 μM (front-face geometry). The BSA-PEG hydrogels were adsorbed on a lamella and the emission spectra were obtained with front-face geometry.

Photoluminescence quantum yields (Φ_{PL}) of samples in solution and in the hydrogel state were measured via the absolute method with a Hamamatsu Quantaurus QY absolute photoluminescence quantum yield spectrometer, model C11347 (integrating sphere) [88,89]. The BSA samples (750 μM) in the absence and presence of PEG (90 mg/mL) were placed in a quartz dish ($V = 180 \mu\text{L}$) and the Φ_{PL} were obtained with an acquisition time of 1000 ms ($\lambda_{exc} = 280 \text{ nm}$) and 40 ms ($\lambda_{exc} = 375 \text{ nm}$).

Fluorescence lifetime images were collected in a Becker and Hickl (GmbH) DCS-120 Confocal fluorescence lifetime imaging microscopy (FLIM) system, equipped with a TCSPC-System module (SPC-150 N), a NIKON Ti2-U inverted optical microscope, controlled by a galvo-drive unit (Becker and Hickl GDA-121) and a hybrid GaAsP photodetector (300–720 nm), controlled by a DCC-100 detector controller card. A $20 \times$ magnification lens (CFI Plan Achromat 20X/0.40/1.20) was used. The DCS-120 confocal microscope system was equipped with a polarizing beam splitter. The excitation source was a cooled picosecond diode laser with 375 (+5/-10) nm working on a pulsed mode (repetition rate: 80 MHz). The instrument response function of the system was found to be less than 100 ps. The total laser power at the sample was set to 40 % of maximum value and the collected emission passed through a 1.5 AU pinhole and two filters (390LP longpass and ET 510/80 bandpass filters). FLIM images were scanned with 512×512 pixels using the 'FIFO imaging' mode of the SPC-150N modules. Data analysis was performed through SPCImage NG data analysis software. Fluorescence intensity decay curves were fitted using the maximum-likelihood algorithm (or maximum-likelihood estimation, MLE) fitting method, in each pixel [90].

2.3.4. Nuclear magnetic resonance (NMR) spectroscopy

High-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) measurements were performed on a Bruker Avance III HD 500 spectrometer, operating at a ^1H frequency of 500.13 MHz. $1\text{D } ^1\text{H}$ NMR experiments were recorded with a 4 mm Bruker TXI $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ HR-MAS probe, equipped with gradient coils, at 25 °C. Spectra were referenced internally to the residual signal of water. $1\text{D } ^1\text{H}$ NMR spectra were collected with 128 transients, acquisition times of 2.40 s, recycle delays of 1.00 s, and spectral widths of 8012 Hz. $1\text{D } ^1\text{H}$ HR-MAS spectra were collected with 1024 transients, acquisition times of 1.36 s, recycle delays of 1.00 s, and spectral widths of 6009 Hz. Data (16k complex points) were processed with zero-filling to 32k, apodization with exponential windowing, and Lorentzian broadening (0.60 Hz). Processing was performed with TopSpin v3.5 (Bruker) and MestReNova v9.1 (Mestrelab) software.

2.4. Characterization of the mechanical properties of the dehydrated hydrogel

Hydrogels (dehydrated by air-drying for 24 h) were subjected to compression and tensile mechanical tests. A texture analyzer was used (TA.XTplusC, Stable Micro Systems, UK) and the parameters of the assays were: 1.2 mm/min extension rate for compression, 4.8 mm/min extension rate for tensile, 50 kg load cell, and the hydrogel was mechanically tested until failure. Samples with a cylindrical shape of approximately 13 mm in diameter and 8 mm in height were used for the compression tests, and a stripe of a film with approximately 0.163 mm in thickness, 5 mm in length, and 10 mm in height was used for the tensile tests. The compression and tensile Young Modulus were obtained from the elastic region of the graphs ($N = 3$).

2.5. Characterization of the hydrogels by scanning electron microscopy

Hydrogels with a cylindrical shape were polymerized and washed thoroughly in water as explained in Section 2.2. The swollen hydrogels were frozen in liquid nitrogen, carefully cut to access the inner of the hydrogel, and lyophilized. The surface properties of the hydrogel fragments were characterized by scanning electron microscopy (SEM) using a Tescan, Vega 3 equipment. Samples were sputter-coated with gold/palladium for 45 s and visualised with an electron beam of 10 kV.

2.6. Ligand binding to the protein in the hydrogel

The binding of the ligands (NBD-Cn, pesticides, and drugs) to the hydrogel was characterized by adding the required hydrogel units to

PBS solutions (pH 7.4) containing the ligands and incubating at 37 °C under orbital agitation at 140 rpm. The hydrogel units were previously washed in water and equilibrated with PBS, as described in Section 2.2. A control sample without the hydrogel was also performed to evaluate possible losses of the ligand from the solution, namely due to binding to the apparatus materials. At specified times, an aliquot of 100 μ L (out of 5 or 10 mL) was taken, and the aliquots were added to HPLC vials containing 200 μ L ethanol, from which 100–150 μ L were analyzed by reverse phase HPLC (Zorbax ODS C18, 150 $\times$ 4.6 mm or 250 $\times$ 4.6 mm, 5 μ m) with methanol:water or methanol:ammonium formate (50 mM, pH = 6.5) as eluent and UV–vis absorption or fluorescence emission detection (Agilent 1200 series G). See Figs. S5–S8 of the SI for further details of the methods and calibration curves for the analytes. A good stability was observed for all ligands at the concentrations used, typical results and conditions are shown in Fig. S9. The binding to the BSA-PEG hydrogels was studied in 10 mL PBS with different amounts of hydrogel units (1–8 units with 4.5 ± 0.1 mg of BSA each, corresponding to a BSA concentration equal to 6.8–54 μ M with respect to the total volume of the solution). In the case of the serum hydrogels, the total volume of the solution was decreased to 5 mL to achieve a similar total concentration of serum albumin with the same amount of hydrogel units. The time dependence of the fraction of ligand in the aqueous media was analyzed with Equation (2) to obtain the rate constant for equilibration (k) and the fraction in the aqueous media at full equilibration ($fL_W(\infty)$). The fraction of ligand associated with the protein in the hydrogel ($fL_{gel}(\infty)$) was then calculated as $1 - fL_W(\infty)$.

$$fL_W(t) = fL_W(\infty) + (fL_W(0) - fL_W(\infty))e^{-kt} \quad (2)$$

In some experiments, the binding of up to 3 ligands was characterized in the same experiment. The binding affinity of ligand i (K_b^i) is given by Equation (3).

$$L_W^i + BSA \leftrightarrow L_{BSA}^i; K_b^i = \frac{[L_{BSA}^i]}{[L_W^i][BSA]} = \frac{fL_{BSA}^i(\infty)}{fL_W^i(\infty)[BSA]} \quad (3)$$

If the concentration of protein is at least 10 times larger than that of all ligands, excess protein may be assumed and the concentration of free protein may be approximated by its total concentration (with the concentration calculated with respect to the total volume of the solution, $[BSA]_T$). This allows the direct calculation of the binding affinity of all ligands in the solution. However, if the protein is only in marginal excess, the concentration of protein without any ligand bound must be calculated from the protein mass balance given by Equation (4).

$$[BSA] = [BSA]_T - \sum_{i=1}^n [L_{BSA}^i] = [BSA]_T - \sum_{i=1}^n [L_W^i]_T fL_{BSA}^i(\infty) \quad (4)$$

The above equation assumes that the ligands compete with each other for binding to the same binding site. If excess protein is used, no significant deviations are expected for the binding affinities obtained if the ligands bind to the same or to different sites. Insights regarding ligand competition may be obtained from experiments performed at excess ligand, through the comparison of the amount of ligand bound when studied individually or mixed with other ligands.

The pesticide malathion does not contain aromatic groups and this leads to a very low sensitivity when detected by UV spectroscopy. Therefore, for this ligand a high concentration was needed, and excess protein could not be achieved. The fraction of ligand bound obtained experimentally showed that protein saturation reached values slightly above one. Accordingly, the model used to analyze the results considered two equivalent and independent binding sites. The binding affinity may be calculated from Equation (5), where $[B]_T$ is the total concentration of binding sites (here assumed to be two equal and independent binding sites *per* BSA), of which $[B]$ are not occupied by the ligand. The right end side of the equation allows the calculation of K_b from the known total concentrations of ligand and BSA, and the fraction of free

and bound ligand obtained in the binding assay.

$$K_b = \frac{[L_{BSA}]}{[L_W][B]} = \frac{[L_{BSA}]}{[L_W]([B]_T - [L_{BSA}])} = \frac{fL_{BSA}(\infty)[L_T]}{fL_W(\infty)(2[B]_{SA}]_T - fL_{BSA}(\infty)[L_T])} \quad (5)$$

The binding affinity of the ligands to the serum hydrogels was calculated assuming binding to serum albumin only and considering a BSA concentration of 21 mg/mL [71]. Contribution from binding to other proteins in the serum hydrogel was accessed through the comparison of the affinities obtained with those observed for the BSA hydrogels.

3. Results and discussion

3.1. Hydrogel preparation, stability and swelling

The hydrogels were prepared from PEG with a low degree of activation (dA, equal to 0.5 or 1 activated PEG terminals *per* PEG molecule). Assuming that both PEG terminals are activated independently and with equal probability, the average value of activated PEG terminals used in this work corresponds to 6 % (or 25 %) of the PEG molecules being activated on both terminals, 38 % (or 50 %) on one of the terminals only, and 56 % (or 25 %) not activated, for an average dA of 0.5 (or 1). The total protein concentration in the serum used in this work was measured using the method of precipitation and staining with amido black [86], being 68 ± 4 mg/mL, which is within the range usually considered for the protein concentration in bovine serum (60–95 mg/mL [72,75,76, 91]). The concentration of albumin in bovine serum depends on age, and values up to 32 mg/mL have been reported for 6 months old calf and cow [72,76]. Recent genome-wide data for bovine serum reported an average value of 21 mg/mL [71], which was the reference value considered in this work. Dilution of serum with the PEG solution leads to a total protein concentration of about 34 mg/mL, of which only 10.5 mg/mL is albumin, in the serum-PEG solution used to prepare the hydrogel. Due to this relatively low protein concentration, the hydrogels were prepared with a PEG degree of activation equal to 1. Less than 1 % of the total protein in the hydrogel leached out during the extensive washing procedure (over 24 h of incubation, with at least 6 washing steps). High protein entrapment efficiencies have been previously observed for BSA-PEG hydrogels prepared at similar concentrations, but with much higher degrees of PEG activation [49]. The results obtained show that a small fraction of crosslinking is enough to stabilize the hydrogel, with most protein being entrapped in the matrix even if not chemically bound.

After extensive washing with water at 4 °C, the mass obtained for the dried hydrogel units was 76($\pm$ 7) % of the initial mass for the hydrogels prepared from PEG with dA = 0.5, and 86($\pm$ 3)% for PEG with dA = 1. Given the very low leaching of protein observed, the mass loss is attributed to leaching of non-activated PEG. The results indicate that almost 40 % of the initial PEG leached out from the hydrogel for dA = 0.5, corresponding to 75 % of the non-activated PEG. In the case of the serum hydrogels, the mass loss is slightly higher than the amount of non-activated PEG, indicating that some of the non-protein components from the serum were not retained in the hydrogel. This result was expected given the complex composition of serum, including several small molecules.

Incubation of the BSA-PEG hydrogel at 37 °C in saline phosphate buffer (PBS) for 2 h led to less than 2 % mass loss (accounting for the mass of the PBS salts retained in the hydrogel), and quantification of the BSA in the PBS showed a very slow BSA leaching of about 0.5 % *per* h. An even lower protein leaching was observed in the case of serum hydrogels, corresponding to ≤ 0.1 % protein *per* h (Table S1). Thus, after washing and conditioning, the hydrogel units contain a similar amount of protein and PEG: 4.5 ± 0.1 mg of each *per* hydrogel unit prepared from 100 μ L of the initial BSA + PEG solution.

The protein content of the aqueous media in the washing steps of a

set of serum hydrogels (3 disks) is shown in the SI, Section S3 -Table S1. An efficient protein retention in the hydrogels is observed, with less than 1 % protein leaching during the extensive washing in water and PBS over about 24 h. The protein composition of the serum and that of the washing solutions was analyzed by SDS-PAGE, the results are shown in Fig. S4. As expected, the most abundant proteins in serum are albumin and IgG [92,93], with several additional proteins being also observed at relatively lower quantities. The SDS-PAGE profile of the washing solutions shows that most of the protein that leaches out from the serum hydrogels has very large molecular weights ($\gg 66$ kDa), higher than expected for the individual proteins present in serum. This proteinaceous material likely corresponds to small fractions of the hydrogel units that are tear-off from the hydrogel thin disks due to the mechanical stress caused by agitation. Nevertheless, the amount of protein leached is always very low. Taking into account the mass loss of the hydrogel units and the amount of protein leached out, at the end of the washing procedure each disk unit prepared from 100 μ L of the initial serum + PEG solution contained 3.5 ± 0.2 mg protein and ≈ 3.8 mg PEG.

The hydrogel showed good water incorporation capacity, with a swelling factor of 16–20 in water for both hydrogels, corresponding to 94–95 % water. In agreement with previous reports for BSA-PEG hydrogels [49], a lower swelling factor was obtained in aqueous solutions with a high ionic strength, being 16 ± 1 and 13 ± 1 in PBS at 37 °C for the BSA-PEG and serum-PEG hydrogels, respectively. Although the maximum swelling is similar to that observed previously for BSA-PEG hydrogels with a higher degree of crosslinking, the maximal swelling occurred much faster for the hydrogels with a low degree of crosslinking reported in this work, less than 2 h compared to several days [50]. This behavior agrees with the high porosity of the hydrogels prepared in this work (Section 3.4), and anticipates a better performance for studies of ligand binding to the protein in the gel.

3.2. Structural properties of the protein in the hydrogel

The BSA-PEG hydrogels were characterized by circular dichroism to assess if the secondary structure of the protein is affected by its incorporation in the hydrogel, the results obtained are presented in Fig. 1A. The first observation is that the far-UV CD spectrum is similar for BSA in solution (at 1 mg/mL) and in the hydrogel. A small decrease is observed in the molar ellipticity of BSA in the hydrogel, most likely due to an overestimation of the amount of protein in the light path, which was calculated from the absorption at 280 nm. To guarantee that the absorption at the wavelengths used to obtain the CD spectra is within the optimal range for those measurements, the hydrogel sample was prepared by polymerization between two squeezed quartz lamellas. The 4-nitrophenylformate present in the activated PEG sample or formed

during polymerization was removed by immersion of the hydrogel (maintained between the quartz lamellas) in water and in PBS, and this may lead to inefficient washing with a significant contribution of 4-nitrophenylformate to the observed absorption at 280 nm.

A detailed analysis of the CD spectra obtained shows a typical profile for the free protein, enriched in alpha-helix [94], as judged by the positive peak at 193 nm and the double negative peaks at 208 and 222 nm. The similar profile obtained for the hydrogel, shows that BSA maintains most of its secondary structure in alpha-helix, as previously reported for BSA-loaded PEG extrudates [47] and for mono- and multi-PEGylated BSA [70]. This agrees with molecular simulations obtained for BSA grafted with low molecular weight PEG (<10 kDa PEG), where grafted PEG behaved almost as free PEG, while BSA maintains most of its solvent accessibility [61,62]. From the far-UV CD data here obtained, a percentage of alpha-helix of nearly 50 % was computed by Program BeStSel for the BSA in solution, as expected for highly concentrated solutions of BSA [44,55]. When in the BSA-PEG hydrogel, the helical content decreased to 34 %, with a concomitant increase in the content of beta-sheet (from 12 % to 22 %). This variation reflects the higher concentration of BSA when in the hydrogel (46 mg/mL in the solution used for polymerization, decreasing to about 30 mg/mL in the swollen hydrogel, corresponding to about 700 and 500 μ M, respectively), and the tendency of BSA to undergo self-oligomerization in solution especially at high protein concentrations [44]. The presence of oligomeric species in concentrated BSA solutions was corroborated by gel filtration chromatography (SEC) presented in Fig. 1B. The SEC chromatogram of fresh PEG-free BSA solutions at concentrations from 5 to 750 μ M registers the immediate presence of monomers (accounting for about 80 % of the total protein mass), dimers (14–17 %), trimers (3–4 %) and also of other oligomers of higher molecular weight (0–4 %). An increase in the concentration of BSA results in a small increase in the fraction of aggregates. Incubation of the BSA solutions at low temperature (4 °C) for 24 h leads to the appearance of large aggregates even for the solution with the lowest concentration, accounting for 4 % of the total protein mass (Fig. S11 and Table S2).

1D ^1H NMR spectra of free BSA and BSA-PEG hydrogels were also performed and are shown in Fig. S12 for the low-field (10.0–6.0 ppm) region. The 1D ^1H NMR spectrum of non-bound BSA in solution shows a profile of signals assigned to the *ortho*- and *meta*-protons of the aromatic moieties (indole, phenol, and phenyl) of Trp, Tyr, and Phe residues. In turn, the HR-MAS-NMR spectrum of BSA-PEG shows a similar profile of peaks, but of lower intensity, given the line broadening of the signals emitted by the larger PEG-bound BSA.

The fluorescence properties of BSA in solution and in the hydrogel were also studied, the spectra are shown in Fig. 2. Although BSA (583-residue protein) is rich in tyrosine (20 Tyr) and phenylalanine (28 Phe)

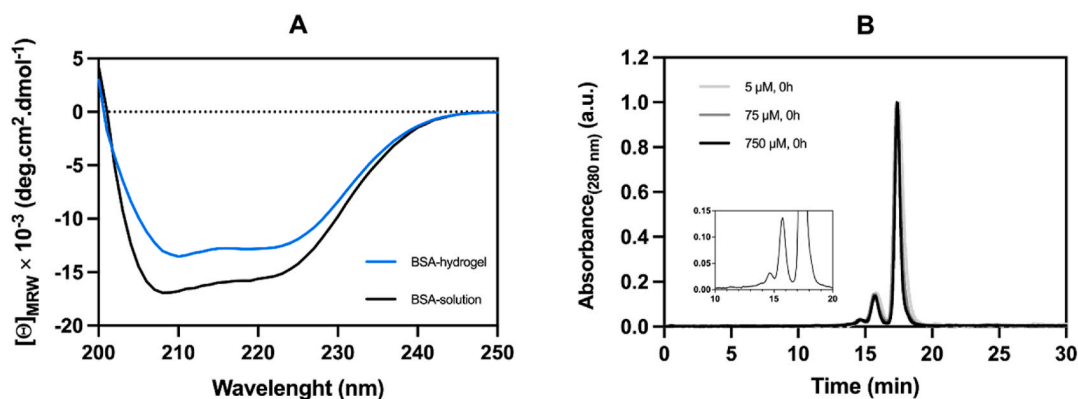


Fig. 1. Structural characterization of BSA in solution and incorporated in the BSA-PEG hydrogel. (A) Far-UV CD spectra of BSA at 1 mg/mL, and BSA-PEG hydrogel (30–46 mg/mL depending on the swelling extent, which has not been characterized for the hydrogel prepared within the quartz lamella and may be smaller than for the hydrogel units free in solution). (B) SEC profile of fresh BSA solutions at 5, 75, and 750 μ M in PBS at pH 7.4. The chromatograms have been normalized by the height of the peak corresponding to BSA monomers. The insert shows a zoom in of the chromatogram obtained for the highest BSA concentration.

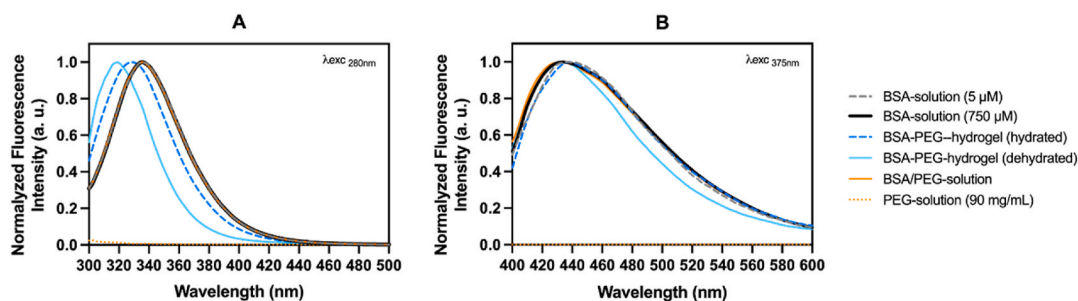


Fig. 2. Fluorescence properties of BSA, PEG, and BSA-PEG solutions and hydrogels. Intrinsic fluorescence emission spectra with excitation at 280 nm (A) and at 375 nm (B). Fluorescence spectra normalized for the maximum emission intensity are shown for free BSA (BSA-solution) at two protein concentrations (5 and 750 μM), for BSA at 750 μM in the presence of 90 mg/mL non-activated PEG (BSA/PEG-solution), and for hydrated and de-hydrated BSA-PEG hydrogels. The non-normalized spectrum obtained for PEG is also shown (PEG-solution).

residues, the fluorescence emission spectrum of free BSA when excited with UV light ($\lambda_{\text{ex}} = 280 \text{ nm}$) is essentially dominated by the fluorescence of its two tryptophan residues (Trp-134, Trp-213). This is typical of proteins in the folded state and is due both to the higher fluorescence quantum yield of Trp (especially that of Trp-134 [95]) and to the efficient fluorescence resonance energy transfer from nearby Tyr residues [96–98]. The maximum fluorescence intensity of free BSA in PBS is at 336 nm, indicating that the fluorescence emission is from Trp residues located in a medium with moderate polarity, as previously observed for BSA in aqueous media in its folded state [44,97,99]. The presence of non-activated PEG does not change the wavelength of maximum emission intensity, while a small shift to lower wavelengths is observed when BSA is incorporated in the hydrated hydrogel indicating that the environment around the Trp residues is less polar and/or more rigid [100]. This effect reflects the increase in the viscosity of the medium, and possibly the stabilization of compact protein structures in the presence of high concentrations of neutral co-solvents that interact strongly with water [101–104]. Consequently, an additional blue-shift (to 320 nm) in the fluorescence spectra is obtained upon dehydration of the hydrogel.

The fluorescence spectrum of BSA excited at 375 nm is shown in Fig. 2B, with fluorescence emission at around 440 nm at all protein

concentrations studied. This is likely due to the presence of protein oligomers that promote the delocalization of peptide electrons through H-bonded networks and/or π - π stacking at the oligomeric interfaces (due to BSA self-oligomerization via intermolecular β -sheet interfaces), as previously reported [43,44]. This agrees with the results from SEC (Fig. 1B and Fig. S11), where protein oligomers are observed even for very low BSA concentrations. As observed for excitation at 280 nm, the wavelength of maximum emission is slightly blue-shifted in the dehydrated hydrogel sample, reflecting the lower water activity [105], and/or a more restricted environment.

Photoluminescence quantum yields (Φ_{PL}) of BSA in solution and in the BSA-PEG hydrogel were also determined (Table S3 in the SI). For excitation at 280 nm, BSA in solution presents a Φ_{PL} in the range of 0.13–0.15, somewhat smaller than that observed for Trp alone (0.20) and typical of proteins containing both Trp and Tyr aromatic residues [100]. Similar values of Φ_{PL} were also obtained for the BSA-PEG hydrogels (0.10–0.12). For excitation at 375 nm, the emission quantum yield was approximately 0.04 for diluted solutions of BSA free in solution and when in the BSA-PEG hydrogels.

BSA-PEG hydrogels were also characterized by FLIM microscopy to visually investigate the spatial distribution of the fluorescence signal.

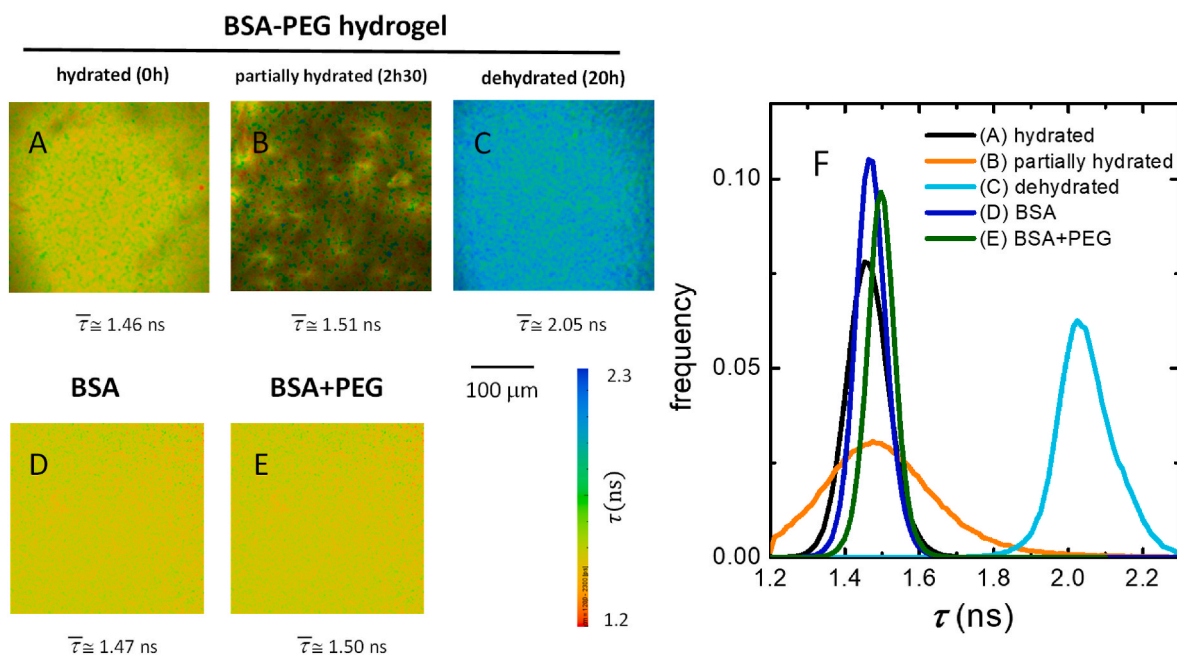


Fig. 3. Fluorescence lifetime images of the BSA-PEG hydrogels fully hydrated (A), 2h30 (B) and 20h (C) after removal from water, an aqueous solution of BSA at 50 mg/mL (D) and a mixture of BSA at 50 mg/mL and PEG at 90 mg/mL (E). The color scale ruler concerns to the mean decay time ($\bar{\tau}$) ranging from 1.2 to 2.3 ns. The distribution of lifetimes for the different conditions is shown in plot (F), and the corresponding average lifetime is indicated below the respective plot.

The results are presented in Fig. 3, together with control samples prepared with BSA alone and BSA-PEG at the concentrations found in the hydrogels. The fully hydrated hydrogel showed a homogeneous fluorescence emission, with small circular areas of non-fluorescent material that should reflect the presence of air bubbles (Fig. 3A). After 2h30 in the microscope, some of the water evaporates, and the partially hydrated hydrogel shows a non-homogeneous spatial distribution of fluorescence lifetimes (Fig. 3B). At complete dehydration (Fig. 3C), a homogeneous distribution of fluorescence and lifetimes is again obtained, with an increase in the fluorescence emission and in the fluorescence lifetime that reflects the less polar and/or more rigid environment. Although the average lifetime in the partially hydrated gel is very similar to that obtained at full hydration, the distribution is wider, with some overlap with the lifetime distribution observed in the dehydrated gel (Fig. 3F). The FLIM images of a solution of BSA alone (Fig. 3D), and BSA with PEG (Fig. 3E) are also shown. A homogeneous distribution is obtained for both samples, with an average fluorescence lifetime very similar to that obtained for the hydrated hydrogel. This shows that the presence of PEG or the reticulation of the protein in the BSA-PEG hydrogel, does not influence the fluorescence properties of the protein oligomers.

3.3. Mechanical properties of the BSA-PEG hydrogel

The mechanical properties of the hydrogels (in their dehydrated form by air-drying for 24h) were characterized regarding their compression and elasticity. The results are shown in Fig. 4 and Table S4. A viscoelastic behavior was observed, with Young's modulus of 6.05 ± 1.10 MPa. The dehydrated hydrogels presented a good elasticity, as stated by the tensile Young's modulus of 2171 ± 600 MPa obtained, and the yield stress of 82.28 ± 8.73 MPa. Permanent deformation of the dehydrated hydrogel only occurred above this stress value, showing an outstanding plastic deformation reaching six times more than the original length. Breakage occurs at 101.6 ± 17.8 MPa. The BSA-PEG dehydrated hydrogels showed increased mechanical modulus capacity when compared with BSA alone [41] or other PEG-based hydrogels [58].

3.4. Morphology of the BSA-PEG and serum-PEG hydrogels

Hydrogel porosity and crosslinking degree has been shown to influence the rate and extent of hydrogel swelling [49,68,69], with impact on the kinetics of drug release from the hydrogels [45,53,56,69]. The porosity of the hydrogels is therefore expected to be of major importance for their performance in the evaluation of ligand-protein binding, directly influencing ligand's access to the protein in the hydrogel matrix and therefore the binding kinetics. Very low porosities (high reticulation degrees) may also influence the structure of the protein and may hinder ligand access to the binding sites in the protein [51,54]. Representative SEM images of the hydrogels prepared in this work are shown in Fig. 5.

Large pores with a diameter close to $10 \mu\text{m}$ are observed on both hydrogels, although a somewhat higher porosity is observed for the serum-PEG hydrogels in agreement with the lower protein concentration in the polymerization reaction. A well-connected pore structure with a uniform network morphology is observed for both types of hydrogel.

The network structure of the hydrogels obtained in this study is similar to that obtained for hydrogels prepared from BSA crosslinked with glutathione [65], and from BSA amyloid fibrils thermally induced [55]. A major difference of the hydrogels prepared in this study is the use of native conditions that lead to maintenance of BSA secondary structure (Section 3.2), making them promising 3D matrices to evaluate drug-protein binding.

3.5. Binding of the NBD-Cn homologous series and bioactive ligands to BSA-PEG hydrogels

A major difference between the hydrogels prepared in this work and other BSA-PEG hydrogels previously reported is the use of a small fraction of cross-linking agent (PEG activated in both terminals), leading to a low degree of crosslinking and a large mesh size. The efficient entrapment of BSA in the hydrogel is due both to crosslinking by PEG activated in both ends, and by the physical entrapment of BSA decorated with PEG activated in only one of its terminals. This decreases significantly the conformational stress imposed on the protein upon gel swelling, with BSA essentially maintaining its native structure (Fig. 1A). Those properties make the hydrogels particularly useful for the evaluation of ligand binding, a property that is explored in this section. The binding affinity and kinetics was first evaluated for ligands structurally similar but with distinct hydrophobicity, to evaluate for eventual effects of the decreased water activity in the hydrogel. The homologous series of fluorescent ligands (NBD-Cn) was selected due to the previous characterization of binding to free BSA [36,106], allowing the quantitative assessment of the effect of the hydrogel environment. Due to the relatively high fluorescence quantum yield of NBD-Cn, low ligand concentrations may be used, allowing the simultaneous characterization of several ligands while maintaining a low level of BSA saturation. The concentration of NBD-Cn in the aqueous medium was quantified by HPLC (details in the SI, Section S4 – Figs. S5 and S6). Typical results are shown in Fig. 6.

The first observation is that the extent of binding to the BSA-PEG hydrogel is strongly dependent on the hydrophobicity of the amphiphile (Fig. 6A, $\text{NBD-C8} \gg \text{NBD-C6} > \text{NBD-C4}$) and increases with the amount of hydrogel added (Fig. 6B). Equilibration occurs relatively fast, being essentially complete after 60 min of incubation, and is mostly independent on the amphiphile and/or number of disk units. The rate of equilibration increases somewhat with the rate of agitation (not shown) and is strongly dependent on the surface/volume ratio of the hydrogel units (being slower for gel units in cones when compared with thin disks,

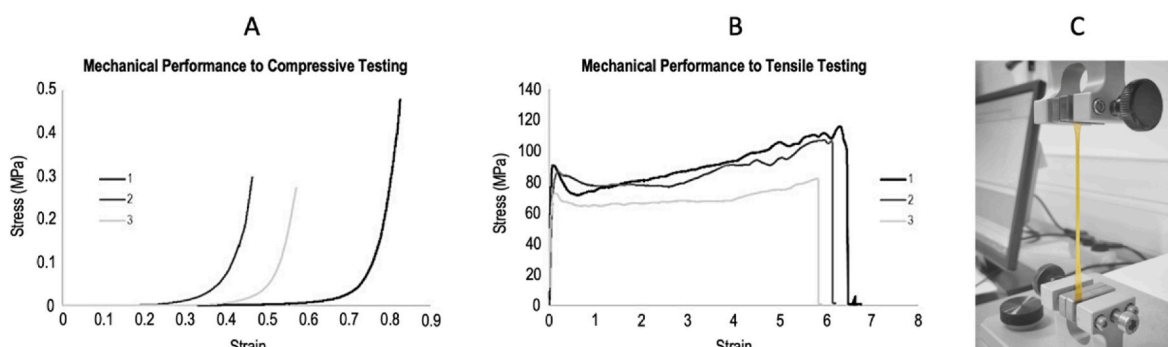


Fig. 4. Stress-strain curves of BSA-PEG dehydrated hydrogels submitted to compression (A) and tensile (B) tests. The curves correspond to three replicates. (C) visualization of the material under the tensile test before permanent deformation.

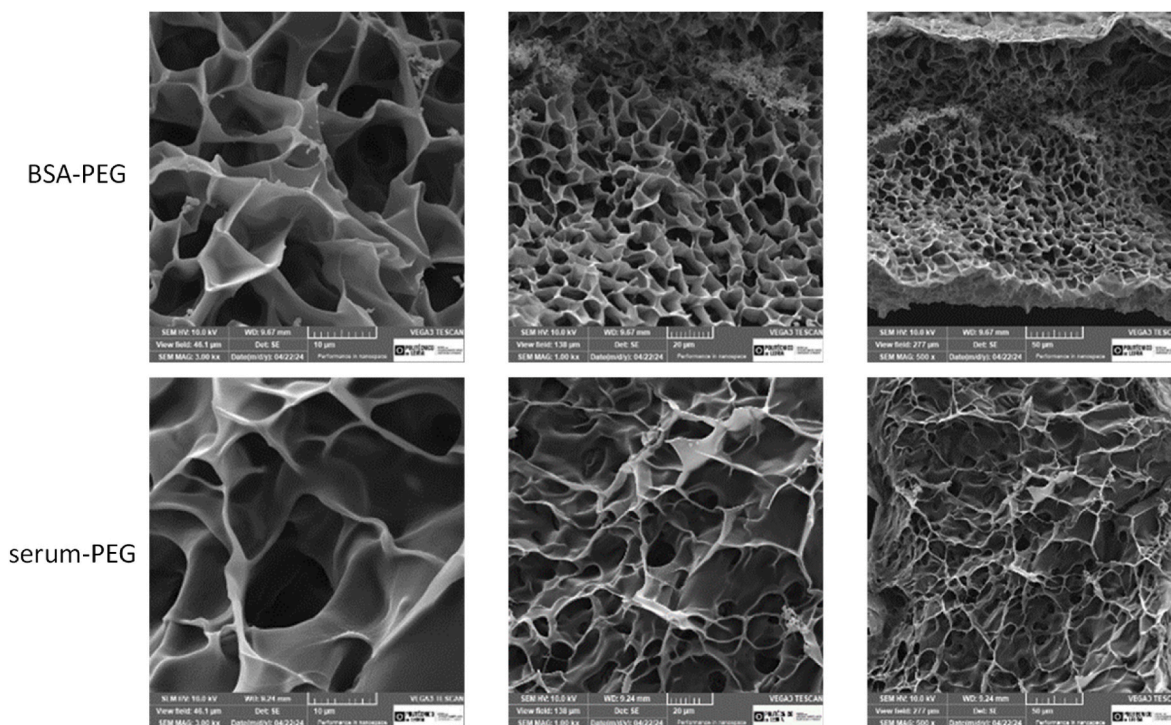


Fig. 5. Representative scanning electron microscopy images of the BSA-PEG (upper images) and serum-PEG (lower images) hydrogels. The scale bar is 10 μm (left panels), 20 μm (middle panels), and 50 μm (right panels).

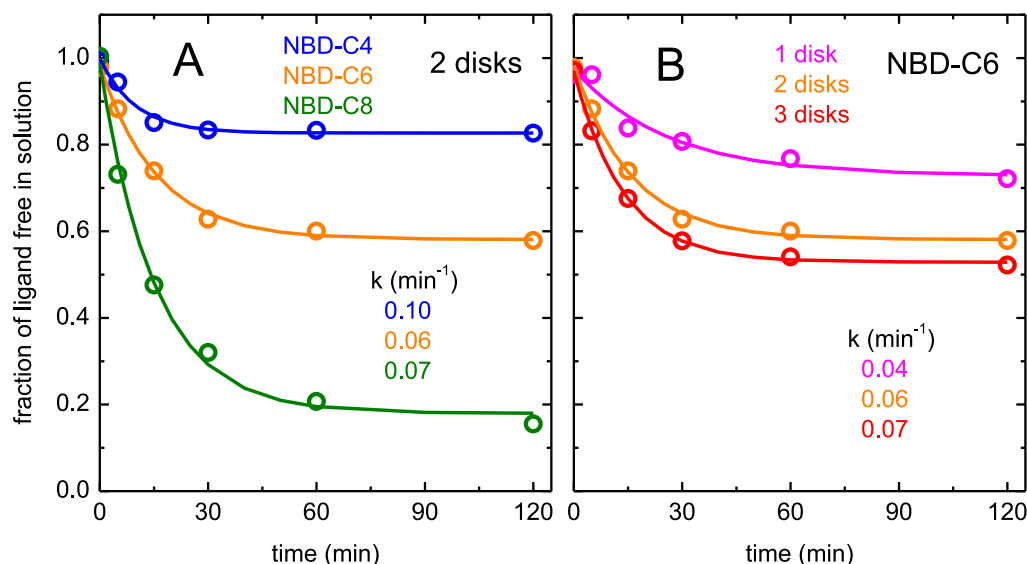


Fig. 6. Time dependence of the fraction of NBD-Cn amphiphiles free in the aqueous solution due to equilibration with the BSA-PEG hydrogel; for the different amphiphiles equilibrated with two hydrogel disk units (plot A), and for NBD-C6 with a different number of hydrogel units (plot B). The lines are the best fit of Equation (2), with the rate constants indicated in the plots.

Fig. S13). This shows that equilibration is limited by the access of the amphiphile to the hydrogel, and not by the rate of association/dissociation of the amphiphile and the protein (which occurs within a few seconds or faster [106,107]).

From the extent of ligand removal from the aqueous phase one may calculate the equilibrium binding constant. The concentrations of NBD-Cn used in the assays ($\leq 0.5 \mu\text{M}$) were much smaller than that of BSA ($\geq 7 \mu\text{M}$), allowing the approximation of a large excess of protein for the estimation of K_b , Equation (3). Due to the low BSA saturation attained,

the values of K_b correspond to binding to the highest affinity binding site, regardless of the possible existence of several binding sites in BSA. The values obtained are collectively presented in Fig. 7, and compared with those obtained for the association with free BSA in solution (taken from Ref. [36] after correction for the different temperature).

The statistical analysis of the values obtained for the binding affinity was performed for $\log K_b$ because this is the variable expected to have a Gaussian distribution [108]. The distribution of the data points (Fig. 7A) shows that the values of $\log K_b$ obtained for the distinct conditions

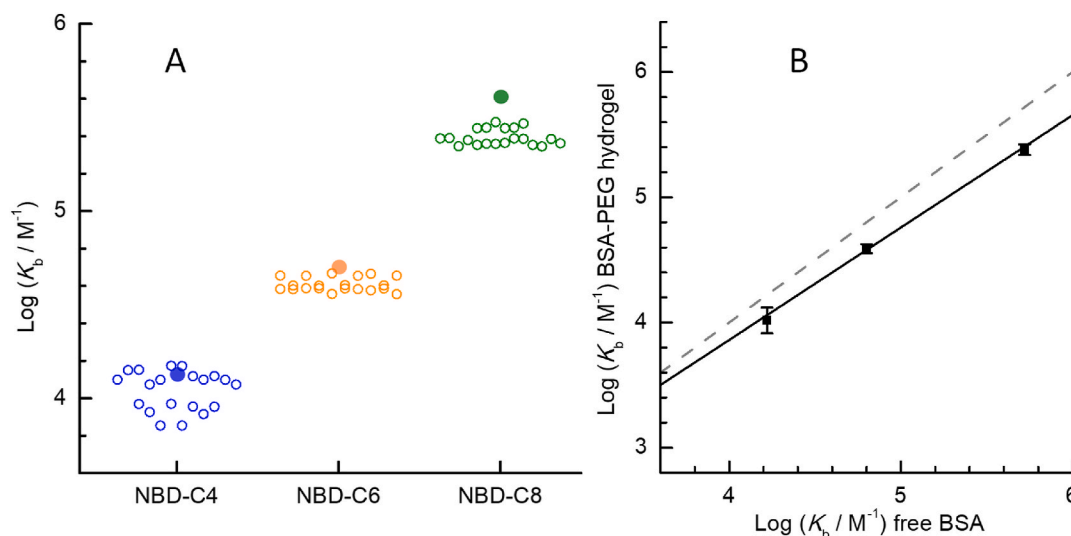


Fig. 7. Affinity ($\log K_b$) of the NBD-Cn amphiphiles to bind to free BSA in the aqueous solution or in BSA-PEG hydrogels. (A) Representation of the values obtained for $\log K_b$ in each experiment with BSA-PEG hydrogels (●, ○, and ●, for NBD-C4, -C6 and -C8 respectively), and the average value of $\log K_b$ obtained with free BSA in solution [36] (●, ○, and ●, for NBD-C4, -C6 and -C8 respectively, the symbol size is equal to the standard deviation). (B) Dependence of the average and standard deviation of $\log K_b$ for binding to the BSA-PEG hydrogels with $\log K_b$ obtained for binding to free BSA in solution. The lines are the relation between the two parameters (— $\log K_b$ (BSA-PEG hydrogel) = $0.27 + 0.90 \log K_b$ (free BSA), $R^2 = 0.999$), and the identity line (---). Data for BSA-PEG hydrogels include replicates from two activated PEG preparations, number of gel units in the assay from 1 to 4, analytes together in the same solution and in independent experiments, using hydrogel units as thin disks and cones, for the best fit of Equation (2) imposing $fL_w(0) = 1$ and with this parameter obtained from the best fit at all times.

(number of gel units, as disks or cones) are statistically equivalent, and they were all included in the calculation of the average and standard deviation for each amphiphile (Fig. 7B). The value obtained for binding of NBD-C4 to the BSA-PEG hydrogels is very similar to that obtained for free BSA. However, the parameters obtained in the two distinct systems become statistically different as the amphiphile hydrophobicity increases, with the affinity obtained for the hydrogels being smaller than that obtained for free BSA. This is reflected in a slope between the two parameters that is lower than one; being equivalent for $\log K_b = 3.2$, with a higher binding affinity for the hydrogels in the case of less hydrophobic amphiphiles, and a lower binding affinity for the hydrogels in the case of more hydrophobic amphiphiles. This behavior is in excellent agreement with the effects of trehalose on the solubility of the amphiphiles in aqueous solutions [103], and likely reflects the presence of PEG in the hydrogel. Given the high concentration of co-solvents in biological fluids, the differences observed correspond to an advantage of this method in comparison with the use of dilute BSA solutions. Significant effects are only observed for ligands with very low solubility in the aqueous medium, as is the case of NBD-C6 and NBD-C8 (13 μM and 1 μM , respectively [36]), and are not expected for common bioactive ligands.

After validation of the binding ability of BSA-PEG hydrogels for structurally related ligands with increasing hydrophobicity, this 3D matrix was evaluated for its general applicability in the characterization of ligand-protein binding. For this purpose, it was characterized the binding affinity of two pesticides, chlorpyrifos (CP) and malathion (MT) and six pharmaceutical drugs, diclofenac (DC), salicylic acid (SA), chlorpromazine (CZ), verapamil (VP), labetalol (LB), and acetaminophen (AC). With the exception of MT, the concentration of the ligands in the stock solutions (in DMSO) was calculated from their absorption spectra in the UV. Whenever available the molar absorptivity used was taken from the literature: $6.8 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 290 nm for CP [109], $3.6 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 306 nm for CZ [110], and $3.5 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 298 nm for SA [111]. The molar absorptivity of the remaining ligands was measured experimentally in this work. The ligand concentration in the stock solutions was first verified by ^1H NMR spectroscopy, as explained in the SI Section S12. The values obtained for the average and standard deviation of the molar absorptivity were: $9.68(\pm 0.39) \times 10^3$

$\text{M}^{-1}\text{cm}^{-1}$ at 278 nm for AC, $1.12(\pm 0.04) \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ at 278 nm for DC, $3.17(\pm 0.30) \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 309 nm for LB, and $5.73(\pm 0.56) \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 278 nm for VP.

The total concentration of ligand in the binding assays was typically 20 μM . A lower ligand concentration (5 μM) was used for CP given the need to use a small number of hydrogel disk units due to the very high binding affinity, and a higher concentration (100–200 μM) was used for MT, due to the low sensitivity in its quantification by UV absorption. The ligand concentration in the aqueous medium after incubation with the BSA-PEG hydrogels was quantified by HPLC following the methods described in the SI (Section S5).

Typical results for the time-dependent evolution of the fraction of ligand in the aqueous phase are shown in the SI (Section S6, Fig. S9). The binding affinities obtained are presented in Table 1 and compared with the values reported in the literature.

The binding affinity of DC, CP and SA for the BSA-PEG hydrogels is in excellent agreement with data from the literature obtained by equilibrium dialysis [112,114,115,117], and a good agreement is also obtained for CZ [118]. The value obtained for AC is also similar to that obtained previously by micro dialysis [122]. For the other ligands studied, no data could be found in the literature with the binding affinity obtained by the method of equilibrium dialysis. The only report encountered in literature for the binding of the pesticide MT to serum albumin uses the method of solid phase extraction [113], and a very similar value was obtained in this work. For the other ligands studied (VP and LB), only data obtained from quenching of the albumin fluorescence and capillary electrophoresis was found. Due to the extremely large uncertainty [124], and the many experimental faults that may compromise the accuracy of the former method [39], only the parameters obtained by capillary electrophoresis are shown in Table 1. It is observed that the binding affinity obtained in this work for VP and LB is somewhat higher than previously obtained from capillary electrophoresis. It is important to note that for the ligands where the binding affinity was characterized by both equilibrium dialysis and capillary electrophoresis, a lower affinity was always encountered with the latter (see e.g. for SA), pointing towards an accurate estimation of the ligand-albumin binding affinity when using the BSA-PEG hydrogels and the methodology followed in this work.

Table 1

Binding affinity of the biologically active molecules to BSA-PEG hydrogels and comparison with data from the literature [112–123].

Ligand	This work (BSA-PEG hydrogels)		From literature	
	K_b (M^{-1})	$\text{Log}(K_b)$	$\text{Log}(K_b)$ ¹	Method ²
Chlorpyrifos	2.2×10^5	5.3 ± 0.1	5.5 5.1	ED [112] SPME [113]
Malathion	1.9×10^4	4.3 ± 0.3	4.0	SPME [113]
Diclofenac	3.7×10^5	5.6 ± 0.1	5.7 5.3	ED [114,115] CE [116]
Salicylic acid	3.8×10^4	4.6 ± 0.2	4.7; 4.3 3.3	ED [115]; [117] CE [117]
Chlorpromazine	5.7×10^4	4.8 ± 0.1	4.3 4.1	ED [118] CE [119]
Verapamil	9.7×10^3	4.0 ± 0.1	3.0 3.3	CE [119] CE [120]
Labetalol	9.0×10^3	3.9 ± 0.1	2.7	CE [121]
Acetaminophen	5.9×10^3	3.8 ± 0.1	4.1 4.2	MD [122] CE [123]

¹ – Values in black correspond to binding to BSA, and those in gray to HSA.

² – ED (equilibrium dialysis), CE (capillary electrophoresis), MD (micro dialysis), SPME (solid phase microextraction)

3.6. Binding of the NBD-Cn homologous series and bioactive ligands to serum-PEG hydrogels

The binding affinity for the serum-PEG hydrogels has been characterized for the NBD-Cn homologous series of fluorescent amphiphiles and 3 drugs, the later including two negatively charged drugs with very distinct hydrophobicity (SA and DC), and one positively charged (CZ). The binding assays have been performed at distinct amounts of hydrogel to allow the identification of eventual binding to proteins present in low quantities. With the same objective, drug binding was characterized for each drug individually and in a cocktail containing all drugs. To evaluate the possible re-use of the hydrogel units, after being used in a binding assay the hydrogels were extensively washed and re-used in new binding assays for the same or distinct ligands. Typical results for the amount of ligand in the aqueous media along the incubation time are shown in Fig. S10, the binding affinities obtained are collectively presented in Table 2.

The first observation from Table 2 is that the binding affinity for the serum hydrogels obtained under the different conditions tested is statistically equivalent. This indicates that at these conditions the analytes do not compete with each other for the association with the binding agents present, and that the method is robust, including the possible re-use of the hydrogels in additional binding assays after proper washing. The data provided in Table 2 also shows that the binding affinity for the serum hydrogels, when considering BSA as the single binding agent, is similar to that obtained for the BSA-only hydrogels across all tested analytes. This indicates that binding to serum albumin is in fact the major determinant of the overall binding to serum proteins. In spite of this overall agreement, some relevant differences are observed. The binding affinity obtained for the uncharged NBD-Cn amphiphiles with the serum hydrogels is 2-to-3 fold higher than that obtained for the BSA hydrogels. A slightly higher affinity for the serum hydrogels is also observed for the cationic drug CZ. This indicates that these analytes have a significant affinity for other binding agents present in the serum. On the other hand, the negatively charged drugs SA and DC associate less efficiently with the serum than with the BSA hydrogels. This suggests that the concentration of BSA considered for the serum hydrogel may be slightly overestimated, or reflect the presence of small amounts of BSA-bound fatty acids in the serum, making the higher affinity observed for the NBD-Cn and CZ analytes even more accentuated.

The distinct behavior observed for the different analytes may be understood given the relatively higher selectivity of serum albumins for negatively charged ligands [125–127], and the significant association of neutral and cationic small molecules to other binding agents in serum

Table 2

Binding affinity of the NBD-Cn and the drugs SA, DC and CZ to the serum-PEG hydrogels and comparison with the affinity obtained for the BSA-PEG hydrogels.

Ligand	Conditions of the serum-PEG binding assays			$\text{Log}(K_b)$ ^d	
	disks use ^a	n ^b	analytes ^c	Serum ^e	BSA
NBD-C4	1st	overall		4.4 ± 0.2	4.0 ± 0.1
		1	all	4.3 ± 0.3	
		3		4.3 ± 0.2	
	3rd	6		4.4 ± 0.1	
		1		4.5 ± 0.3	
		3		4.3 ± 0.1	
NBD-C6	1st	overall		5.0 ± 0.1	4.6 ± 0.1
		1	all	5.0 ± 0.1	
		3		4.9 ± 0.1	
	3rd	6		5.0 ± 0.1	
		1		5.1 ± 0.1	
		3		4.9 ± 0.1	
NBD-C8	1st	overall		5.7 ± 0.1	5.4 ± 0.1
		1	all	5.7 ± 0.1	
		3		5.5 ± 0.1	
	3rd	6		5.8 ± 0.1	
		1		5.7 ± 0.1	
		3		5.5 ± 0.1	
Salicylic Acid	1st	overall		4.4 ± 0.2	4.6 ± 0.2
		6	alone	4.4 ± 0.2	
		6	all	4.3 ± 0.2	
	2nd	6		4.3 ± 0.1	
		6		4.4 ± 0.1	
		6		4.4 ± 0.1	
Chlorpromazine	1st	overall		5.0 ± 0.1	4.8 ± 0.1
		6	alone	5.0 ± 0.1	
		6	all	5.0 ± 0.1	
	2nd	6		4.9 ± 0.1	
		6		5.0 ± 0.1	
		6		5.0 ± 0.1	
Diclofenac	1st	overall		5.4 ± 0.1	5.6 ± 0.1
		6	alone	5.4 ± 0.1	
		6	all	5.5 ± 0.1	
	2nd	6		5.3 ± 0.2	
		6		5.3 ± 0.1	
		6		5.3 ± 0.1	
	2nd	1	alone	5.4 ± 0.1	
		3		5.4 ± 0.1	
		6		5.4 ± 0.1	

^a 1st/2nd/3rd times that the hydrogel units have been used in binding assays.

^b Number of disk units in the binding assay.

^c Binding assay performed with the analyte indicated only (alone) or in a mixture of analytes (all). The mixtures contained the 3 NBD-Cn or the 3 drugs.

^d An uncertainty in $\text{Log}(K_b)$ smaller than 0.1 units was often obtained, 0.1 is however indicated due to the limited number of experiments at each condition (2–3).

^e Considering binding to BSA only, and 21 mg/mL of BSA in the serum [71].

such as lipoproteins and α_1 -acid glycoprotein [121,128]. The binding to the serum hydrogels provides therefore a more accurate estimation of drug plasma-protein binding than binding to the serum albumin alone, this being particularly relevant for the case of neutral and cationic ligands.

4. Conclusions

The BSA-PEG and serum-PEG hydrogels displayed high protein retention with minimal leaching, even under extensive washing, confirming their stability for ligand-binding studies in crowded protein environments similar to physiological conditions. Notably, these hydrogels showed a high swelling degree, resulting in a matrix with substantial water content that closely mimics the environment found in blood plasma. This high water retention is achieved through the use of PEG with a low activation degree, leading to minimal crosslinking and thus imposing little constraint on the entrapped proteins during swelling. The native structure of proteins within the BSA-PEG hydrogel

was verified through circular dichroism and UV–vis fluorescence spectroscopy. Together, the high porosity and aqueous content of these hydrogels create a physiologically relevant environment for evaluating plasma protein binding.

Compared with standard BSA or BSA-PEG hydrogels, serum-PEG hydrogels capture the complexity of whole serum, making them a superior model for assessing drug interactions in blood plasma. Both types of hydrogels demonstrated robust binding capacity for a wide range of bioactive molecules, including model drug-like amphiphiles, pesticides, and pharmaceutical drugs, underscoring serum albumin's central role in ligand binding within serum matrices. While emphasizing the role of ligand hydrophobicity in the binding affinity, the results show that this unspecific ligand-protein association is somewhat reduced in the molecularly crowded environment of blood serum, which the hydrogels effectively replicate.

Serum-PEG hydrogels also demonstrated an advanced capability to capture a broader spectrum of interactions present in whole serum. Uncharged and cationic ligands showed higher binding affinities in serum-PEG compared to BSA-PEG hydrogels, highlighting the importance of additional binding agents in serum, such as lipoproteins and α 1-acid glycoprotein. Conversely, negatively charged ligands displayed relatively weaker associations with the serum hydrogels, likely due to competition with fatty acids in the serum that preferentially bind to serum albumin.

Both hydrogels represent physiologically relevant matrices that offer a viable alternative to traditional methods for evaluating drug plasma-protein binding, circumventing the limitations of traditional methods like equilibrium dialysis by avoiding membrane artifacts while delivering faster and more accurate binding profiles. By offering an efficient, high-throughput, and accurate approach, these hydrogels can support pharmacokinetic and ADME/Tox analyses, particularly for studies on drug bioavailability and accumulation risks.

This study validates BSA-PEG and serum-PEG hydrogels as powerful, adaptable platforms bridging the gap between simplified *in vitro* models and the complex binding environments *in vivo*, offering valuable insights for drug discovery and toxicology research. The high porosity, stability, and mechanical resilience of these hydrogels further expand their potential applications into areas such as smart materials for drug delivery, biosensing, nanoencapsulation, coatings, wound dressings, and tissue regeneration.

CRediT authorship contribution statement

Carlos D.F. Coelho: Investigation, Formal analysis. **Victor S. Paiva:** Investigation, Formal analysis. **Zaida L. Almeida:** Writing – original draft, Investigation, Formal analysis. **João A. Jesus:** Investigation. **Madalena Marteleira:** Investigation. **Cristiana V. Ramos:** Investigation. **Pedro F. Cruz:** Investigation, Formal analysis. **Telma Costa:** Methodology, Investigation. **Carla S. Moura:** Resources, Investigation, Formal analysis. **Daniela Trindade:** Investigation, Formal analysis. **Rui M.M. Brito:** Resources, Funding acquisition. **Ricardo Lagoa:** Resources, Funding acquisition, Conceptualization. **Daniela C. Vaz:** Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Maria João Moreno:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Associated content

The following file is available free of charge. Raw data and additional results not included in the manuscript: Section S1 - Characterization of the degree of PEG activation (Fig. S1); Section S2 - Visualization of the BSA-PEG hydrogels (Fig. S2 and links to movies); Section S3 - Protein leaching from disks of the BSA-PEG and Serum-PEG hydrogels (Figs. S3 and S4, Table S1); Section S4 - Quantification of NBD-Cn by HPLC

(Figs. S5 and S6); Section S5 - Quantification of the bioactive ligands by HPLC (Figs. S7 and S8); Section S6 - Typical raw data obtained in the assays for ligand binding to the hydrogels (Figs. S9 and S10); Section S7 - Analysis of oligomeric species in BSA solutions by SEC (Fig. S11 and Table S2); Section S8 - 1D ^1H NMR spectra of free BSA and BSA-PEG hydrogels (Fig. S12); Section S9 - Photoluminescence quantum yields of BSA in solution and in the BSA-PEG hydrogel (Table S3); Section S10 - Mechanical properties of the BSA-PEG dehydrated hydrogels (Table S4); Section S11 - Effect of hydrogel geometry on the kinetics of NBD-Cn binding to BSA-PEG hydrogels (Fig. S13); Section S12 - Molar absorptivity of the pharmacological drugs in the UV (Figs. S14 and S15). (PDF)

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

ADME/Tox	absorption, distribution, metabolism, excretion and toxicity
PEG	polyethylene glycol
BSA	bovine serum albumin
NBD-Cn	7-nitrobenz-2-oxa-1,3-diazol-4-yl with aliphatic chain lengths containing n carbons (for n = 4, 6 or 8)
CD	circular dichroism
NPCF	4-nitrophenyl chloroformate
DMAP	4-dimethylaminopyridine
PBS	saline phosphate buffer
SEC	size exclusion chromatography
FLIM	fluorescence lifetime imaging microscopy
CP	chlorpyrifos
AC	acetaminophen
CZ	chlorpromazine
DC	diclofenac
LB	labetalol
MT	malathion
SA	salicylic acid
VP	verapamil

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mtchem.2025.102565>.

Data availability

Data will be made available on request.

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