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BSA-PEG Hydrogel: A Novel Protein-Ligand Binding 3D Matrix †

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Abstract: Hydrogel materials have good biomimetic properties and high potential for biomedical and bioanalytical applications. In this work, a hydrogel of serum albumin crosslinked with poly-(ethylene glycol) was prepared and characterized for its water content, protein structure and stability. The ability of the hydrogel to bind small molecule ligands with different hydrophobicity was evaluated using a homologous series of amphiphiles (NBD-Cn, n = 4, 6 and 8) and the calculated binding affinities were similar to that of free protein in solution. Overall, the results indicate this type of hydrogel system as a convenient tool for studying the binding of xenobiotics to tissue proteins.

Keywords: ligand binding; serum albumin; hydrogel; protein structure

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1. Introduction

The evaluation of non-specific binding of xenobiotics to proteins is of high relevance in pharmacology and ecotoxicology due to the effects of sequestration in the bioactivity of the xenobiotics, influencing their circulation time in the blood and the permeability through biological barriers. Binding to serum albumins is particularly relevant due to their high concentration in the blood and interstitial fluids [1]. Serum albumins are also good models to evaluate non-specific binding to protein enriched matrices such as the skin [2], allowing the evaluation of xenobiotic retention in matrix. Protein hydrogels are of particular relevance in this respect due to their high water content, which imposes low conformational constraints in the protein, and facilitate the diffusion of the xenobiotic and the rapid equilibration between the aqueous medium and the protein [3]. Additionally, in comparison with the protein in solution, the use of protein hydrogels facilitates the evaluation of xenobiotics binding due to direct access to the aqueous medium and the possibility of regeneration and re-use in different binding assays.

In this work, we report the preparation and characterization of a hydrogel obtained by copolymerization of poly-(ethylene glycol) 6000 (PEG) and bovine serum albumin (BSA). The hydrogel was characterized regarding swelling and stability. The structural properties of the protein were also characterized by circular dichroism (CD) to evaluate the possible effects of incorporation in the hydrogel. The binding affinity of a homologous series of fluorescent amphiphiles (7-nitrobenz-2-oxa-1,3-diazol-4-yl with different aliphatic chain lengths; NBD-Cn with n = 4, 6 and 8) to the hydrogel was also characterized. The comparison with the binding affinity previously reported for NBD-Cn and BSA in solution [4] allows the evaluation of the hydrogel adequacy as a model system in binding assays for xenobiotics with distinct hydrophobicity.

2. Materials and Methods

Materials. Bovine Serum Albumin (BSA) was from Fischer Scientific (Lisboa, Portugal), poly-(ethylene glycol) 6000 (PEG) from Merck (Algés, Portugal), 4-nitrophenyl chloroformate 97% purity from Acros Organics (Gell, Belgium) and 4-dimethylaminopyridine > 99% purity from Fluka Analytical. The fluorescent amphiphiles NBD-Cn were synthesized and purified following the procedure described in the literature [4]. The aqueous medium was a 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 (Sintra, Portugal).

Hydrogel preparation and characterization. The gel was prepared following the method described by Fortier and co-authors [5]. Briefly, a solution of BSA in PBS at pH 8.5 was mixed with a solution of PEG previously functionalized with nitrophenyl chloroformate and dissolved in water, for a final concentration of 50 and 90 mg/mL of BSA and PEG, respectively. These concentrations were chosen in order to give the OH/NH_2 molar ratio of activated PEG hydroxyl groups versus free amino groups of BSA, taking into account that two activated hydroxyl groups are present on each molecule of PEG and 27 accessible free amino groups are available on each molecule of BSA. Aliquots of 100 µL were pipetted onto a plastic dish to obtain a thin disk-shaped gel or onto a cone-shaped platform, and allowed to polymerize. After about 24 h, the gel was polymerized and dried, being easily detached from the dish/platform. The dried gel units were immersed in PBS at 4 °C, and maintained under agitation to remove the p-nitrophenol formed upon polymerization and eventual non-polymerized material. The wash solution was changed frequently (and whenever it became strongly yellow) until the hydrogel became fully translucent (usually within a couple of hours).

Hydrogel swelling was determined as the ratio between the mass increase in the fully hydrated gel and its weight upon vacuum-drying [5]. To evaluate for eventual denaturation of BSA during the hydrogel preparation, they were characterized by circular dichroism. For this purpose, a thin hydrogel was prepared by polymerization in situ between two quartz surfaces. This was required to obtain a small optical pathlength and a corresponding low absorption in the UV compatible with the characterization of the CD absorption spectra. The pathlength was calculated from the protein absorption at 280 nm assuming that all protein is retained in the polymerized gel, thus leading to a concentration of 50 mg/mL in the swollen hydrogel. The stability of the hydrogel at 4 °C, as also evaluated by measuring the amount of protein released into PBS.

Binding experiments. The binding of the (NBD-Cn; n = 4, 6 and 8) to the hydrogel were performed by adding the hydrogel to PBS (pH 7.4) solutions containing the amphiphiles and incubating at 37 °C under agitation. A control without the hydrogel was also performed to evaluate possible losses of the amphiphile from solution, namely due to binding to the apparatus materials. A good stability was observed at the concentrations used, in agreement with previous studies [4]. At specified times, an aliquot of 0.5 mL (out of 10 mL) was taken, replaced by fresh PBS, and the aliquots were analyzed by reverse phase HPLC with fluorescence detection [4]. The binding was studied with two different amounts of hydrogel gel (2 or 4 units with 5 mg of BSA each, corresponding to a BSA concentration equal to 15 or 30 µM). The time dependence of the fraction of amphiphile in the aqueous media was analyzed with Equation (1) to obtain the rate constant for equilibration (k) and the fraction at full equilibration ($f_{\mathrm{NBD_W}(\infty)}$), from which the fraction associated with the hydrogel ($f_{\mathrm{NBD_{Gel}}(\infty)}$) was calculated. The association binding constant (K_a) was then calculated using Equation (2), which assumes that the protein is in large excess relative to the ligand (the concentration of BSA was always at least 30 times higher than that of NBD-C4, and 150 times higher than that of NBD-C8):

$$f_{\mathrm{NBD_W}(t)} = f_{\mathrm{NBD_W}(\infty)} + \left(1 - f_{\mathrm{NBD_W}(\infty)}\right) e^{-k\,t} \quad (1)$$

$$K_a = \frac{f_{\mathrm{NBD_{Gel}}(\infty)}}{f_{\mathrm{NBD_W}(\infty)}[\mathrm{BSA}]_T} \quad (2)$$

3. Results and Discussion

The hydrogel showed good water incorporation capacity, with a swelling factor of 10, corresponding to 91% water in the hydrogel. This behavior is similar to that obtained previously for BSA-PEG hydrogels at high ionic strength [5]. The stability of the hydrogel in PBS at 4 °C was accessed through the quantification of protein leaching from the gel using its fluorescence intensity at $\lambda_{ex}/\lambda_{em}$ 280/340 nm. A small but continuous increase in the amount of BSA in the aqueous medium was observed, with about 2% of the total hydrogel protein being released after 20 days (results not shown). For much longer incubation periods, the hydrogel integrity is compromised and the suspension becomes turbid. For the binding assays, the gels were used within 5 days after preparation, corresponding to less than 1% of the total protein being in the aqueous media.

For the use of the hydrogels as model systems to evaluate protein binding, it is mandatory that the protein retains its tertiary structure. This was evaluated by circular dichroism. It was observed that the shape of the CD spectra of the protein in the hydrogel was exactly the same as for the protein dissolved in PBS (Figure 1A). The small decrease observed in the molar ellipticity mostly reflects the uncertainty in the value considered for the concentration of protein and the optical path of the thin hydrogel film used. This was estimated directly from the absorption of the hydrogel film at 280 nm, assuming that absorption is only due to the protein.

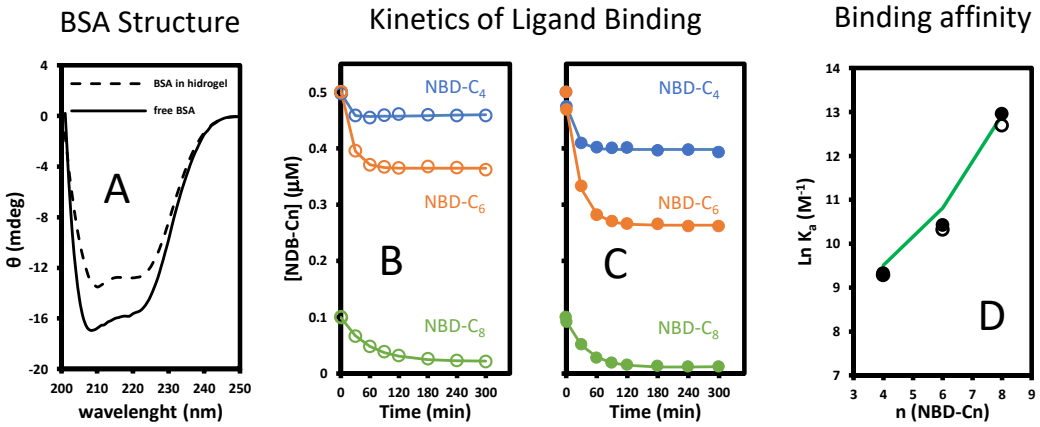

Figure 1. CD spectra of BSA dissolved in PBS pH 7.4 (—) or incorporated in the BSA-PEG hydrogel (—), Plot A. Decrease in the amount of NBD-Cn (C4, C6 and C8) in the aqueous media due to binding to the BSA-PEG hydrogels prepared as thin films, corresponding to a concentration of BSA equal to 2.7 mg/mL (Plot B) or 5.4 mg/mL (Plot C). Effect of the length of the alkyl chain in NBD-Cn on their binding affinity to BSA free in the aqueous media (—), or incorporated in the BSA-PEG hydrogel (○ and ● for the binding experiments with 2 or 4 disks respectively), Plot D.

The results presented in Figure 1A show that the secondary structure of BSA is not affected by its incorporation in the hydrogel, strongly suggesting that its binding properties are also maintained. Additionally, the very high aqueous content of the hydrogel allows for fast diffusion of the ligand through the gel, making this system a promising tool for the easy evaluation of the binding of xenobiotics to BSA. The adequacy of the hydrogels was further evaluated through the characterization of the binding of a homologous series of fluorescent amphiphiles with increasing hydrophobicity (NBD-C_n, n = 4, 6 and 8), which have previously been characterized for their binding affinity to BSA free in solution [4]. The results obtained are shown in Figure 1 (plots B to D).

The first observation from Figure 1B,C is that the magnitude of the decrease in the fraction of amphiphile in the aqueous medium depends on the length of the alkyl chain, being smaller for NBD-C_4 and larger for NBD-C_8. This shows that NBD-C_8 binds to the gel with higher efficiency, in agreement with the relative binding affinities to free BSA [4]. It is also observed that the fraction of amphiphile bound to the gel increases with the amount

of gel in solution (plot C compared to plot B). The experiments were performed with the hydrogels as thin disks or cones. The result obtained show that although a similar behavior is observed at long incubation times, the rate at which the equilibrium is attained is faster for the thin disks (results not shown). This suggests that the rate of equilibration with the hydrogel is dependent on the surface area between the gel and the aqueous medium.

The time dependence of the decrease in the concentration of NBD-Cn in the aqueous media was well described by Equation (1), and the value obtained for fraction at equilibrium was used to calculate the binding affinity to the hydrogel, see Equation (2). The results obtained are shown in Figure 1 plot D. An excellent agreement was obtained between the binding affinity of all NBD-Cn to BSA free in solution and to the BSA-PEG hydrogel. This shows that the properties of BSA as a binding agent are retained, and that PEG does not contribute significantly for the retention of the amphiphiles. In the future, this hydrogel will be applied to study the binding of xenobiotics, namely pesticides, to BSA.

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