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Fluorescence probe techniques to monitor protein adsorption-induced conformation changes on biodegradable polymers

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Abstract

The study of protein adsorption and any associated conformational changes on interaction with biomaterials is of great importance in the area of implants and tissue constructs. This study aimed to evaluate some fluorescent techniques to probe protein conformation on a selection of biodegradable polymers currently under investigation for biomedical applications. Because of the fluorescence emanating from the polymers, the use of monitoring intrinsic protein fluorescence was precluded. A highly solvatochromic fluorescent dye, Nile red, and a well-known protein label, fluorescein isothiocyanate, were employed to study the adsorption of serum albumin to polycaprolactone and to some extent also to two starch-containing polymer blends (SPCL and SEVA-C). A variety of fluorescence techniques, steady state, time resolved, and imaging were employed. Nile red was found to leach from the protein, while fluorescein isothiocyanate proved useful in elucidating a conformational change in the protein and the observation of protein aggregates adsorbed to the polymer surface. These effects were seen by making use of the phenomenon of energy migration between the fluorescent tags to monitor interprobe distance and the use of fluorescence lifetime imaging to ascertain the surface packing of the protein on polymer.

Keywords: Fluorescence; Anisotropy; Time resolved; Albumin; Protein conformation; Fluorescence lifetime imaging; Synchronous scan; Tryptophan; Nile red; Fluorescein isothiocyanate

1. Introduction

An implant will encounter proteins when inserted into the human body [1,2] and, under certain circumstances, adsorption to the implant surface can induce protein conformation changes [3,4]. The process of protein adsorption and subsequent conformation changes might affect the host response to any implant [5–7]. This has led to a vast body of research being done on materials that are ultimately aimed at finding biocompatible materials for implantation. Some of these works have used fluorescence to study changes in protein conformation on adsorption [8–18], although to our knowledge, little has been reported regarding the fluorescence of proteins adsorbed to biodegradable polymers (including those in the present study) that are intended to be used in implants or tissue engineering constructs.

Some protein adsorption studies have used the intrinsic fluorescence of proteins, mainly that emanating from the amino acid tryptophan [9,11,19–21]. The advantage with the fluorescence of tryptophan is that it is very sensitive to the polarity of the local environment in that the emission peak will shift depending on the dielectric constant of the local environment. Thus, if the conformation of a protein changes, as to modify the local environment around the tryptophan, this will be reported by a change in the position of the fluorescence peak.

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Although the use of intrinsic fluorescence can be advantageous, as no addition to the protein is required that may perturb its conformation, it can be complex to resolve if the background fluorescence is high or if there is more than one fluorophore in the chosen protein. The use of a suitable extrinsic probe molecule offers enhanced specificity, as it can be chosen to absorb and emit in a specified wavelength range and with known fluorescence characteristics, allowing environmental changes to be monitored.

The well-studied fluorescence probe Nile red (NR) is highly sensitive to the polarity of its local environment [22–24] and absorbs and emits in a wavelength region longer than that of intrinsically fluorescent amino acids. Its usage in conjunction with proteins can therefore prove beneficial when studying their adsorption to polymer constructs, which fluoresce in a similar wavelength range to the intrinsic protein fluorophores. As Nile red is only attached with hydrophobic interactions, it cannot be guaranteed to remain inside the core of a protein even if it is predominately hydrophobic. Opting for a covalently bonded probe could be a way around this obstacle. A common covalently bound probe absorbing in the same wavelength region is the fluorescein derivative, fluorescein isothiocyanate (FITC). However, it lacks the highly solvatochromic response of Nile red and its fluorescence emission spectrum is sensitive to pH [25] (reducing drastically in acid conditions). This probe exhibits a sizable overlap between its absorption and emission spectra, a fact that can be made use of to monitor interprotein distances via energy migration [26–30]. Thus, deliberately tuning the labelling ratio of a protein could provide information about the packing and/or conformation of adsorbed proteins.

Proteins adsorb as a thin layer to the construct surface and thus, even a small amount of background fluorescence can prove to be significant in relation to the fluorescence originating from the (labelled) protein. In the situation where the background signal is as strong and overlaps with the probe fluorescence it is possible to opt for synchronous scan spectroscopy, in which the excitation and emission monochromators are incremented simultaneously at a constant wavelength difference, to aid the resolution of the different fluorescing species [31]. With a good choice of offset between the monochromators it is often possible to eliminate the background signal. The interpretation of the shape and position of the peaks is not so straightforward and this form of fluorescence spectroscopy often is used to obtain a fingerprint of a mixture of fluorophores [32,33].

The current study was aimed at evaluating the use of Nile red and covalently bound FITC, in order to ascertain their ability to detect protein conformation changes on some biodegradable polymers, which are currently evaluated for use in biomedical applications. The polymers chosen were polycaprolactone (PCL), and to some extent starch–PCL (SPCL) and starch–ethylene vinyl alcohol (SEVA-C), all of which were found to be fluorescent themselves. To resolve their fluorescence from that of the labelled protein (bovine and human serum albumins were used in this study) time-resolved and steady-state fluorescence methods were employed as well as fluorescence imaging techniques.

2. Materials and methods

Polycaprolactone (starch–PCL (50:50 wt%) and starch–ethylene vinyl alcohol (50:50 wt%, SEVA-C) were injection-moulded in-house into 1 cm diameter disks and were sterilised with ethylene oxide (Pronefro, Portugal). For intrinsic tryptophan fluorescence measurements 1 mg/ml bovine serum albumin (BSA, Fraction V, 96%, Sigma) was used. For measurement with Nile red (Sigma) essentially immunoglobulin and fatty acid-free (to avoid Nile red–fatty acid aggregation) human serum albumin (HSA, Sigma) was used. The Nile red was first dissolved at 1 mM in DMSO (Sigma) in a NR:HSA ratio not higher than 1:5. In addition, BSA was labelled with fluorescein isothiocyanate (Sigma, mixed isomers) in 0.1 M carbonate buffer at pH 9 and room temperature in the dark for 4 h. The molar FITC–BSA ratio (F/P) ranged from one decade below to one decade above unity. The FITC–BSA solutions were dialysed with cellulose membranes (Sigma, cutoff 12 kDa) against 1 L of 0.01 M phosphate buffered saline at pH 7.4 (PBS) four times for 1 day each in the dark at 8 °C. Light absorption at 495 nm was finally below 0.003 for the supernatant. The dialysis membranes were washed in distilled water for at least 4 h with extensive rinsing once each hour. The concentration and FITC–BSA (F/P) ratio was determined according to the methods described by the manufacturer. Albumin absorption was at 280 nm, 0.66 mg/ml, and ratio of FITC absorption at 280 nm versus 495 nm, 0.35. Background values at 600 nm were subtracted prior to the ratio and concentration calculations.

The disks were immersed in PBS for a few minutes and then a solution with the (labelled) albumin was added to final concentration around 0.1 mg/ml at room temperature and if not otherwise stated incubated for 1 h. These were then rinsed with distilled water with flow toward the forceps and directly transferred (with a drop of water on the side to be measured to avoid side effects of drying the adsorbed proteins) to the measurement cuvettes already with PBS buffer in order to avoid signal from labelled BSA that would otherwise have adsorbed to the cuvette prior to fluorescence measurements in PBS at pH 7.4 and room temperature, unless stated otherwise.

Spectra were recorded using a Shimadzu UV-3101PC or UV-1601 spectrophotometer for light absorption and a SPEX Fluorolog spectrophotometer for a number of fluorescence modes, namely; emission, excitation, synchronous scanning, and steady-state anisotropy. For time-resolved measurements a single-photon counting apparatus equipped with a NanoLED excitation source (HORIBA, Jobin Yvon, excitation at 490 nm) was used. The fluorescence decays, selected using a 550 nm cutoff filter were analysed by using a sum of exponentials (IBH DAS6 software). The distance between FITC molecules was calculated according to the Förster [29,30] approach using data from the time-resolved measurements using an unquenched lifetime of 4.05 ns [26].

Fluorescence lifetime imaging (FLIM) [34,35] was used to detect the distribution of adsorbed proteins and was performed using an inverted confocal microscope (Leica TCS SP2) with excitation from a pulsed diode laser (Hamamatsu LP-10 470) with an optical pulse width 90 ps and wavelength 467 nm

using a 20× objective, NA = 0.5. The fluorescence was detected through a (525 ± 25) nm interference filter using a cooled PMC100-01 detector (Becker & Hickl, based on a Hamamatsu H5772P-01 photomultiplier), with the data collected with a Becker & Hickl SPC 830 card using 64 time channels in a 3 GHz, Pentium IV, 1 GB RAM computer running Windows XP. The FLIM images were acquired for 1200 s with an average fluorescence count rate below 10k cts/s. SPCImage software 2.8 (Becker & Hickl) based on a Levenberg–Marquardt fitting algorithm was used to fit a monoexponential decay to the fluorescence decay curve in each pixel of the image. A false colour scale was assigned to each fluorescence lifetime value (blue for a short lifetime and red for a long lifetime), yielding FLIM maps.

3. Results and discussion

3.1. Nile red labelling

As shown in Fig. 1, excitation at 295 nm (used to excite tryptophan and not the other fluorescent amino acids) for BSA adsorbed to the polymers caused the polymers to fluoresce to such a degree that it precluded the resolution of the low level of intrinsic protein fluorescence. However, relatively weak fluorescence was found for PCL when it was excited at 490 nm; thus, it was decided to choose the dye Nile red (solvatochromatic emission) with an absorption peak at this wavelength. Fig. 2a shows the fluorescence spectra of NR-HSA in solution and when adsorbed onto PCL, SPCL, and SEVA-C. There is a distinct change in peak position between NR-HSA in solution and when adsorbed to all of the polymers, with little difference noted between PCL and SPCL. The SEVA-C displayed a double peak, one of which is due to the emission of the SEVA-C itself. Synchronous scan fluorescence spectra were taken in an attempt to resolve the NR emission from any polymer fluorescence and discriminate between NR-HSA adsorbed to PCL and SPCL (Fig. 2b). Using this technique the SEVA-C contribution to the spectra was eliminated, but no obvious differences between NR-HSA on PCL and SPCL were seen. However, it should be noted, that normal and synchronous scan spectra of NR itself adsorbed to these polymers (data not shown) gave exactly the same spectra. Decomposition of the spectra into a sum of Gaussian component spectra [36] allowed the change in dielectric constant experienced by the NR to be estimated [23]; in the cases of PCL and SPCL it was halved (from ca. 4 to ca. 2), with only a slight decrease observed in the case of SEVA-C.

Time-resolved fluorescence measurements (data given in Table 1) also exhibited similarities between the adsorbed NR-HSA and NR directly adsorbed to PCL. The data for NR-HSA in solution are comparable to those previously reported [37,38], but the shorter decay time has earlier been shown to decrease on NR-BSA adsorption to quartz [38]. In the presence of PCL the subnanosecond component apparently lengthens and its relative amount reduces. Apart from a trace of a longer lived component, within error, the data with and without HSA in the presence of the polymer seem equal. This can be indicative of the fact that the NR has leached from the HSA and transferred

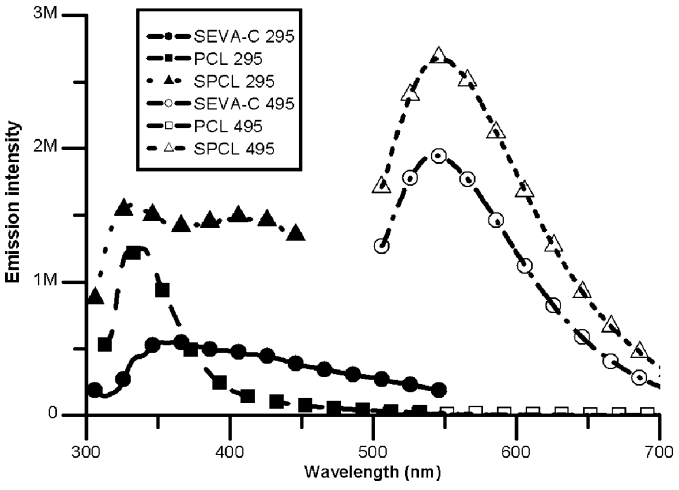


Fig. 1. Fluorescence emission spectra of disks of SEVA-C, PCL, and SPCL immersed in PBS and excited at 295 and 495 nm, respectively.

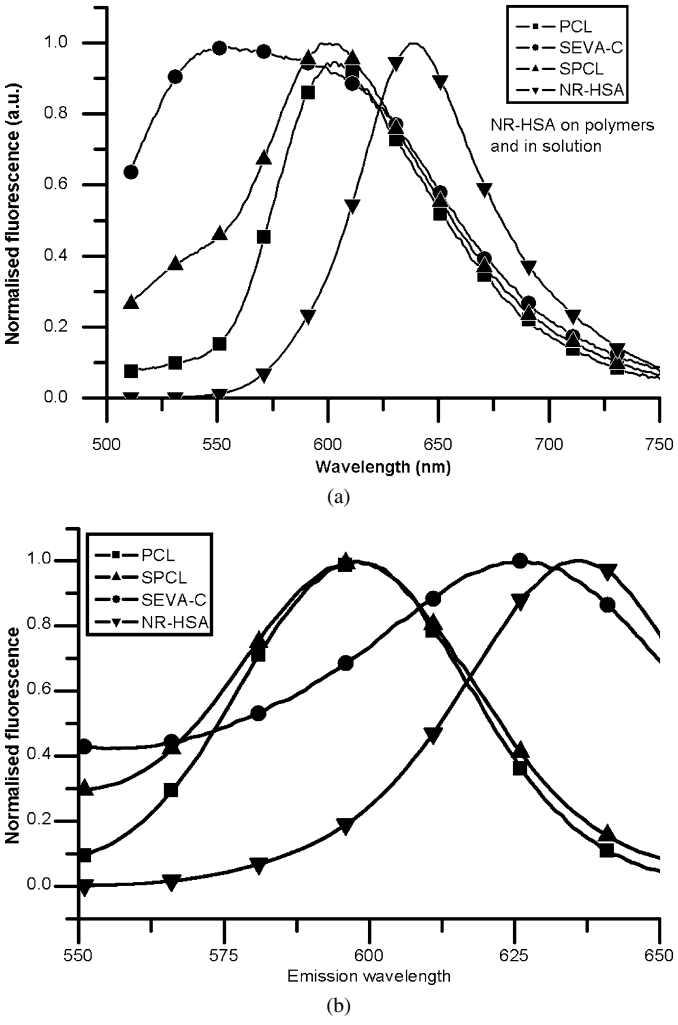


Fig. 2. (a) Normalised fluorescence emission spectra of NR-HSA in solution and adsorbed to PCL, SPCL, and SEVA-C. (b) Normalised synchronous scan (offset 50 nm) fluorescence emission spectra of NR-HSA in solution and adsorbed to PCL, SPCL, and SEVA-C.

Table 1
Fluorescence decay data for Nile red in HSA, both in solution and adsorbed to PCL plus Nile red adsorbed to PCL

System	Time-resolved parameters						
	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	α_1	α_2	α_3	χ^2
NR-HSA	0.84	4.39	6.78	0.13	0.83	0.04	1.08
NR-PCL[a]	2.10	4.05		0.07	0.93		1.13
NR-HSA PCL	2.05	3.95	19.6	0.08	0.92	t[b]	1.14

NR was excited at 490 nm and the emission was selected using a 550-nm cutoff filter.
[a] Dry, no buffer.
[b] t, trace

into the polymers. As the NR is hydrophobic the leaching most likely occurred from protein adsorbed to the polymers, thus the NR gives a "footprint" of protein adsorption. This may be useful in giving an indirect measure of protein distribution, but fails in the aim of assessing changes in protein conformation, although the fact that leaching has happened could indicate that a conformational change during or after adsorption took place.

3.2. FITC labelling

To avoid the possibility of the fluorescent probe leaving the protein, the covalently coupled probe FITC, excited at nearly the same wavelength as NR, was chosen. FITC is not very sensitive to environmental polarity in terms of changes in peak wavelength position [39]. However, making use of fluorescence anisotropy and its proven sensitivity to energy migration, i.e. distance dependent nonradiative energy transfer due to the large overlap of its absorption and emission spectrum [26], might be useful for studying protein packing and conformational changes on adsorption. This would be a function of the labelling ratio, as this has influence on the degree of energy migration by altering the average separation between FITC tags.

The fluorescence spectra of FITC conjugated to BSA at different F/P ratios and adsorbed to PCL are given in Fig. 3a. In the main features of the spectra, the peak position and its ratio to a shoulder manifest close to 570 nm, two distinct trends are seen depending on whether the protein contains on average more (overlabelled) or less (underlabelled) than one FITC label. In the underlabelled samples the relative intensity of the shoulder in relation to the main peak is greater than that for the overlabelled samples and the peak is at longer wavelengths (Fig. 3b). Fig. 3c shows a comparison of the peak emission wavelength compared with that seen for FITC–BSA in solution [26]. This wavelength shift in the underlabelled samples is unlikely to be caused by any underlying PCL fluorescence as the addition of this should produce a shift to shorter wavelengths and could be indicative of FITC–FITC interactions. The observation of a shoulder has previously been linked to dimer formation [40–43]. If indeed these effects are related to interactions between different FITC labels, then because of the labelling ratio (on average less than one per each protein) the origin can only be explained by protein aggregation. Considering the overlabelled samples, intraprotein FITC–FITC interactions probably dominate, which is why the deviation from the solution data is not so

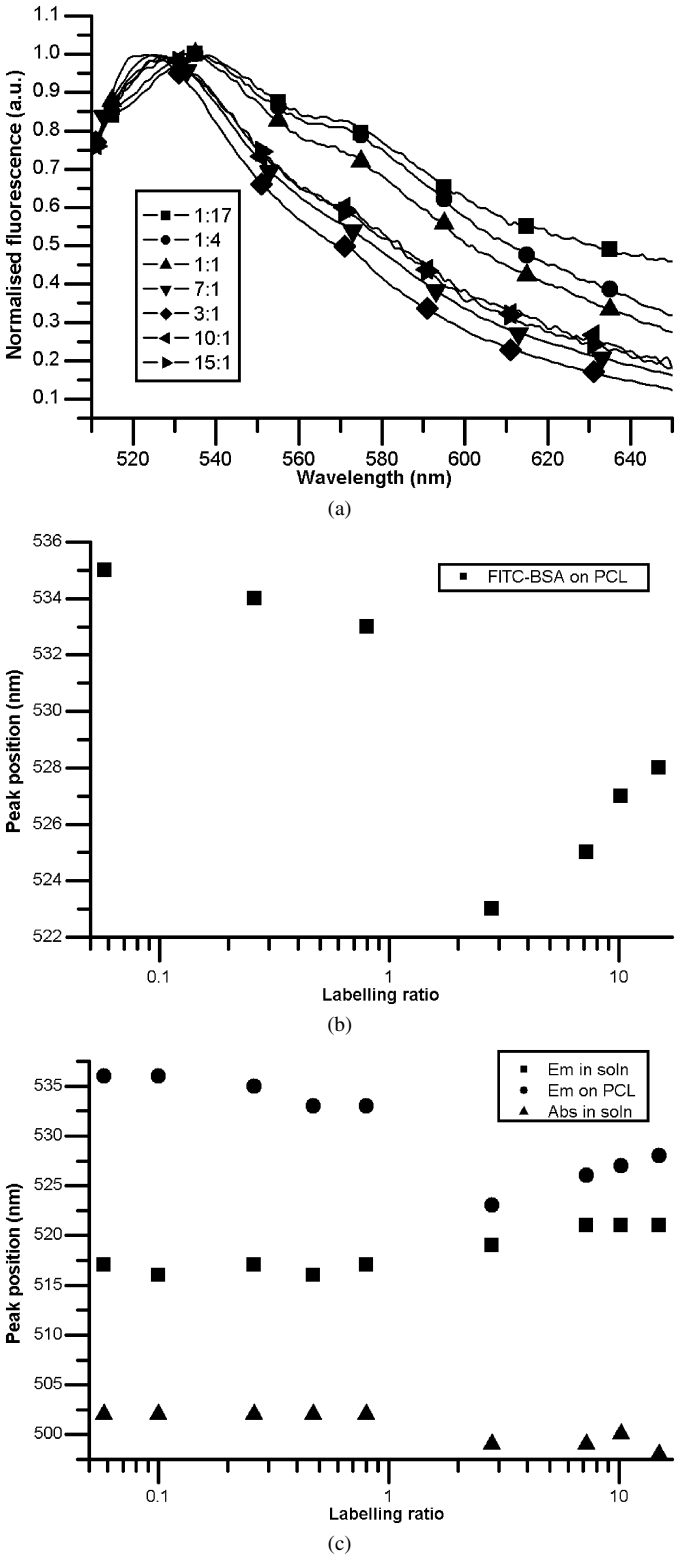
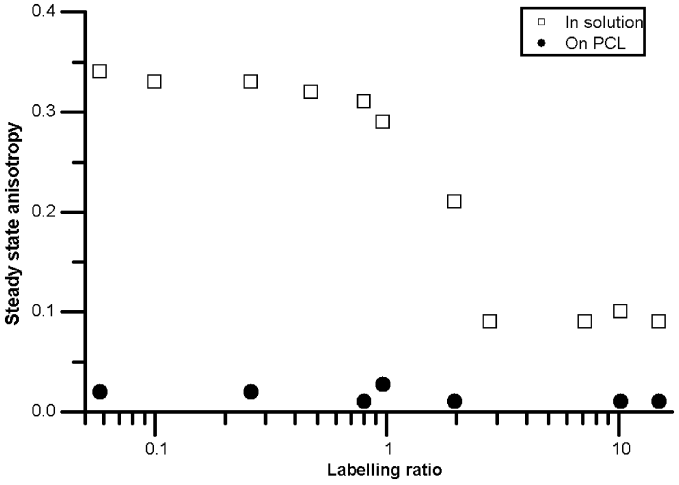
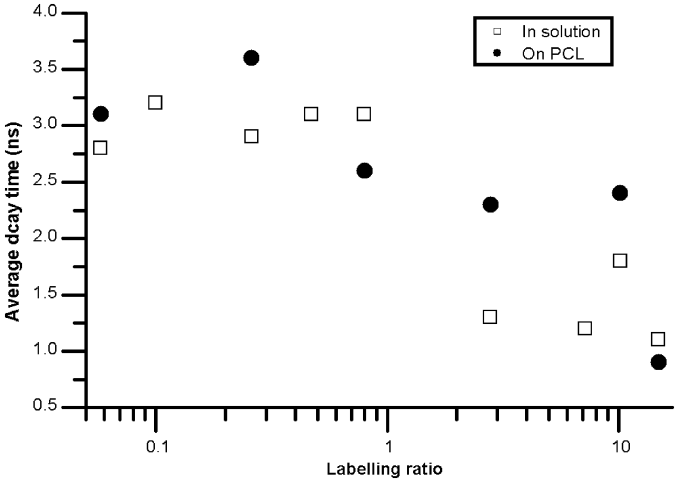


Fig. 3. (a) Normalised fluorescence of FITC–BSA adsorbed to PCL. (b) Ratio of fluorescence at (peak + 40 nm)/peak for FITC–BSA adsorbed to PCL versus labelling ratio. (c) Peak positions of emission in solution and on PCL and absorption in solution of FITC–BSA as a function of labelling ratio.

enhanced. However, a difference and increasing departure from the solution data are seen with increasing labelling ratio. These trends are mirrored in the main peak to shoulder ratio (Fig. 3b).



(a)



(b)

Fig. 4. (a) Steady-state anisotropy versus labelling ratio for FITC–BSA in solution and on PCL. (b) Average decay time versus labelling ratio for FITC–BSA on solution and on PCL.

This behaviour can relate to competition between inter- and intraprotein FITC interactions.

The steady state anisotropy of FITC–BSA in solution is near 0.35 (see Fig. 4a) for the underlabelled FITC–BSA and rapidly goes down to 0.1 for F/P above unity [26]. The higher value is close to that previously found for FITC-labelled proteins [44,45] and the S-type curve itself has also been observed before, although without explanation [45]. The anisotropy of PCL itself was found to be practically zero (0.007). When FITC–BSA is adsorbed to PCL, the anisotropy of the label drops to below 0.02 (Fig. 4a) and no trend with labelling ratio is observed, thus showing a constant high degree of fluorescence depolarisation. This is further evidence for an energy migration process, as it is unlikely that the FITC tag will exhibit total rotational freedom. One might argue that the relatively small volume of protein to polymer would affect this value, although that is contradicted in a study in which pyrene–BSA adsorbed to polyethylene and polyvinyl alcohol gave nonzero anisotropy [46]. It should be kept in mind that the critical Förster distance is close to 50 Å for FITC–FITC resonance energy transfer [47–50] and it has been

Table 2a
Fluorescence lifetime data for FITC–BSA, at different labelling ratios, in solution

FITC: BSA	Time-resolved parameters							
	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_{ave} (ns)	α_1	α_2	α_3	χ^2
1:17	4.01	0.69		2.8	0.63	0.37		1.14
1:10	4.04	0.91		3.2	0.73	0.27		1.08
1:4	4.02	0.74		2.9	0.66	0.34		1.06
1:2	4.01	0.8		3.1	0.7	0.3		1.16
1:1	3.98	1		3.1	0.72	0.28		1.05
3:1	3.61	1.85	0.48	1.3	0.14	0.25	0.61	0.99
7:1	3.59	1.75	0.45	1.2	0.13	0.22	0.65	1.03
10:1	3.8	1.83	0.45	1.8	0.29	0.24	0.47	1.09
15:1	3.53	1.52	0.36	1.1	0.15	0.22	0.63	1.13
FITC	4.05			4.1	1			1.10

Table 2b
Fluorescence lifetime data for FITC–BSA, at different labelling ratios, adsorbed to PCL

FITC: BSA	Time-resolved parameters							
	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_{ave} (ns)	α_1	α_2	α_3	χ^2
1:17	4.67	1.5	32	3.1	0.41	0.58	0.01	1.10
1:4	5.54	2.12	41	3.6	0.33	0.66	0.01	1.12
1:1	4.12	1.14	22	2.6	0.12	0.57	0.01	1.19
3:1	4.42	1.35	25.2	2.3	0.26	0.73	0.01	1.11
10:1	3.74	1.16	17	2.4	0.43	0.56	0.01	1.10
15:1	3.59	1.58	0.24	0.9	0.11	0.22	0.67	1.10
PCL	4.87	1.77	20.1	3.5	0.44	0.54	0.02	1.13

proposed that efficient resonance energy transfer takes place at a acceptor density of 0.1 per R_0^2 [51]. With the "diameter" of BSA in the order of 80 Å [52] and assuming that BSA is lying down, close packed, on the surface, then that limit would be reached for F/P = ca. 1:4 and higher. That is, one would expect a detectable interprotein interaction of FITC for all the overlabelled FITC–BSA.

The time-resolved fluorescence data for FITC–BSA in solution yield, on average, a fluorescence decay time that decreases when the labelling ratio exceeds unity (Fig. 4b). There only appears to be a weak trend, when comparing the average decay time for FITC–BSA in solution and when adsorbed to PCL (see Supplementary material), with slightly longer decay times observed for the adsorbed proteins. There is only one decay time for uncoupled FITC in solution (4.05 ns, Table 2a) in agreement with earlier results [25,44] and in earlier studies of FITC-labelled proteins it was assumed that two different locations of FITC gave rise to the two major decay times. These were FITC on the outer rim of the protein and for FITC inside the protein [44,53]. Lewitzki et al. set these to 4 and 1 ns, respectively [53], which are close to the values that we have observed for underlabelled BSA in this study. When FITC is attached to BSA either in solution or adsorbed to PCL 2 or 3 decay times are needed to obtain a good fit with the time-resolved data (Tables 2a and 2b), also in agreement with earlier studies [53–57]. For underlabelled FITC–BSA in solution the major decay component is similar to that of uncoupled FITC in solution, but appears to decrease slightly with increasing the number of tags per pro-

tein. This could relate to the increased possibility of the tag binding to a site favourable for quenching FITC emission, as has been noted before for various amino acids (mainly tryptophan) with FITC in solution [54] or FITC-labelled protein [58]. For overlabelled FITC–BSA in solution three time components are needed for a good fit and the main component decays with a subnanosecond (0.5 ns) lifetime. Previous work labelling different sized silica spheres with FITC has used a decrease in the average lifetime, attributed to energy transfer, to measure inter molecular distance between the FITC labels [30]. The results that we obtain are consistent with this idea, but with the energy transfer occurring between different FITC molecules in the same BSA molecule. For all FITC–BSA adsorbed to PCL three decay time components are needed, although the longer lived component probably is due to the PCL itself and is very weak. The second time component (1.1 to 2.1 ns) shows no obvious trend relating to the labelling ratio. It is the predominant component in all cases, except for the highest overlabelled (15:1) FITC–BSA conjugate. This exhibits a short-lived (0.24 ns) major component.

By making use of synchronous scan fluorescence spectroscopy it was possible to resolve the emissions from PCL and FITC–BSA (Fig. 5a). With a Stokes' shift of ca. 25 nm an equal offset is expected to give a high signal for the FITC. Fig. 5a shows that using this offset the presence of adsorbed conjugate is clearly ascertained, although emission from PCL itself is still detectable. With a larger offset of 75 nm the spectrum of FITC–BSA itself looks quite different compared to 25 nm offset. However the relative signal from adsorbed FITC–BSA is now much lower (Fig. 5b).

3.3. Assessment of protein conformation and packing on PCL

In an attempt to quantify the results of the fluorescence measurements the energy transfer efficiency using the Förster model [29,30] was calculated from the time-resolved data. This employs an admittedly simplistic approach of using the average lifetime and taking the underlabelled samples in solution for unquenched reference data. By this method and making use of previous work [26] differences between the average FITC distance attached to BSA in solution and adsorbed to PCL can be seen, as illustrated in Fig. 6. In this figure it is apparent that the average distance between FITC tags is larger in the adsorbed protein than in solution, with the exception of the highly overlabelled sample (15:1). In this case the values are similar. These results can be indicative of a slight unravelling of the protein structure on adsorption to the polymer.

To further elucidate the effect of interaction between probes on different proteins a means of visualising the packing of the proteins would be helpful, since it is known that albumin can aggregate [16,17,59,60] and to ascertain if protein aggregates are indeed formed. To this end fluorescence lifetime imaging was performed making use of confocal microscopy. The images obtained are shown in Fig. 7 for 1:1 FITC–BSA on PCL. This ratio was chosen so that on average every BSA molecule should contain a probe. Naked PCL (data not shown) gave a uniform fluorescence intensity. When the PCL was incubated

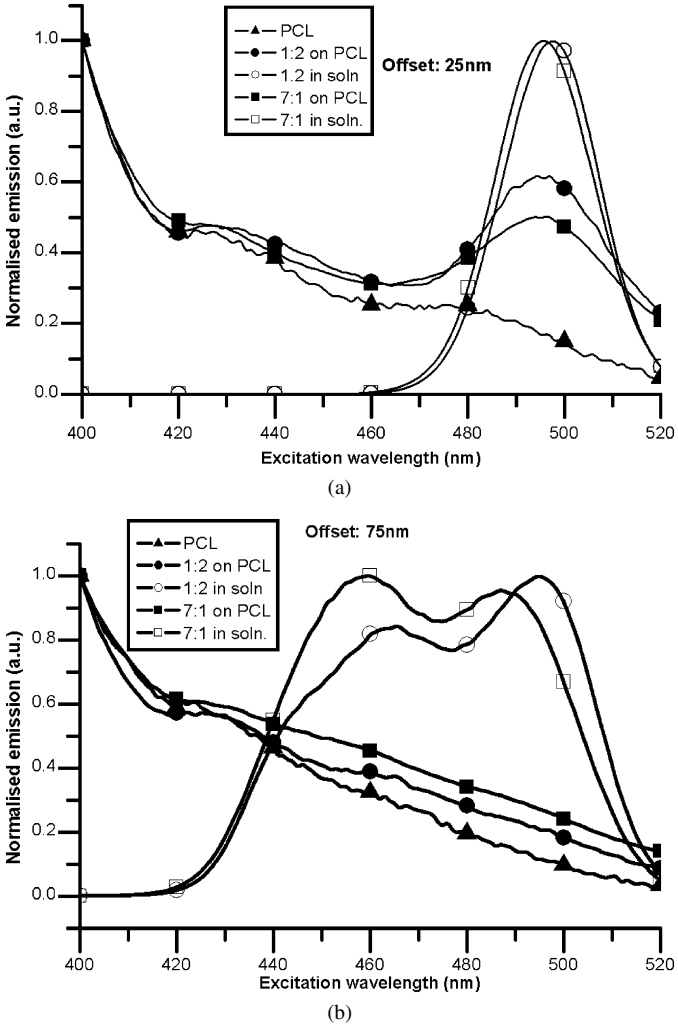


Fig. 5. (a) Normalised emission of synchronous scan at offset 25 nm of PCL and FITC–BSA in solution and adsorbed. Results from labelling ratio 1:2 and 7:1 and PCL alone are shown. (b) Normalised emission of synchronous scan at offset 75 nm of PCL and FITC–BSA in solution and adsorbed. Results from labelling ratio 1:2 and 7:1 and PCL alone are shown.

with the FITC containing BSA, a nonuniform distribution of protein aggregates or clusters was observed, as indicated by the bright spots on a dark background (Fig. 7a) that would confirm that if there are probes on the outer rim of the proteins they potentially can interact, i.e., quench each other. There is a good correlation between the fluorescence obtained from the intensity image (Fig. 7a) and the shorter lived decays (Fig. 7b). It should be kept in mind that the decay time of FITC alone in PBS buffer is close to 4 ns and for F/P 1:1 has two components 4 and 1 ns in FITC–BSA [26]. Note that the average fluorescence decay times obtained by FLIM would correspond more to the average lifetimes obtained via conventional single-photon counting and it is reasonable to compare with the data presented in Fig. 4b. Some representative decays from the protein clusters and the PCL (see Supplementary material) show that the fluorescence from the protein clusters decays faster than that of the PCL. FLIM is valuable in obtaining contrast between areas with and without adsorbed protein and in providing, through

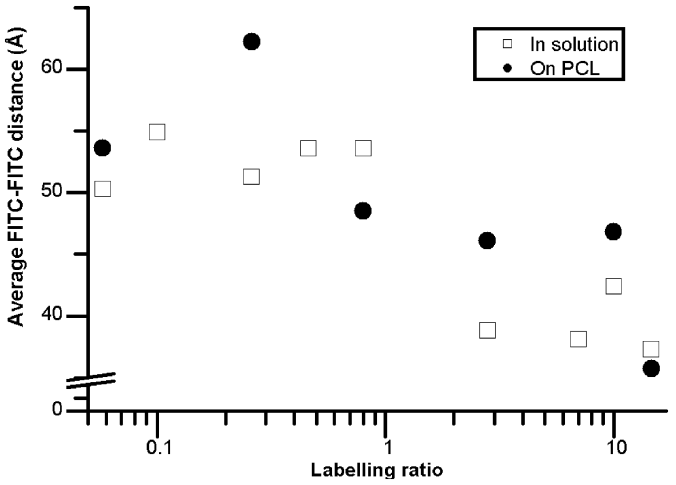
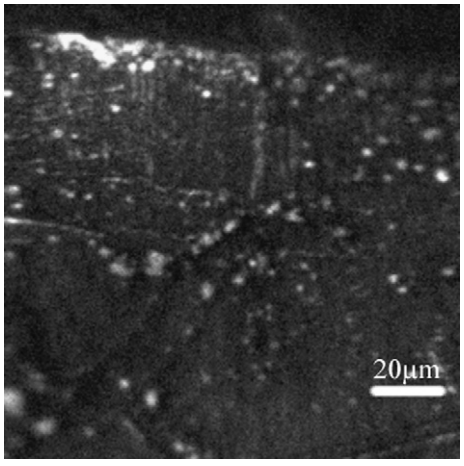
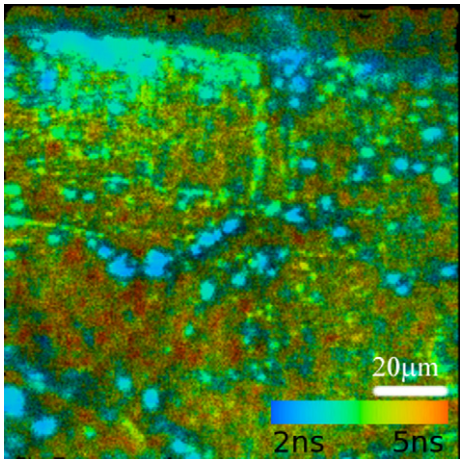


Fig. 6. Average FITC–FITC distance on FITC–BSA with labelling ratio above unity in solution and adsorbed to PCL.



(a)



(b)

Fig. 7. (a) Intensity image of PCL incubated for approximately 2 h in FITC–BSA (1:1) solution. (b) FLIM image of the same area as in panel (a). The FITC–BSA lifetime is clearly shorter than the one of PCL.

the average lifetime value, an indication of any interaction between fluorescence probes. As areas with average decay times close to 2 ns are seen this may help support evidence for inter-protein probe interactions, which are present in the steady-state data. This goes well in hand with previous findings that aggregates adsorb to a larger degree to latex than monomers of albumin [16]. Atomic force microscopy images of the surface topography of naked PCL (see Supplementary material) gave no obvious connection between image topography and cluster formation.

As always when using probes for detecting changes in interaction with other proteins and interfaces the introduction of probes (in this case Nile red and fluorescein) might by virtue of their respective size and hydrophobicity affect the conformation of the labelled proteins. This could make labelled proteins behave in a different manor to unlabelled ones. In our case, especially for the overlabelled FITC–BSA, this could be a source of misinterpretation, making it difficult to translate the results to native proteins. However, the labels are relatively small in comparison with the protein (molecular weight ca. 400 Da for FITC compared to ca. 65000 for BSA), thus making the impact of each FITC negligible. But for the overlabelled FITC–BSA the sheer number of extra hydrophobic points could probably increase the propensity of the overlabelled proteins to adsorb, especially to hydrophobic surfaces, in comparison with unlabelled proteins. Notably, the results show that a labelling ratio of much above 3:1 does not convey extra useful information from the fluorescence behaviour, so that the problem with probes can be minimised by choosing a ratio in the range 2 to 3 per protein.

4. Conclusion

Since the polymers studied exhibit sufficient fluorescence to mask that from the fluorescent amino acids of a thin layer of protein, the use of intrinsic protein fluorescence was precluded for studying protein adsorption. The experiments with Nile red indicated that the probe leaked out from the protein and thus was only suitable for providing a “footprint” of protein adsorption, although an estimate of polymer polarity could be made. This gave a rationale for using a covalently linked probe and it should be noted that because the peak emission wavelength depends on the chosen polymer a range of labels might be needed when comparing an array of biodegradable polymers.

The covalently bound tag, FITC, employed at different labelling ratios and used in conjunction with a variety of fluorescence techniques has proved useful in elucidating the presence of adsorbed proteins and the possibility that conformational changes and aggregation of BSA occurred on adsorption to PCL disks. The exploitation of different labelling ratios proved advantageous in relation to these aspects, allowing the usage of the interaction between FITC molecules to elucidate differences between FITC–BSA in solution and adsorbed to PCL. FLIM measurements also helped to ascertain the presence of protein aggregates or clusters adsorbed on the PCL. The combination of these techniques, with the possibility of using other linked fluorophores, should allow for the interaction of proteins a range of different (fluorescent) biomaterials to be studied.

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Supplementary material

The online version of this article contains additional supplementary material.

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