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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