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A fluorescent spectroscopy and modelling analysis of anti-heparanase aptamers–serum protein interactions

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ABSTRACT

Aptamers are short, single stranded oligonucleotide or peptide molecules that bind a specific target molecule and can be used for the delivery of therapeutic agents and/or for imaging and clinical diagnosis. Several works have been developed aiming at the production of aptamers and the study of their applications, but few results have been reported on plasmatic dynamics of such products. Aptamers against the heparanase enzyme have been previously described. In this work, the interactions of two constructs of the most promising anti-heparanase aptamer (molecular weights about 9200 Da and 22000 Da) to human and bovine serum albumins were studied by fluorescence quenching technique. Stern–Volmer graphs were plotted and quenching constants were estimated. Stern–Volmer plots obtained from experiments carried out at 25 °C and 37 °C showed that the quenching of fluorescence of HSA and BSA by the low molecular weight aptamer was a collisional phenomenon (estimated Stern–Volmer constant: 3.22 (±0.01) × 10^5 M^{-1} for HSA at 37 °C and 2.47 (±0.01) × 10^5 M^{-1} for HSA at 25 °C), while the high molecular weight aptamer quenched albumins by static process (estimated Stern–Volmer constant: 4.05 (±0.01) × 10^5 M^{-1} for HSA at 37 °C and 6.20 (±0.01) × 10^5 M^{-1} for HSA at 25 °C), interacting with those proteins constituting complexes. Linear Stern–Volmer plot from HSA titrated with the low MW aptamer suggested the existence of a single binding site for the quencher in this albumin. Differently, for aptamer 2, the slightly downward curvature of the Stern–Volmer plot of the titration for that albumin suggested a possible conformational change that led to the exposition of lower affinity binding sites in HSA at 25 °C. Similarly, although short aptamer does not appear to form a stable complex (collisional interaction), the longer aptamer is found to form a stable complex with HSA. In addition, the behaviour of quenching curves for HSA and BSA and values estimated for ratio R_1/R_2 from model developed by Silva et al. suggest that the primary binding site in both aptamers is located closer to the tryptophan residue in sub domain IIA. It is likely that both aptamers are competing for the same primary site in albumin.

1. Introduction

The enzyme heparanase is a b-1,4-endoglycosidase compound that plays important role in extracellular matrix (ECM) degradation and remodelling. The nascent polypeptide is a 543 amino acid pre-proenzyme which, after removal of the signal peptide sequence in the endoplasmic reticulum, undergoes proteolytic processing in late endosomes/lysosomes by cathepsin-L like proteases at sites Glu109-Ser110 and Gln157-Lys158, yielding an N-terminal 8 kDa polypeptide, a C-terminal 50 kDa polypeptide and between them; a 6 kDa linker polypeptide [1,2]. The 50 and 8 kDa polypeptides associate to form a heterodimeric active enzyme, whilst the 6 kDa linker is excised and degraded [3]. The enzyme is secreted by activated CD4 + T cells [4–6], platelets [3], neutrophils and metastatic cells [7] and its activity is associated with activated leukocytes, mast cells, placental tissue and macrophages.

Upon specific conditions, as we can find when metastic tumour cells secretes heparanase, the enzyme hydrolyses the glycosidic bonds of heparan sulphate chains attached to proteoglycans to products of 10–20 sugar units in length [8], leading to penetration of the endothelial cells of blood vessels and target organs by the tumour cell. Liberation of bound cytokines and growth factors sequestered by heparan sulphate chains in tissues [9] further facilitates growth of the tumour and promotes angiogenesis and proliferation of secondary tumours [10]. The heparanase expression levels in tumour cells correlate with their metastatic potential. Elevated levels of heparanase mRNA and protein have been found in

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cancer patients who show significantly shorter postoperative survival times than patients whose heparanase levels are normal [10,11]. In addition to its function in cancer progression, heparanase enzyme also plays a major role in the activity of inflammatory cells. The enzyme has been detected in a variety of immune cells including T and B cells, macrophages, neutrophils and mast cells. It has been shown to mediate extravasation through the endothelial barrier via the remodelling of ECM heparan sulphate, which then allows trafficking to the sites of inflammation [7,12,13]. Expression has been linked to tumorigenesis in a number of different cancers, for example, acute myeloid leukaemia [14], bladder, brain [15], breast [16], colon [17], gastric [18], oesophageal [19], oral [20], and pancreatic [21], suggesting that it may be a suitable target for drug therapy. Currently available inhibitors of heparanase include neutralising antibodies [22], peptides [23] and small molecules [24] as well as heparin [25] and sulphated oligosaccharide mimics of heparan sulphate [26,27]. Aptamers are short DNA or RNA oligonucleotides developed for diagnostic and therapeutic use that display high binding affinity and specificity for target molecules [28–30]. The affinity of aptamers has been compared with that of antibodies (i.e. in the nanomolar range), but as aptamers are smaller (8–25 kDa compared to 150 kDa), they can both penetrate tissues and be cleared from the plasma within minutes of intravenous administration, without triggering an immune response, which can be useful when using them as diagnostic agents [31]. For therapeutic use they are able to retain their function and binding characteristics upon modification with other moieties to improve their stability and solubility, whilst reducing their toxicity and plasma clearance [32–37].

Typically, aptamers are from 22 to 100 bases in length, and contain a region of variable sequence, flanked by known primer ligation sequences, which are used for amplification and identification purposes. Aptamers have been previously selected against the heparanase enzyme and have been shown to bind with high affinity and selectivity and have the ability to inhibit its enzymatic function and prohibit tissue invasion [1]. In this study, these aptamers have been used to characterize their potential interaction with sera proteins as a means of identifying their binding mechanisms, potential bioavailability and predict the drug administration protocols necessary.

It is known that the balance between free and bound-to-protein concentrations of a substance in plasma influences directly its biodistribution, which gives to albumin an important role on the therapeutic action of any drug [38–41]. Albumin represents 52–60% of the total plasmatic protein content and transports endogenous ligands as well as xenobiotics mostly through the formation of non-covalent complexes at specific binding sites, actuating in the regulation of their plasmatic concentrations [42–44]. From this perspective, the study of the interaction of aptamers with albumin is particularly important, especially as there are very few studies on the bioavailability of aptamers in circulation.

Quenching measurement of albumin fluorescence is an important method for the study of interactions between several substances and this protein [45–47]. It can reveal the accessibility of the quenchers to albumin's fluorophore groups, help understand albumin's binding mechanisms to drugs, and provide clues about the nature of the binding phenomenon. Intrinsic fluorescence of proteins, emitted when the protein is excited by ultraviolet light at a wavelength around 290 nm, is mainly due to the presence of tryptophan residues. Another frequent fluorescent amino acid in proteins is tyrosine, which shows high emission especially when pure in solution.

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. Quenching may result from a variety of processes such as excited state reactions, energy transfers, ground-state complex formation and collisional processes. Collisional quenching, or dynamic quenching, results from the collision between fluorophores and a quencher. Static quenching is due to ground-state complex formation between fluorophores and a quencher. Collisional quenching is described by the Stern–Volmer equation, which gives the ratio between fluorescence intensities in the absence or the presence of a quencher as a function of its concentration, taking into account the fluorophore lifetime in the quencher's absence. From this equation, the Stern–Volmer, or quenching constant, can be calculated, and it is possible to distinguish static from dynamic quenching by their differing dependence on temperature, as well as by measurements of their life duration time [48,49].

In the present work we studied the *in vitro* interactions of two constructs of a clinically promising anti-heparanase aptamer, previously described as long and short aptamers (molecular weight 9254 and 22,348 g/mol) [1] with fatty acid and globulin free HSA and BSA, using the quenching of intrinsic fluorescence of tryptophan residues. We estimated the Stern–Volmer quenching constants, and discussed the nature of the highest affinity binding sites of aptamers to the HSA and BSA based on our modelling studies. The behaviour of quenching curves for HSA and BSA and values estimated for ratio R_1/R_2 from model developed by Cortez et al. [50] suggest that the primary binding site in both aptamers is located closer to the tryptophan residue in sub domain IIA. Thus, we modelled the binding of the aptamer to this site, close to a long stretch of arginine residues in this subdomain. Our studies show that the short aptamer interacts with sera albumins via collisional processes, and does not form a complex. This is in agreement with previous studies published [51], where we have used the same method to study the interactions of the MUC1 aptamers against serum proteins. There, too, aptamers of the same length, but quite different binding properties and recognition epitopes showed similar properties with the short aptamer examined in this study. On the other hand, the longer aptamer, which contains the two primer recognition sites, forms a complex with the albumins, a fact that has direct implications on its bioavailability and time it remains in circulation.

2. Materials and methods

We studied *in vitro* the interactions of two aptamer constructs (Eurofins MWG, UK), one of short chain (25 bases, MW 9254 g/mol) and the other of long chain (72 bases, MW 22,348 g/mol) with sequences 5′-ACTTTTGAATGTGGCAACAAATTCGACAGG-3′ (short aptamer) and 5′-GGGAGACAAGAATAAACGCTCAAATGGACTTTTGAATGTGGCAACAAATTCGACAGGAGGCTCACAACAGGC-3′ (long aptamer) [1], with HSA and BSA (Fatty acid and globulin free; Sigma, UK) in 37 °C and 25 °C. Fluorescence measurements were performed on a JobinYvon Horiba Fluoromax-P System, and UV light at 290 nm was used to excite the albumin fluorophore group triggering the emission of intrinsic fluorescence.

Both aptamer constructs were fluorescent, as it can be seen in Fig. 1. This shows the fluorescence spectra (excitation wavelength of 290 nm and emission wavelengths between 300 and 400 nm) obtained from gradual addition of short (Fig. 1a) and long aptamer (Fig. 1b) in 10 mM phosphate buffer pH 7.4, at 37 °C. According to Lakowicz [49], the nucleotide chain fluorescence is very low, but the presence of any aromatic ring in their molecular chain can turn the aptamer fluorescent. The presence of aptamer fluorescence represents a challenge for the study of its interaction with serum albumins. It required finding an adequate concentration range to be used, and earlier tests showed that aptamer concentrations ranging from 0.1 μg/ml to 8 μg/ml did not interfere in the evaluation of albumin quenching. In addition, that aptamer range is in accordance to the plasmatic level measured by some authors [52].