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Studies on Interactions of Human Serum Albumin with Hot Spots and Peptidic Inhibitors of Insulin and Amylin Aggregation

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Introduction

Human serum albumin (HSA), being a molecule of an increasing number of applications, shows great potential for becoming a safe, multifunctional delivery vehicle for important medications (e.g. phenylbutazone, ketoprofen, liraglutide, warfarin) [1]. HSA has two main binding sites, localizing in IIA (Sudlow site I) and IIIA subdomains (Sudlow site II), with a highly ordered secondary structure, composed mainly of α-helix and stretched chains (Figure 1). The conformational changes of HSA are regulated by pH alteration of the external environment, influencing its transport properties [2]. Nowadays, amyloidosis caused by undesirable polypeptide/protein aggregation is a huge challenge for scientists. Amyloidosis includes neurodegenerative diseases (Alzheimer's disease, Parkinson's disease), diabetes, cardiac amyloidosis, and others. Scientific work is being intensively carried out on the search for new methods of treating amyloidosis [3,4,5]. It is supposed that HSA could be applied as a drug delivery system (DDS) for inhibitors of pathological protein/polypeptide aggregation, with the aim to alleviate the symptoms and detrimental effects of neurodegenerative and metabolic disorders. On the one side, these inhibitors should be precisely delivered to the target site but on the other side, their immune activity should be minimal. The aim of the study was to check whether HSA can be used as a drug carrier for *N*-methylated analogous amyloidogenic cores (so-called hot spots) [6] of insulin and amylin, which significantly inhibit the aggregation of hormones regulating carbohydrate metabolism and are directly involved in the development of diabetes. The implementation of the research goal required the synthesis of amyloidogenic cores of insulin and amylin hormones and peptidic inhibitors of undesirable aggregation. For synthesis of peptides, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium p-toluenosulphonate (DMT/NMM/TosO$^-$) [7] was used as coupling reagent. Moreover, all peptides were labeled with 4-(1-pyrenyl)butyric acid (PBA), fluorescent dye permitting to monitor their docking to HSA. For selected fragments, it was planned to carry out research using a microscope, microscale thermophoresis (MST), and circular dichroism techniques (CD). Additionally, it was planned to check the effects of glucose and cholesterol concentrations on the inhibitor release from the HSA.

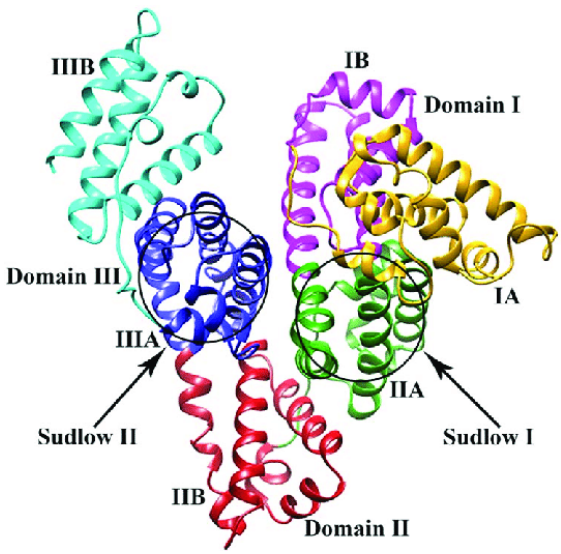

Fig. 1. HSA structure with indicated potential binding sites of external ligands.

Results and Discussion

All analyzed peptides were synthesized on the chloro-2'-chlorotrityl resin according to Fmoc/tBu methodology. The application of DMT/NMM/TosO$^-$ as a coupling agent allowed us to obtain final products with the required efficiency and purity, even in the case of hot-spots fragments showing a

strong tendency to aggregation, as described in the literature [8]. In the next step, all fragments were labeled by using 4-(1-pyrenyl)butyric acid as a fluorescent probe. The optimized reaction conditions let us synthesize peptide-probe conjugates with preserved spectroscopic properties of pyrenyl moiety (Figure 2). Observing the changes in the absorbance and fluorescence spectra of the conjugates, it was possible to assume that they would be able to interact with HSA.

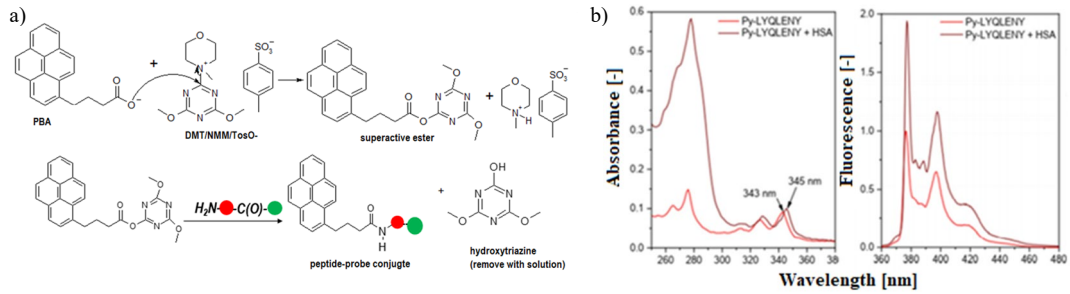

Fig. 2. a) Synthesis of fluorescent labelled conjugates, red spot - peptide chain, green spot - solid phase; b) absorption and fluorescence spectra recorded for labelled amyloidogenic core of insulin in the presence and without addition of HSA.

It has been found that the fluorescent-labeled conjugates derived from insulin and amylin hot spots have shown a tendency to aggregate in the absence of HSA. The addition of HSA resulted in an increase in absorbance, the intensity of the emission of the fluorescence probe, and the visualization of the entire oscillatory structure of the fluorescence spectrum (Figure 2b). These observations indicated changes in the microenvironment within the probe, probably caused by the docking of the conjugate within the HSA region. Only for a labelled amyloidogenic fragment of amylin (Py-FGAIL) on the recorded spectra, was not observed above changes that could point to the lack of persistent interactions of this fragment with HSA or on different places of its docking within plasma protein (Table 1).

Due to the possible influence of pyrenyl moiety on interactions peptides with HSA we also performed studies for unlabelled hot spots fragments of insulin and amylin. Congo red (CR) and thioflavin T (ThT) were used as dyes to study the amyloid structures. The inhibitory effect HSA on the aggregation process for unlabelled amyloidogenic cores of metabolic hormones was observed (Table 2). However, the exception was again amylin fragment (FGAIL) for which on the recorded both absorption spectra for CR as well as fluorescent spectra for ThT we noticed changes characteristic for amyloids formation even in the presence of HSA (the bathochromic shift and CR absorbance decrease as well as the irregular changes fluorescence intensity of ThT). The obtained results were also confirmed in circular dichroism and microscopic studies. The lack of increase in α-helix content on recorded CD spectra for FGAIL fragment after incubation with plasma protein indicated the preservation of the structure typical for amyloid fibers (mainly β-sheet conformation). Moreover, on the microscopic photo, the highest content of amyloid-like fibers in the presence of HSA was observed for amylin fragment. In the case of two tested insulin fragments, noticeable inhibition of their aggregations in the presence of HSA was observed, indicating promising potential of plasma protein in metabolic disorders treatment. If the HSA could limit amyloid formation, it could also act as an effective DDS for *N*-methylated peptidic inhibitors of insulin and amylin aggregation. To test the influence of glucose and cholesterol on the behaviour of the HSA-inhibitor complex, three different glucose solutions and one cholesterol solution were prepared. These were designed to mimic the natural ratios of HSA to the above-mentioned biomolecules in the case of hypoglycemia (1) (<7 mg/ml), normal (2) (7-9.9 mg/ml), and hyperglycemia (3) glucose (10-12.6 mg/ml) as well as a normal (<20 mg/ml) cholesterol level (4). Since the concentration of HSA in human blood ranges from 34 to 50 mg/ml, 40 mg/ml was chosen as the reference value. In the case of diabetes treatment concentration of glucose could have a crucial influence on the possible release of *N*-methylated peptidic inhibitor from HSA, which finally decides about the desired activity of a drug. On the other hand, lipids concentration in the diabetic's bloodstream could also modify HSA structure, as a result

of the protein lipidation process [9]. For examination of the release process of the *N*-methylated fragments of insulin (L'YQLENY, LYQ'LENY, L'YQ'LENY), 2 ml of a given HSA-inhibitor solution were mixed with 2 ml of each glucose/cholesterol solution respectively. All samples were incubated at 37°C for 7 days. The recorded CD spectra for analyzed samples were presented in Figure 3.

Table 1. Spectroscopic changes observed for analyzed conjugates in the presence of HSA.

Analyzed fragment	*Fluorescent conjugates*	*Absorption spectrum*	*Fluorescence spectrum*	
		Bathochromic shift	Increase the fluorescence intensity	Oscillatory structure visualization
Insulin hot-spots	Py-LYQLENY	+	+	+
	Py-VEALY	+	+	+
Amylin hot-spot	Py-FGAIL	-	-	-
N-methylated fragments of insulin	Py-L'YQLENY	+	+	+
	Py-LYQ'LENY	+	+	+
	Py-L'YQ'LENY	+	+	+
	Py-VE'ALYL	+	+	+
	Py-VEAL'YL	+	+	+
	Py-VE'AL'YL	+	+	+

'Y,'L,'A – N-methylated amino acids

Table 2. Spectroscopic studies for unlabelled amyloidogenic cores in the presence of HSA.

Studied hot-spot	*Decrease in CR absorption*	*Irregular changes on ThT fluorescence spectra*	*Increase in α-helix structure on CD spectra*	*Presence of amyloid fibres on microscopic photos*
LYQLENY	-	+/-	+/-	+/-
VEALYL	-	+/-	+/-	+/-
FGAIL	+	+	-	+/-

The spectrum obtained for HSA buffer solution confirms the dominant contribution of α-helix structure in protein conformation, two characteristic minima were observed around 209 and 222 nm as well as a maximum at 195 nm. Based on the rest curves shown in Figure 3a it could be assumed that glycosylation and lipidation of HSA lead to a loss of heart-shape structure of HSA and a reduction in α-helix content. The most similar changes were also recorded for HSA-L'YQLENY sample incubated with glucose solution as well as with cholesterol solution, respectively. It was found that for this *N*- methylated fragment in analyzed conditions its release from HSA structure was the most effective. In the case of two other inhibitors, namely LYQ'LENY and L'YQ'LENY, their interactions with HSA seemed to be stronger, the recorded bands showed totally different spectral shapes in comparison to the first inhibitor. The results obtained from CD measurements were additionally compared with microscale thermophoresis studies

Table 3. The K_d values of inhibitor-HSA complex.

The studied sample	K_d
L'YQLENY + HSA	19.2 nM
LYQ'LENY + HSA	15.6 nM
L'YQ'LENY + HSA	8.07 nM

performed in buffer solution for *N*-methylated inhibitor-HAS complexes. The MST technique allowed us to calculate dissociation constant (K_d) for possible interactions between inhibitors and HSA. It has been found that L'YQLENY-HSA complex showed the highest K_d value which indicated the most effective ligand release from the serum protein structure (Table 3). For the rest inhibitor-HSA complexes, estimated K_d values were lower but still on the same order of magnitude. It could be assumed that also for these inhibitors possible interactions with HSA showed reversible character. This is a very important factor that should be monitored in all paths of developing innovative drug delivery systems allowing for more effective and controlled transport of drugs to the affected sites.

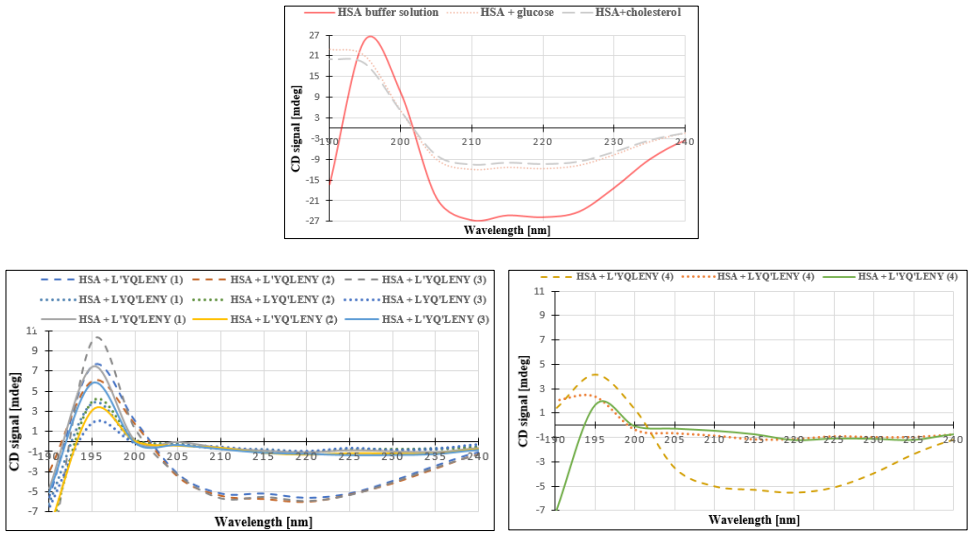

Fig. 3. The CD spectra recorded for: a) HSA in different environments; b) HSA-inhibitor complex: (1) hypoglycemia, (2) normal concentration of glucose, (3) hyperglycemia; c) HSA-inhibitor complex in presence of cholesterol.

Conclusions

The results presented in this paper have shown that all peptide-pyrenyl conjugates with their unlabelled analogues could interact with HSA. The inhibition of the aggregation process for peptides forming amyloid structures suggests that HSA may be an internal stabilizing factor of insulin and its fragments, and therefore could offer a natural, internal inhibitor of the process of their aggregation. Thus, HSA shows the potential to be used as an effective inhibitor for undesirable aggregation of amyloidogenic cores of peptides in the bloodstream, especially from the point of view of diabetes treatment. Moreover, the performed studies provide the first evidence that HSA could be also applied as a drug delivery system for *N*-methylated analogues of hot spots of insulin, to inhibit/limit the aggregation of this hormone within the tissue. The studies on the preparation of HSA complexes with peptide inhibitors of insulin aggregation and the use of HSA as a transporter are ongoing.

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