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Bence-Jones protein as the form of nano-scaled β-stacked supramolecular aggregates

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Abstract. *The formation in β-structured protein aggregates in tissues and fluids of the body is one of the most dangerouse complications of various diseases. The most famous of them are amyloidoses, but they such deposits are observed at other, much more widespread, diseases. The generally accepted approach to amyloids'detectionis based on high-specific coloring by Congo Red dye. However, the Abbe's diffraction limit excludes the seeing of the objects smaller than 0.61 wavelengths (about 240 nm). Such nanoscale formations are capable to disrup the functioning of surrounding tissues, to causethe complications and recurrences of the disease, and to pass through biological barriers with the following accumulation in body's fluids. It's likely that these conditions are the cause of the urinary congophilia, that is associated with preeclampsia at pregnancy and chronic kidney disease. Nor the less suspicious object is the Bens-Jones protein that appears in the urine at multiple myeloma and some other diseases, which are in more or less extent,are related to the disturbance of protein metabolism.*

The purpose of this study was to clarify the aggregate state of the Bens-Jones protein as a possible β-structured supramolecular associate.

Methods.The subject of the study was the freshly received urine from a patient with multiple myeloma. The presence of the Bens-Jones protein was checked by thermopacification of the acidified sample. For control, the urine was used by a healthy person with the addition of certain amounts of human serum albumin ("Reanal", Hungary) with a concentration of 0, 0.01, 0.1 and 1%.

Result. The obtained data testify to the appropriateness of such a point of view and create preresquites for the expanding of diagnostic possibilities.

Conclusions.The results obtained during the study testify to the peculiarity of the structure of the Bens-Jones protein, which is nano-sized beta-structured supramolecular

Key words: *protein aggregation, amyloidoses, β-aggregates, congophilia, Bence-Jones protein.*

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Білок Бенс-Джонса як різновид β-структурованого супрамолекулярного агрегату

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Резюме. *Формування в тканинах та рідинах організму β-структурованих білкових агрегатів належать до найтяжчих ускладнень різноманітних захворювань. Провідне місце серед них посідають амілоїдози, однак подібні депозити спостерігаються й за інших, значно поширеніших, захворювань. Класичний підхід до виявлення амілоїдів ґрунтується на їх високовибірковому забарвлення барвником Конго червоним. Однак дифракційне обмеження Аббе робить неможливим виявлення агрегатів, менших за 0.61 довжини світлової хвилі (порядку 240 нм). Подібні нанорозмірні утворення повній мірі здатні порушувати функціонування оточуючих тканин, викликати ускладнення та рецидив захворювання, проходити через біологічні бар'єри та накопичуватись в рідинах організму. Ймовірно, що саме цими обставинами обумовлено уринирну конгофілію, пов'язану з прееклампсію вагітних та хронічним захворюванням нирок. Не менш загадковим об'єктом є білок Бенс-Джонса, що з'являється в сечі за мієломної хвороби та групи інших захворювань, в тій чи іншій мірі пов'язаних з порушенням білкового обміну.*

Метою даного дослідження є дослідження агрегатного стану білка Бенс-Джонса як можливого β-структурованого супрамолекулярного ассоциата. Отримані дані свідчать про слушність подібної точки зору та обумовлюють нові діагностичні можливості.

Методи. Предметом дослідження була щойно отримана сеча пацієнта з множинною мієломою. Наявність білка Бенс-Джонса перевіряли термопацікацією підкисленого зразка. Для контролю використовували сечуздорової людини з додаванням певних кількостей людського сироваткового альбуміну («Reanal», Угорщина) з концентрацією 0, 0,01, 0,1 і 1%.

Результат. Отримані дані свідчать про доцільність такої точки зору та створення попередніх умов для розширення діагностичних можливостей.

Висновки. Отримані в ході дослідження результати свідчать про особливість структури білка Bens-Jones, який є нанорозмірним бета-структурованим супрамолекулярним

Ключові слова: *агрегація білків, амілоїдоз, β-агрегати, конгофілія, білок Бенс-Джонса.*

Introduction. Determination of protein content in urine belongs to the most mass clinical trials. The norm for urinary protein excretion does not exceed 30 mg, which is not registered by normal laboratory methods and is defined as «traces or lack of protein in the urine.» The protein content of more than 150 mg / l is evaluated as proteinuria, which may indicate significant impaired functioning of the kidneys, organs and tissues of the body [1]. Not only quantitative, but also qualitative definition of protein in the urine is of great diagnostic value, since it, together with clinical signs, allows both to detect the presence of trouble in the body, and to specify the nature of the disease [2].

It is known that the formation and maintenance of proteins in the native conformation is a complex energy-intensive process, which leads to the formation of the only true structure for each protein. An integral circumstance of many diseases is the development of endogenous intoxication caused by the formation and accumulation of various structurally damaging derivatives of native proteins. Such structures are prone to aggregation. We have emphasizethat β-structured protein aggregates are a kind of «energy bottom» for an immense number of conformational states of misfolded proteins [3]. It is the energy utility that gives the formation of such associate self-sufficient autochthonous nature. Similar processes are observed both for pathological changes, and for the normal state of many living organisms [4,5]. The most severe manifestations of the formation of β-structured protein deposits in the body include amyloidosis – a rather large group of diseases that are not curable [6,7]. Formation of amyloid fibrils and aggregates formed by them is a regular multistage process due to the formation of several levels of supramolecular structures [8].In the association thus formed, the distance between adjacent peptide chains in the β-sheet is 4.7 Å, and the distance between individual sheets is 10 Å [9]. Aggregated β-fold structures are capable of sorption and rearrangement on their own kind of soluble proteins, which ensures the growth of

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the aggregate [10]. In the case of most amyloidosis, the starting matrix of this process is the damaged structures of insoluble proteins – collagen and reticulin, which is responsible for the periclogenic and periereticular nature of most amyloidosis [11]. The starting matrix can be formed as a result of membrane holding of unstructured or structurally damaged proteins [12]. Structurally unbalanced proteins are also capable of spontaneous β-aggregation from the solution [13]. In the latter case, the transition from a pool of individual molecules to an insoluble aggregate must pass through the formation of nanosized groups [8]. Due to Abbe's diffraction limit (0.61λ), similar supramolecular associatives are not visible in the optical wavelength range. It means that the protein aggregates become visible on exceeding their linear dimensions more than 240 nm. The total molecular weight of such an aggregate is at least 7,000,000 kDa and it becames visiblein the optical wavelength range on exceeding of this value only [14]. It is known that nanoscale particles easily overcome biological barriersand affect significantly the course of various physiological processes [15].

Therefore, the identification of such deposits is of considerable scientific, educational and practical interest.The most generally accepted approaches to identifying β-stacked structures are based on their interaction with specific dyes.Congo Redand Thioflavin T are the most used among them (Figure 1).

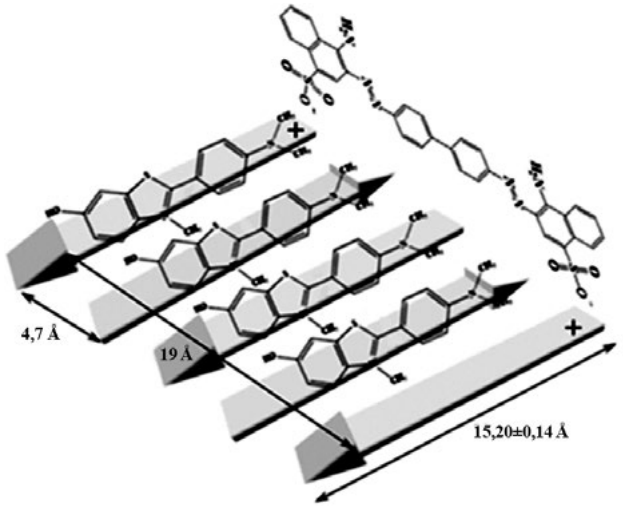

Fig. 1. Structural model of conjugation of the Congo red (top right) and Thioflavin T with the β-folded sheet of the protein aggregate[16].

The most widespread method for amyloid testingis based on Congo red staining [17]. Absorption level of this dye on intravenous injection is considered as faultless diagnostic criterion of amyloidosis [18]. This dye stains amyloids in different shades of red. However, there is a risk to obtain false-positive results during direct visualization at histological research, because Congo red is able to stain elastin. In addition, intact and hemolyzed erythrocytes and various cell constituents stained with Congo red give similar color. Hence, along with examination of stained tissues with using of light microscope, utilization of polarizing microscopy is required [19]. Due to regular structure, which is a feature of amyloid aggregates, they exhibit an optical activity. Consequently, amyloid formations stained with Congo red are observed in polarizing microscope as apple-green crystal-like structures that drastically differ from shaded tissues, which have no stereospecificity and thus are not able to deflect polarized light [20]. That is the cause why the reliable identification of amyloid entities with using of Congo red staining should combine both light and polarizing microscopy techniques.In addition, Congo Red is capable of effectively depositing microparticles of artificially-generated β-folded protein aggregates [21]. The latter circumstance prompts a closer look at the case of the unary congophilia, which constitutes the essential circumstance of preeclampsia, chronic kidney disease and lupus nephritis [22]. The degree of binding of Congo red to urine proteins not only makes it possible to diagnose the corresponding complications, but also suggests the presence of nanoscale protein aggregates in the urine.No less suspicious in this regard is the Bence-Jones protein(B-JP). This protein is absent in healthy people, and its appearance in the urine is the result of prerenal proteinuria caused by myeloma disease, as well as Waldenstrom macroglubulanemia, plasmacytoma, primary amyloidosis, idiomatic monoclonal gammopathy, chronic lymphocytic leukemia, endotheliomas, paraproteinemic hemoblastomas, and lymphogranulomatosis. B-JP is able to provoke urolithiasis due to the formation of protein cylinders in the renal tubules, that makes it similar with damage-associated molecular patterns.

Quantitative B-JP in urine and blood plasma is an important indicator for assessing the patient's condition and the effectiveness of treatment. In clinical and laboratory practice B-JPdetermination is is expedient at the positive probe for total protein by precipitation of sulphosalicylic acidonly. It destroys the hydrated cower of the protein molecules, that cause the aggregation and precipitation. The classic method for detecting B-JP is based on the thermal precipitation in a slightly acidic medium. Filtered urine in a quantity of 4 ml is mixed with 1 ml of 2 M sodium acetate buffer pH 4.9 and heated for 15 minutes in a water bath at 56°C. In the presence of the Bens-Jones protein it rapidly precipitates, but dissolves againwhen cooled. B-JPconsists of the light chains of immunoglobulins, that are monoclonal mainly [23]. But the monoclonal light chains are the basis of protein deposits for the most common type of amyloidosis – AL [6,7]. The reverse nature of the thermoprecipitation, the molecular composition, and the damage of the nephron tubules by the protein deposits suggests for the possibility of staying B-JP in a water-soluble high-stabilized state of β-structured protein aggregates. All these considerations led us to test the aggregate state of B-JP by simple and accessible laboratory methods.

The purpose of our study was to verify the possible aggregate state of the Bens-Jones protein as a set of nanosized β-folded groups.

Materials and methods.To accomplish this task, it was considered appropriate to use Congo Red and Tioflavin T dyes that are specific for the β-folded structures. Unlike the small-specific dyes, which are used more or less successfully for colorimetric or turbidimetric determination of protein content in urine [1], as Congo red, and Thiophlavin T are characterized by exceptionally high selectivity of affinity precisely to β-structured protein groups [19, 24]. Therefore, the definition of amyloid-like inclusions in the tissues with the help of these dyes by light, polarization and luminescent microscopy refers to classical methods of histology.

The subject of the study was the freshly received urine from a patient with multiple myeloma. The presence of the Bens-Jones protein was checked by thermopacification of the acidified sample as discussed above. For control, the urine was used by a healthy person with the addition of certain amounts of human serum albumin («Reanal», Hungary) with a concentration of 0, 0.01, 0.1 and 1%. The total protein precipitation was performed by adding of an equial volume of 10% trichloroacetic acid to the test samples, followed by centrifugation and dissolwing of the precipitate in 0.1% solution of sodium hydroxide for the determining of the protein amount of by Lowry method [25]. Specific dyes of Congo Red and Thioflavin T (Acros Organics, USA) were used in the form of a 0.1% solution in 0.9% sodium chloride.

Results. When added to the filtered sample of the urine of a patient with multiple myeloma of equal volume of 5% sulphosalicylic acid, the protein aggregate wasprecipitated. For quantitative evaluation of the protein content in urine, an equal volume of 10% solution of trichloroacetic acid was added to the urine specimen and the precipitate was separated by centrifugation using an OPN-8 centrifuge for 15 minutes at 3000 rpm. The precipitate was dissolwed in 0.1 N solution NaOH and the protein content was determinewd by the Lowry method. It was 0.740 mg / ml of patient's urine. None protein deposition was observed in the urine specimen of a healthy person. On addition of 1 m1 of 3 M sodium acetate buffer pH 4.9 to 4 ml of the patient's filtered urine and incubation of the mixture at 56°C in thewater thermostat, the formation of a loose precipitate was achieved. This precipitate dissolved quickly at room temperature that is typical for Bens-Jones protein. The heating of the same sample for 15 minutes in a boiling water bath deposition of the protein was slow, but irreversible. When added to the sample of urine equal to 0.1% of the Congo solution, there were no immediate changes, but at heating to 56°C an irreversible deposition of a dark red sediment was observed. The same deposites were formed at the storage of the mixture at 2°C for two days. Both precipitates when viewed in a light microscope were turned to be an array of red-colored particles that had a bright green color in the polarization microscope (Figure2).

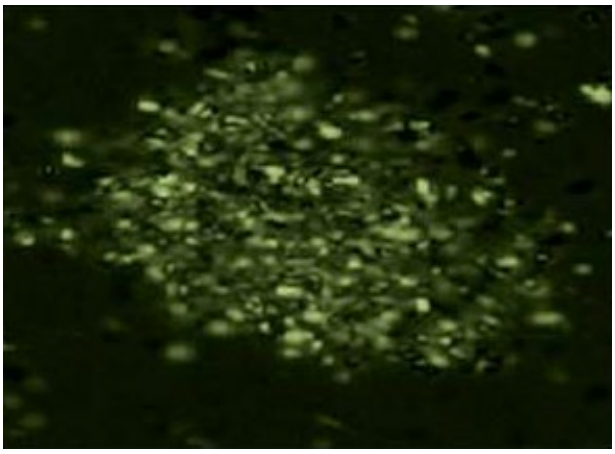

Fig. 2. Aggregates of the Bens-Jones protein precipitated by Congo Red dye. Polarization microscopy, ×200.

In other words, the formed precipitate gave a color reaction, typical for amyloid proteins. The attempt to conduct a fluorimetric study of the interaction of the Bens-Jones protein with Tioflavin T was unsuccessful, since the optical properties of the urine themselves blocked the wavelength of the emission of fluorophore formed by the interaction of this dye with amyloid structures. At the same time, it has been noted that adding the patient's urine to an equal volume of 0.1% Thioflavin T at room temperature or at store for 2 days at 20°C were resulted in the formation of a precipitate. In the case of a warming of such a sample at 45°C the formation of the precipitate was significantly accelerated, and at 55°C it was immediate and irreversible. The heating of the same samples in a boiling water bath, both with the Congo Red, and with Thiophlavin T, led to a rapid and irreversible deposition of proteins.

For control, 1%, 0.1% and 0.01% of human serum albumin solutionsin the urine of the healthy person wwere used. Adding to the test samples equal volumes of 0.1% solutions of both Congo Red and Thiophlavin T at room temperature, neither when heated to 56°C, nor with prolonged storage at 2°C did not lead to precipitation. The heating of the same samples in a boiling water bath for 15 minutes both with dyes and in their absence led to precipitation of denatured protein. The precipitate sorbed Congo red, but no optical activity was detected in the polarization microscope. On these reasons we may conclude about the absence of any induction effects of the used dyes on the confirmation status as a Bens-Jones protein in the urine of the fpatien with multiple myeloma and human serum albuminin the urine of a healthy person.

Discussion. The formation of structured fibrils is a complex, multi-step and long process (Fig.3). Therefore, instant precipitation of the protein by the dye when heated to 56°C is a consequence of the violation of the hydratation shell of the ready nanoaggregates.

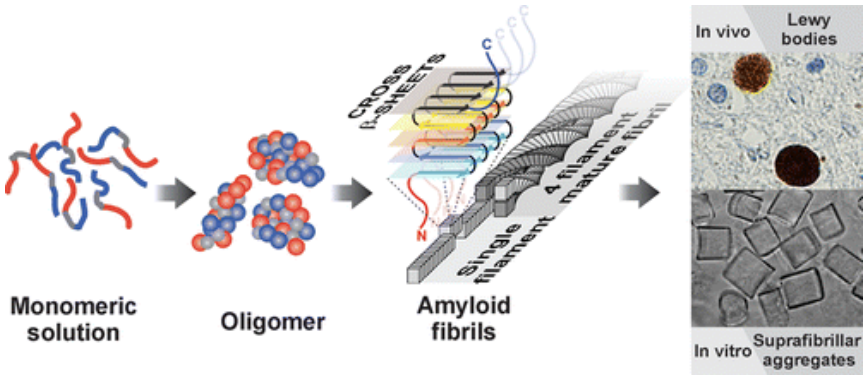

Fig. 3. The key stages of self-assembly of -structured protein aggregate [8].

Due to the peculiarities of the nano-sized objects, such aggregates are not visible in the optical range and are not precipitated from the aquatic environment by themselves. The addition of the dye leads to the formation of macro-aggregates and sedimentation, similar to that described in the literature for artificial beta-structured microparticles [26]. The revealed features of the interaction of Congo Red and Thioflavin T with the Bens-Jones protein can be recognized as evidence of the existence of the latter in the form of nano-sized β-structured aggregates. In favor of this point of view, the abolition of the nature of the depositional action of both dyes indicates that they are in good agreement with the given data regarding the specificity of their interaction with β-folded structures [16].

It is probable that the continuation of the aggregation processes B-JPis the molecular basis for the formation of protein cylinders in the renal tubules and due to their urolithiasis. The differences in the precipitation of B-JP byCongo Red and Thioflavin T may be explained by the differences in the mode of interaction of these dyes with β-stacked structures (Fig. 1). These peculiarities may be used both for the following studies of B-JP and for its quantifying in urine. The resulting precipitate can be investigated for the total protein content by any method that does not coincide with the optical range of the precipitating dye. It should be emphasized that the formation of nanosized β-aggregated supramolecular structures is unlikely to be limited to the Bens-Jones protein. The possible presence of such kind of nanosized protein aggregates in medicated drugs and food products [27] deserves close attention. No less significant cjnsequences may be caused by the presence of such particles in body's tissues were as the possibility of a violation by them of the normal course of physiological processes is jbvious. In particular, the formation and incorporation of highly stabilized protein aggregates into the structure of cell membranes may be the main cause of fibrosis, that is the routine characteristic of processes associated with a violation of the synthesis and exchange of proteins. No less important is their possible role in the relapse of various abnormal tissues. It is clear that the study of these tissues requires for the use of a set of physico-chemical methods that aren't limited by Abbe diffraction limit. Thus, the great attention has been paid to the creation of new fluorescence probes for the determination of β-structured protein aggregates, first of all – with Alzheimer's disease [28]. The use of this kind of probes for the determination of nanosized protein aggregates in blood circulation can also be used to quantify the excessive amounts of such formations, which appears to be a promising direction of molecular diagnostics.

Conclusions. The results obtained during the study testify to the peculiarity of the structure of the Bens-Jones protein, which is nano-sized beta-structured supramolecular aggregates. The possible participation of such kind of formations in the disruption of the normal course of physiological processes is under consideration.

Conflict of Interest: The authors state that there is no conflict of interest.

Information about the contribution of each participant.

N.M. Voroshylova: precipitation of proteins, determination of their concenteratuion, and participation in the writing of manuscript,

M.D. Timchenko: microscopic examination of samples and participation in the writing of manuscript,

S.V.Verevka: general research guides and participation in the writing of manuscript.

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