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TECHNOLOGY FOR TEST-SYSTEMS FOR DIAGNOSIS OF MODIFIED PROTEINS (PROTEOPATHY) UNDERLYING SOCIAL DISEASES (Report 1)

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A new experimental technique for obtaining diagnostic test systems is introduced based on conjugates "polymer microsphere–bioligand" for rapid diagnosis of autoantibodies to the modified protein on example of thyroglobulin, which consists of preliminary blocking of aldehyde groups of polymer microspheres by glycine amine groups, and then of the covalent interaction between carboxyl group of glycine and the amino groups of thyroglobulin. This way of creating test systems has enhanced the sensitivity of the reaction of latex agglutination (RLA) by extending the boundaries of titer.

***Key words**: diagnostic test system, the polymer suspension, thyroglobulin, latex agglutination reaction.*

Introduction

A promising approach to improve the preventative and drug treatment is early diagnosis of multifactorial proteopathies underlying social diseases. Such diseases include autoimmune thyroid disease (Hashimoto's thyroiditis, Graves' disease and other) [1–3], due to disruption of the structure of thyroglobulin (proteopathy), leading to its accumulation and antigenic effects on the body.

Development of modern highly sensitive diagnostic test systems that can detect autoantibodies to thyroglobulin can be an important criterion for immunological diagnosis of autoimmune thyroid diseases.

Among a variety of laboratory diagnostic methods (radioimmunoassay RIA) [4], enzyme-linked immunosorbent assay (ELISA) [5], immunochromatographic assay (IHA) [6], etc., a special place in immunodiagnostic practice takes the reaction of latex agglutination (RLA) [7–9]. This is the easiest screening method of testing which belongs to the category of qualitative or semi-quantitative methods of analysis. The relative cheapness of analysis, high sensitivity, specificity, and reproducibility of the RLA method, simplicity and the ability to test in almost any conditions allow for rapid diagnosis of diseases, both in single and in mass trials.

RLA tests are based on a visual assessment of the nature of agglutination "clumping" of polymer particles containing specific surface bioligands (antibodies or antigens) that can bind to a detectable affinity component (antigen-antibody).

The purpose of this study was to develop methodological bases for the construction of highly sensitive test systems based on conjugates "polymer microsphere-bioligand" for rapid laboratory diagnosis of autoantibodies to thyroglobulin thyroid underlying social diseases (autoimmune disease).

Experimental

Materials

Styrene, purified from the stabilizer with 5% aqueous sodium hydroxide solution, washed with water until neutral, dried over calcined calcium chloride and twice distilled in a vacuum. The fraction boiling at 41°C (2.1 kPa) was used, d_4^{20} = 0.906 g/cm^3. Mass fraction is of 99% of the main substance.

Styrene sulfonate sodium, mass fraction is of 99% of the main substance.

Acrolein, a technical product of «Fluka», three times distilled at atmospheric pressure. The fraction boiling at 52°C was used, d_4^{20} = 0.806 g/cm^3.

Azobisisobutyronitrile — initiator, was purified by recrystallization from methanol. The melting point is 101°C.

Potassium persulfate — initiator, without further purification. Mass fraction is of 99% of the main substance.

Polyvinylpyrrolidone, applied with a molecular weight of 40,000, used the mark "reagent grade" without further purification.

(N-Ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride) — «Merck» company. Purity — 98%.

Thyroglobulin — was obtained at the Mechnikov Research Institute of Vaccines and Sera, the molecular weight of 600 kDa. Purity is not less than 98%.

Serum samples of patients with diseases of the thyroid gland against thyroglobulin (titer immunosorbent assay (ELISA) — 1:3240) were obtained from the Diagnostic Center Mechnikov Research Institute of Vaccines and Sera.

Phosphate-buffered saline (pH 7.2), prepared by mixing 720 mL of the solution (11.876 g) Na_2HPO_4 in 1000 mL of distilled water and 280

mL (9.078 g), KH_2PO_4 in 1000 mL of distilled water to the total volume of the solution phosphate salts (1000 mL), 9 g NaCl. Water - bidistillate.

Methods

Seed polymerization method

A seed polymerization of the monomers (acrolein, styrene) was performed in 1.5 L reactor. The reactor is charged bare polystyrene suspension injected stabilizers (styrenesulfonate sodium and polyvinylpyrrolidone) and distilled water. Degassing of the reaction mixture in the reactor was carried out by a nitrogen purge 200–250 rpm stirring for 5–10 minutes. In parallel, an oil-soluble initiator dissolved (azobisisobutyronitrile) in the monomer, and injected into the reactor. Conduct degassing the reaction mixture with stirring, as described above, for 15–20 minutes.

First there is swelling of polystyrene seed particles in the reaction mixture containing monomer, at room temperature (20±0.5°C) for 24 h. The reactor was connected to the thermostat, the temperature was adjusted to 70±0.5°C and intersect the start of polymerization. 3 hours after the start of the polymerization initiator is introduced additional potassium persulfate dissolved in distilled water.

The polymerization was carried out at a temperature of 70±0.5°C at stirring 200–250 rpm to complete monomer conversion. For the control of monomer conversion in the suspension samples from the reactor was carried out every 2 h. After 24 h, the polymerization is stopped.

Cleaning polymer suspensions of residual components of the polymerization

Cleaning polymer slurry method is carried out using micro filtration cell. To clean the polymer slurry used 3 cell filtration capacity of 1 liter. In each cell using a funnel and a measuring cylinder filled polymer suspension was added distilled water and mixing included.

Microfiltration was carried out at a pressure of 117–137 kPa, using the compressor. Particles polymer slurry, which are on the filter membrane of the cell, add distilled water and filtered, spent 2 more times. After filtering the contents of 3 filtration cells were fused into the tank for the treated slurry and solids content was determined in the resulting polymer slurry.

Polymer suspension handed to the stage of the depolimerisation by γ-radiation beams (for 6 hours, the dose rate of 100 rad/s).

Aggregate stability of polymeric microspheres in a buffer solution (phosphate-buffered saline (pH 7.2)) was determined using light and electron microscopes, and deposition in the wells of 96-well microplates (firms «Lenmed Polimer», Russia, «Greiner», Germany).

Determination of aggregate stability of the particles in the wells of 96-well plates was performed by the following procedure:

The first row is contributed to 25 mL of buffer solution. Prepared 4% albumin solution in a buffer solution and measured it at 25 mL in the first row microplates, the concentration of albumin in the first hole is equal to 2% of the first series of wells in the next two steps (reducing the concentration of albumin 2-fold) was performed breeding albumin, leaving only the last row with a buffer solution (as zero control).

4% → 2 → 1 → 0.5 → 0.25 → 0.125 → 0.06 → 0.03 → 0.015 → 0.008 → 0.004 → 0.002%

Next to all the wells of row added to 25 mL 0.3% strength polymer slurry plate was shaken and left at room temperature for 5–6 hours. After 6–7 hours (time of sedimentation of particles of polymer slurry) on the bottom of the wells formed precipitate point in aggregate stability of particles, the particles of aggregate instability agglutinates formed in an inverted umbrella. The minimum concentration of albumin, in which there is no aggregation of particles, is determined by the final hole with a point precipitate.

Detection of autoantibodies to thyroglobulin (Tg) immunoassay (ELISA)

Antibodies to Tg were determined by indirect ELISA using polystyrene plates sensitized with highly purified preparation Tg (purification of at least 98%). Since antibodies to Tg belong mainly to the IgG class (95%), it was used as a conjugate of protein A labeled with horseradish peroxidase. The reaction showed a mixture of substrate tetramethylbenzidine («Clinical Science Products Inc», USA). Tg antibody levels were evaluated using the calibration curve. To take account of the reaction was carried out on a multi-channel photometer «Anthos» (Austria) at a wavelength of 450 nm.

Detection of thyroglobulin (Tg) immunoassay (ELISA)

Tg taped a "sandwich" ELISA: the surface of the wells of polystyrene plates sorbed affinity isolated rabbit polyclonal antibodies to thyroglobulin. Formed in the first step of the reaction complex "thyroglobulin autoantibody" identified conjugate antibody Tg-labeled horseradish peroxidase. Tg concentration in the samples was calculated from the calibration curve of the optical density on the concentration of triglycerides in the calibration samples. Sensitivity is not less than 3 ng/mL, accuracy — 90-100%, range of detectable concentrations — 5–500ng/mL.

Covalent binding of functional groups bioligands with functional groups of the polymer

Covalent binding bioligands with carboxyl groups located on the surface of polymer microspheres was as follows: after centrifugation polymer suspension was transferred to phosphate-buffered saline (pH 7.2) and adjusted to the

concentration of the polymer slurry to 0.5%. 1 mL of 0.5% of the second suspension was mixed with a solution of 1 mL of carbodiimide, the concentration of which was equal to 0.1 mg/mL. After incubation for 30 min at room temperature, it was washed three times with the suspension of unreacted carbodiimide phosphate buffer (pH 7.2) and conducted three times by centrifugation for 15 min at 4000 rpm. Polymer slurry with particles contained on the surface, activated carboxyl groups of the polymer, mixed with an equal volume of solution bioligands (various concentrations), taken in 2 mL. After incubation for 4–6 hours at a temperature of +37°C are not connected bioligands washed by centrifuging the solution three times for 15 min. at 4000 rpm, and diluted with phosphate buffer (pH 7.2), slurry concentration of 0.1%.

Reaction of latex agglutination (RLA) was performed in 96-well microplates

Quantitative determination of the detected component (antibody/antigen). Additions to the wells of 96-well polystyrene plates were carried out according to the scheme shown in Table 1.

Table 1. Addition to the wells

	1	2	3	4	5	6	7	8	9	10	11	12	Dilution
A	**NC**	**PC**	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**1:10**
B	**NC-1**	**PC-1**	**C1-1**										**1:20**
C	**NC-2**	**PC-2**	**C1-2**										**1:40**
D	**NC-3**	**PC-3**	**C1-3**										**1:80**
E	**NC-4**	**PC-4**	**C1-4**										**1:160**
F	**NC-5**	**PC-5**	**C1-5**										**1:320**
G	**NC-6**	**PC-6**	**C1-6**										**1:640**
H	**NC-7**	**PC-7**	**C1-7**										**1:1280**

1. As well A1 contributed 50 ul serum negative control (NC). NK-serum containing no antibody/antigen.

2. As well A2 contributed 50 ul of serum with the positive control (PC). PC serum containing antibodies/antigens.

3. To wells A3–A12 was introduced into 50 mL test sera (C1–C10) in a 1:10 dilution in accordance with Table 1.

4. Into the wells of rows B to H was introduced into 25 mL phosphate-buffered saline (0.01 M, pH = 7.4).

5. From the first of row A (at 25 mL) in the next series of wells B → C → D → E → F → G → H (for disposal) in increments of 2 (2-fold dilution) was performed dilution solutions.

6. To each well of the plate was introduced into 25 mL 0.3% suspension (diagnostic test kits), the plate was shaken and left at room temperature for 1.5–2 h.

With positive RLA is formed at the bottom of the wells in the agglutinates inverted umbrella with a negative - point precipitate.

In the analysis of the studied serum their maximum dilution giving a positive reaction is taken.

Under a positive reaction in a dilution of sera studied understand the value of credits in two or more times the value of credits in the negative control serum (NC).

The lowest concentration tested agglutinate determined by the final hole with a positive (agglutination unit (AU)). The next hole, which is not observed RLA is called — (sub agglutination unit (SU)).

Determination of particle size and particle size distribution were determined by photon – correlation spectroscopy on the device «Malvern» (England) [10]; the concentration of aldehyde groups on the surface of the polymer microspheres were determined by conductometric titration [11]; the protein concentration was determined by the Lowry method [12]; for determination of the monomer was carried out by spectrophotometry [13].

Results and Discussion

General technology of creating diagnostic test kits based on conjugates "polymer microsphere-bioligands" includes the following process steps (Fig. 1).

The first stage of the technological process (TP.1) — synthesis of functional polymeric suspensions with a narrow particle size distribution, stable with immobilized specific bioligands.

The second stage of the process (TP.2) is to clean polymer suspensions of residual impurities (monomer, initiator, stabilizer, etc.) capable of interacting with a detectable component, which negatively affects the registration and conduct of immunochemical RLA.

The third and fourth stages of the process are the most labor-intensive and consist of isolation and purification of specific bioligands (TP.3), and selection of the conditions of immobilization of bioligands on the surface of particles of the polymer suspension (TP.4).

On the first (TP.1) and second (TP.2) stages of the process polymer suspension was obtained with particles used as carriers of bioligands (Tg) in the development of test systems for the detection of autoantibodies to thyroglobulin by the RLA method (Fig. 2).

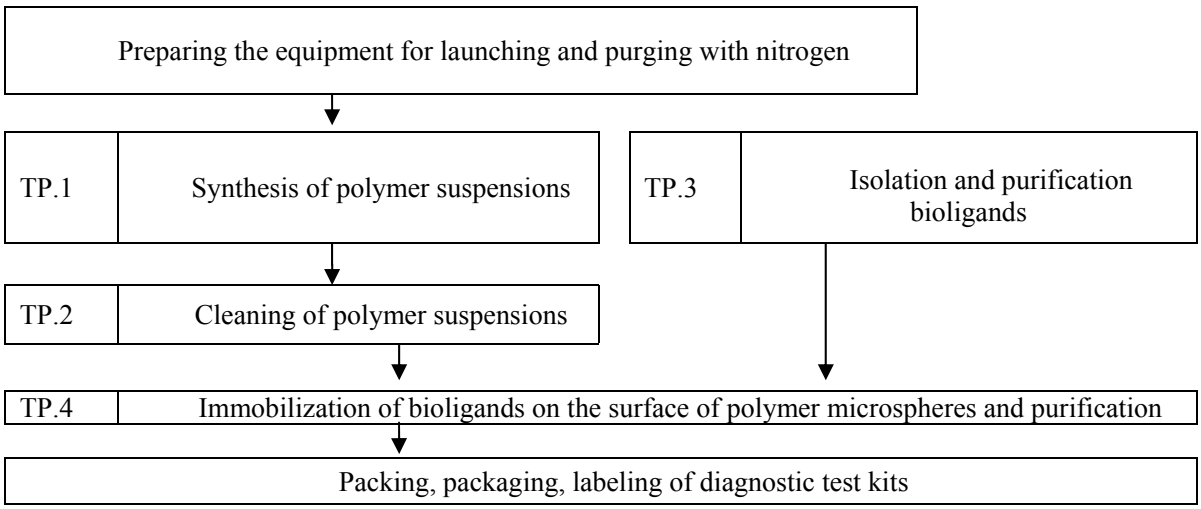

Fig. 1. Flow sheet of diagnostic test kits using polymeric microspheres and specific bioligands.

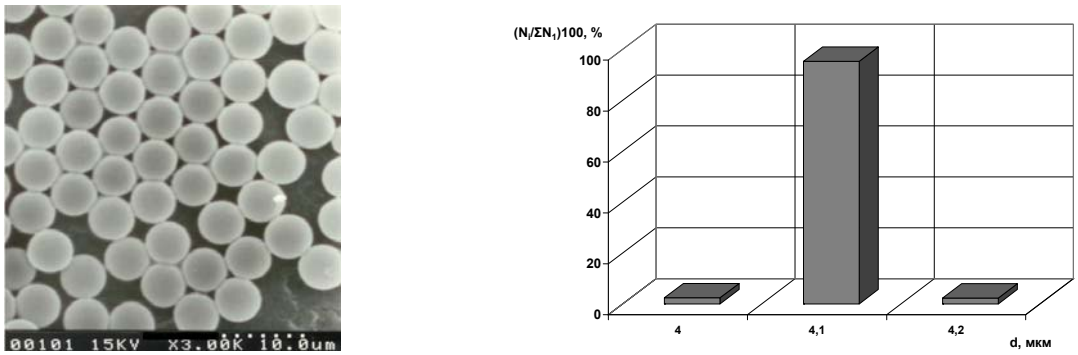

Fig. 2. Microphotograph of polymeric microspheres and a histogram of particle size distribution. Average diameter — 4.1 micrometer.

Polymer particles had an average diameter of microspheres - 4.1 microns, was characterized by a narrow particle size distribution (coefficient of variation less than 3%), and contained aldehyde groups on the surface at $1.2*10^{-3}$mol/g of polymer [11]. Mass fraction of residual monomer is no more than 0.05%. Particles retained aggregate stability in 0.25 M solution of NaCl. The polymer content of the suspension was 0.4% (dry residue).

Synthesized polymer microspheres met the requirements for the carriers of bioligands required to construct on their basis diagnostic test systems, namely:

• were characterized by a narrow particle size distribution, which allows to accurately determine the surface area of the carrier and determine the extent of its coverage by bioligands, which in turn causes the similar nature of their behavior during the reaction of latex agglutination (RLA);

• contained functional groups (aldehyde groups) on the surface of polymer microspheres needed to interact with amino groups of bioligands;

• had aggregate stability in buffer (phosphate-buffered saline pH 7.2) and in biological solution (serum) which prevents non-specific aggregation of particles;

• had a high rate of sedimentation in the wells of 96–well microplates (30–40 min), which allows for express testing;

• did not contain residual components of polymerization (monomer, initiator, surfactant, etc.) that affect the immobilization of bioligands on the surface of the polymer particles, and the stability of test systems for storage.

In the third stage of the process (TP.3) were obtained molecules of specific bioligand (Tg), which was isolated from the supernatant by ultracentrifugation of homogenate thyroid tissue. Pure thyroglobulin was isolated by gel filtration on a column of Sepharose 6B. Elution of thyroglobulin was performed in 0.01 M phosphate buffer pH 7.2–7.4. First collected fraction (peak 1) was concentrated by ultrafiltration using membranes «Amicon» Brand X-300. The degree of purification of thyroglobulin was controlled by electrophoresis in 5% polyacrylamide gel. Thyroglobulin is a dimer with a molecular weight of 600000 Da. The degree of purification of thyroglobulin was 98%.

The fourth stage of the process (TP.4) is the most high-tech process. The difficulty lies in the fact that, in each case it is needed to immobilize bioligands of different nature, size, molecular weight, etc. (viruses, antigens, antibodies, hormones, etc.) on the surface of polymer microspheres. This is largely due to the lack of systematic research to identify the factors, that allow the native conformation of bioligands when immobilized on the surface of polymer microspheres and,

therefore, to provide high sensitivity of RLA.

To study the immobilization of thyroglobulin (three-dimensional structure of which it is currently unknown) on the surface of polymer microspheres a computer model of its site was constructed homologous to acetyl cholinesterase, which clearly shows the location of the amino groups of lysine residues (Lys), and half of the known determinants of thyroglobulin recognized by autoantibodies [1, 14] (Fig. 3, Table 2).

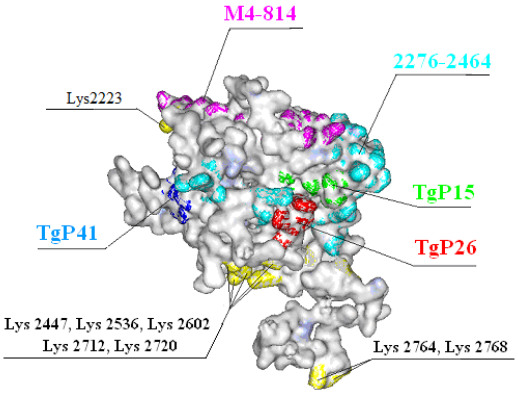

Table 2. Name determinants and their amino acid length, which are located on thyroglobulin site homologous to acetyl cholinesterase

№	Name of determinants Tg	Length of amino acid residues
1	M4-814	2188-2242
2	TgP15	2339-2358
3	2376-2464	2376-2464
4	TgP26	2471-2490
5	TgP41	2651-2670

Fig. 3. A computer model of the site thyroglobulin homologous to acetyl cholinesterase.

It is evident that the amino groups of lysine residues are concentrated in three places of biomolecule, each of which can react with the aldehyde groups of polymeric microspheres, including those groups that are close to the sites of a determinant responsible for binding to the active sites of autoantibodies.

The computer model allowed to assume that the interaction of amino groups contained in the residues Lys2764 and Lys2768 with aldehyde groups of polymeric microspheres, will not escape the antigenic determinants of thyroglobulin, and possible interactions of amino groups contained in the residues Lys2447, Lys2536, Lys2602, Lys2712, Lys2720 Lys2223 will lead to blocking them.

To obtain conjugates "polymer microsphere-thyroglobulin" covalent binding of amino groups of thyroglobulin was conducted with aldehyde groups present on the surface of polymer microspheres. The scheme of covalent interaction of aldehyde groups contained on the surface of polymer microspheres (PM) with the amino groups of thyroglobulin (Tg) is shown in Fig. 4.

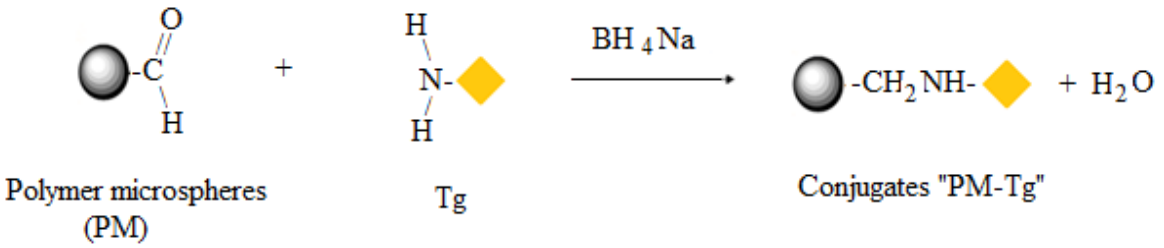

Fig. 4. Scheme of interaction of aldehyde groups contained on the surface of polymer microspheres (PM) with the amino groups of thyroglobulin (Tg).

The interaction of amine and aldehyde groups result in Schiff base which is unstable at low pH, so it was reduced with sodium borohydride (temperature 25°C, 30 min).

It was found that in 3 hours at 37°C 80% of thyroglobulin binds with the surface of the polymer from its original amount (500 μg/mL), and at 4°C in 12–14 hours (Fig. 5). Further exposure does not increase the amount of bound thyroglobulin, due, apparently, to saturation of particle surface with protein molecules. The calculation of the concentration of bound thyroglobulin with the surface of polymer microspheres were carried out on the basis of the determination of thyroglobulin in solution (Lowry method) before the immobilization process of thyroglobulin to the surface of the polymer, and after the cleaning process of the particles of the polymer suspension.

But the reaction of latex agglutination (RLA) with the developed test system did not take place. Reducing the number of molecules of thyroglobulin in the interphase layer of polymer microspheres from 80% to 4% did not lead to the expected result.

It was suggested that after the restoration of a Schiff base with sodium borohydride may occur decontamination of determinant sites of thyroglobulin responsible for binding to the active sites of autoantibodies. Violation of the native conformation of bioligands can occur as a result of a strong multi-point adsorption of Tg on the surface of polymer microspheres.

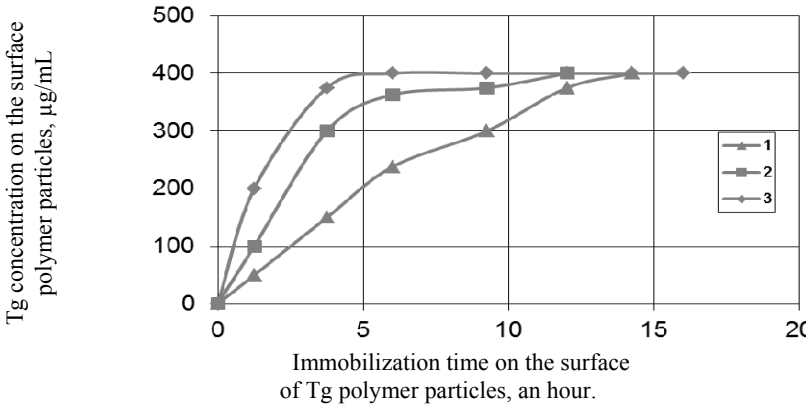

Fig. 5. Effect of temperature on the rate of immobilization of thyroglobulin (Tg) on the surface of polymer microspheres (1 – at 4°C, 2 – at 20°C, 3 – at 37°C).

It was proposed initially to block aldehyde groups of polymeric microspheres with amino glycine, which was used as a spacer, and then conduct a covalent interaction between the carboxyl groups of glycine and amino groups of thyroglobulin (Fig. 6).

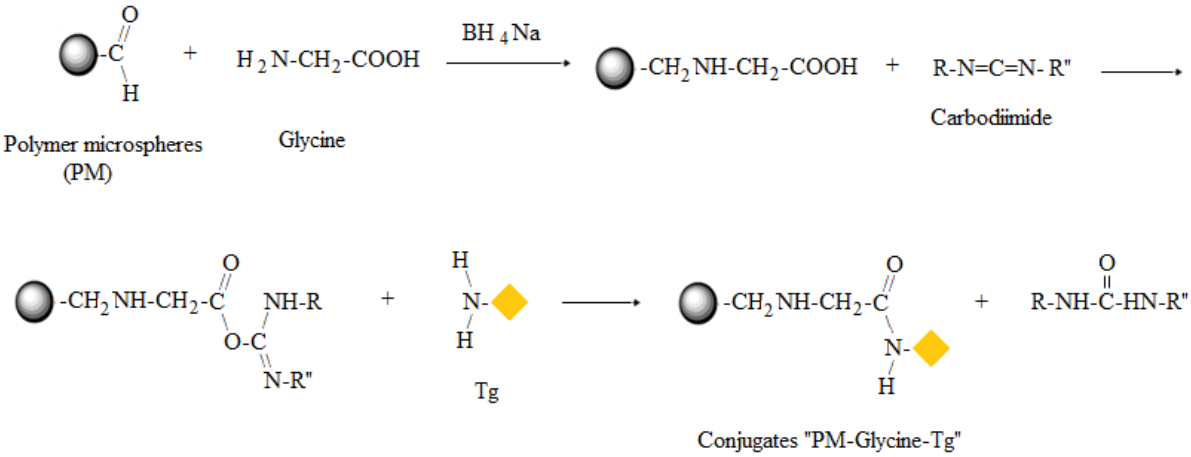

Fig. 6. Scheme of interaction between aldehyde groups contained on the surface of polymeric microspheres and amino groups of glycine with subsequent activation of its carboxyl groups by carbodiimide, and accession of the amino groups of thyroglobulin.

Immobilization of glycine on the surface of polymer microspheres was performed from hydrochloric acid solution of glycine, taken in the concentration range of 0.1–1.0 M at different pH values from 3.0 to 5.0.

Fig. 7 shows the change in the concentration of aldehyde groups on the surface of polymer microspheres on incubation time of polymeric microspheres in a solution of glycine.

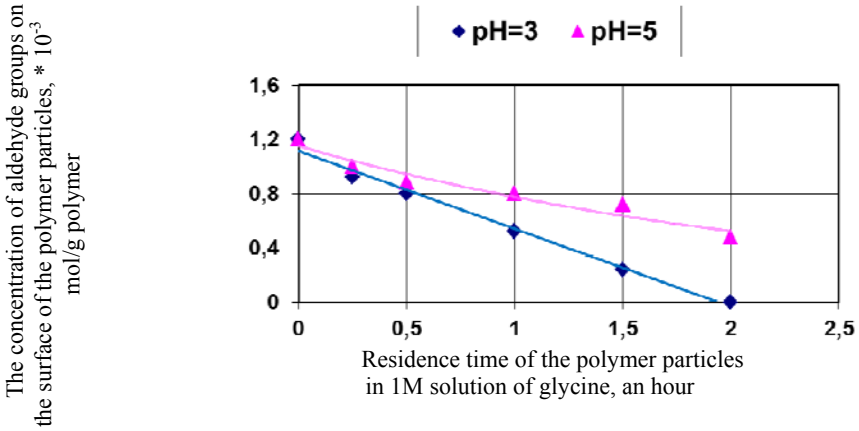

Fig. 7. Changing of the number of aldehyde groups on the surface of polymeric microspheres on the time of immersion in a solution of 1 M glycine.

From the data presented in Fig.7 we see that after exposure of the suspension for 1 hour at 37°C the concentration of aldehyde groups decreased 2 times, and when after exposing polymeric microspheres in a solution of glycine for 2 hours there is a complete blockage of glycine.

Carboxyl groups of glycine on the surface of polymer microspheres, were activated by carbodiimide (N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride) and attached amino groups of thyroglobulin to them to form peptide bond. In this case, the amount of spent thyroglobulin was 8% of its original amount (500 μg/mL).

Created diagnostic test-systems "PM-glycine-TG", using a spacer-glycine, provided clear differences in testing serum samples with high 400-1700 IU/mL (titer RLA - 1:640 and >), an average of 100-400 IU/mL (titer RLA - 1:80-1:640) and a low of 20-100 IU/mL (titer RLA - 1:10-1:80) content of autoantibodies to thyroglobulin.

For the assessment of the sensitivity of test systems "PM-glycine-TG", a comparative analysis of the detection of autoantibodies to thyroglobulin by the RLA and the traditional methods of testing - enzyme immunoassay (ELISA), hemagglutination (using red blood cells) (IHA).

Table 3 presents the results of detection of autoantibodies to thyroglobulin RLA different methods of analysis, ELISA and IHA.

Table 3. The identification of autoantibodies to thyroglobulin methods RLA, ELISA and IHA

Group surveyed	**Number of sera tested**	**The range of detection of autoantibodies to thyroglobulin by RLA, IU/mL**	**Number of sera matched with data RLA**	
			according to ELISA	**according to IHA**
Donors	30	10–30	28	29
Patients with autoimmune thyroid disease	10	30–100	9	10
	30	100–400	27	28
	20	400–1000	19	19
	10	1000–1700	9	10
Percentage of matches			**92%**	**96%**

Note: for a negative result in RLA adopted titer autoantibodies thyroglobulin equal 1:10.

Agreement of the results to identify autoantibodies to thyroglobulin by RLA and by ELISA or IHA noted in 92% and 96%, respectively. Differences are associated with both initial properties of used thyroglobulin from different manufacturers (the firm "Alcor-BIO" or obtained from Mechnikov Institute of Vaccines and Sera), and with the characteristics of the antigenic structure of thyroglobulin after its immobilization on a solid phase polymer plates (ELISA), or on the surface of biological media – erythrocyte (IHA) [15].

The above results led to the establishment of low-waste technology for the test of the "PM-glycine-TG" with the use of polymer microspheres and thyroglobulin, meeting the requirements of their use in modern clinical and diagnostic laboratories in the identification of auto-antibodies to thyroglobulin thyroid cancer (see Report 2).

Conclusion

A new experimental technique for obtaining diagnostic test systems is introduced based on conjugates "polymer microsphere-bioligand" for rapid diagnosis of autoantibodies to the modified protein on example of thyroglobulin, which consists of preliminary blocking of aldehyde groups of polymer microspheres by glycine amine groups, and then of the covalent interaction between carboxyl group of glycine and the amino groups of thyroglobulin. This way of creating test systems has enhanced the sensitivity of the reaction of latex agglutination (RLA) with extension of the boundaries of titer.

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