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TECHNOLOGY FOR TEST-SYSTEMS FOR DIAGNOSIS OF MODIFIED PROTEINS (PROTEOPATHY) UNDERLYING SOCIAL DISEASES (Report 2)

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A high-tech technology for highly sensitive test systems is proposed based on conjugates "polymer microsphere-bioligand" for express diagnosis of autoantibodies to the modified protein (proteopathy) for example, thyroglobulin, using the method of latex agglutination reaction (in vitro).

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

Introduction

In laboratory practice for immunochemical analysis is often used the reaction of latex agglutination (RLA), using diagnostic test systems derived from polymeric microspheres and specific bioligands [1–4].

RLA is different from traditional testing methods (double radial immunodiffusion [5] and immunoelectrophoresis [6], radioimmunoassay (RIA) [7], enzyme-linked immunosorbent assay (ELISA) [8], immunochromatographic assay (IHA) [9]) in its simplicity, it requires no special equipment and allows to carry out a visual record of results in a short time. The relative cheapness of analysis, high sensitivity, specificity, and reproducibility of the RLA method, simplicity and the ability to test performance in almost any conditions allow for express diagnosis of diseases, both in single and in screening studies.

Test systems for RLA are polymeric microspheres containing on its surface specific bioligands capable to affinity binding with a detectable component, thus forming a spatial grid agglomerates, which are easily visualized.

The proposed methodology for the diagnostic test systems based on conjugates "polymer microsphere-bioligand" for express diagnosis of autoanti-bodies to the modified protein (proteopathy), the example of thyroglobulin, is described in detail in the Report 1.

The purpose of the work was to develop high-tech technology for highly sensitive test systems based on conjugates "polymer microsphere-bioligand" for express 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. Used the fraction boiling at 41°C (2.1 kPa), d_4^{20} = 0.906 g/cm^3. Mass fraction is of 99% of the main substance.

Sodium styrene sulfonate, mass fraction is of 99% of the main substance.

Acrolein, a technical product of «Fluka», three times distilled at atmospheric pressure. Used the fraction boiling at 52°C, 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

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 h. After 6–7 h (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.

To control the specificity of the diagnostic test systems a method of inhibition of the reaction of latex agglutination (RTLA) in 96-well microplates is proposed

In a number of 96-well polystyrene microplates 25 μL saline or buffer solution is inserted.

In the first well of the row insert 25 μL of buffer solution (phosphate-buffered saline pH 7.2) containing thyroglobulin with concentration 100 μg/μL. From the first well in a row dilution is carried out of 25 μL buffer containing thyroglobulin in sub-sequent wells with step 2.

Introduced into each well of the row human serums with high titer in an amount of 25 μL, containing not less than 400 IU/μL of autoantibodies to thyroglobulin.

Keep the system within 3-5 min.

After 3-5 min in each well introduce 25 mL of 0.3-0.4% suspension (diagnostic test systems), than shake it and leave at room temperature for 5-6 h.

In the experimental range of diagnostic test system agglutination is not taking place or take place with increase of serum titer. This indicates that the diagnostic test system is specific.

With a positive reaction (RTLA) at the bottom of the microplate a spot precipitate is formed, with the negative reaction agglutinate is formed in form of inverted "umbrella".

For quantitative control of diagnostic test systems a method of reaction of latex agglutination (RLA) in 96-well microplates is proposed

Quantitative detection of autoantibodies to thyroglobulin is proposed to be conducted in 96-well polystyrene plates according to the scheme:

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

1. In well A1 add 50 μL serum with negative control (NC).

2. NC-serum containing no antibody/antigen.

3. In well A2 add 50 μL of serum with the positive control (PC). PC-serum containing antibodies/antigens not less than 100 IU/μL.

4. To wells A3-A12 introduce 50 μL test sera (C1-C10) in a 1:10 dilution.

5. Into the wells of rows B to H introduce 25 μL phosphate-buffered saline (0.01 M, pH 7.4).

6. From the first wells of row A (at 25 μL) in the next wells of rows B→C→D→E→F→G→H→ (for disposal) in increments of 2 (2-fold dilution) perform dilution of solutions.

7. To each well of the plate introduce 25 μL of 0.3-0.4% suspension (diagnostic test systems), shake the plate and leave at room temperature for 1.5-2 h.

With positive RLA at the bottom of the wells is formed the agglutinates in the form of inverted umbrella with a negative – spot precipitate.

In the analysis of the studied serum their maximum dilution giving a positive reaction is taken. As a positive reaction in a dilution of studied sera understand the value of titers in two or more times greater than the value of titers in the negative control serum (NC).

The minimum tested concentration of agglutinate determined by the final well with a positive reaction (agglutination unit (AU)). The next well, which is not observed RLA is called subagglutination unit (SU).

Synthesis of polymeric suspensions were determined by the seed polymerization of the monomers in the polymer seed particles [10,11], cleaning polymer suspensions of residual components of the polymerization was performed by microfiltration [12], for determination of residual monomer in a polymer suspension spectrophotometry was carried out [13], determination of particle size and particle size distribution were determined by photon – correlation spectroscopy on the device «Malvern» (England) [14], the concentration of aldehyde groups on the surface of the polymer microspheres were determined by conductometric titration [10] and the protein concentration was determined by the Lowry method [15], detection of autoantibodies to thyroglobulin (Tg) immunoassay (ELISA), detection of thyroglobulin (Tg) immunoassay (ELISA) [8], covalent binding of the amino groups with carboxyl groups bioligands glycine contained on the surface polymeric microspheres was performed using the soluble carbodiimides [16].

Results and Discussion

Each of the stages of the technological process: the synthesis of polymeric suspensions (TP.1), cleaning polymer suspensions of residual components of the polymerization (TP.2) isolation and purification of specific bioligands (TP.3) bioligands specific immobilization on the surface of polymer microspheres (TP.4) requires special attention and a number of requirements in the development of test systems based on conjugates "polymer microsphere-bioligand" for express laboratory diagnosis of autoantibodies to thyroglobulin of the thyroid.

In the first (TP.1) and second (TP.2) stages of the process, obtaining polymer suspensions, it was necessary to determine the quality criteria of the polymer particles and methods of control that would meet the requirements for carrier particles bioligands (Tg) for the development of diagnostic test system for autoantibodies to thyroglobulin.

Quality criteria of polymer microspheres and methods of control are shown in Table 1.

Table 1. Quality criteria of polymeric microspheres, which are used in the development of test systems for the detection of autoantibodies to thyroglobulin, and methods of their control

Indicator	Method	Display
The mass fraction of the polymer (dry basis),%	Determination of polymer content in the suspension (dry basis), the mass fraction of the polymer in %	For seed polymerization not less than 0.4, not more than 0.6
The size of polymer microspheres and particle size distribution	Photon-correlation spectroscopy	Microsphere diameter 4.0-4.2 microns particle size distribution narrow (coefficient of variation less than 3%)
Concentration of functional groups	Conductometric titration	The concentration of aldehyde groups $1.2–1.4\times10^3$ mol/g of polymer
Residual monomer	Determination of residual monomer in a polymer suspension	Mass fraction of residual monomer is no more than 0.05%
Aggregate stability	Determination of aggregate stability of polymeric microspheres in a solution of NaCl	Aggregate stability in the 0.15–0.25 M solution of NaCl

In the third stage of the process (TP.3) upon receiving a specific bioligand molecule (Tg) were determined bioligands quality criteria and methods of control, which are presented in Table 2.

Table 2. Quality criteria of specific bioligands (Tg), which are used in the development of test systems to detect autoantibodies to thyroglobulin, and methods of control

Indicator	Method	Display
The degree of purification of thyroglobulin	Electrophoresis in 5% polyacrylamide gel	The degree of purification of thyroglobulin at least 98%
The molecular weight of thyroglobulin	Ultrafiltration using membranes «Amicon» Brand X-300	Dimer with a molecular weight of 600000 Da

The fourth stage of the process (TP.4) is one of the most high-tech processes (method of immobilization on the surface of specific bioligands polymer microspheres). Developed and presented in the Report 1 method of immobilization of specific bioligands (Tg) on the surface of polymer microspheres allowed to obtain a highly sensitive test system "PM-glycine-TG", which provided a clear difference in the test serum samples with high 400–1700 IU/mL, the average 100–400 IU/mL and a low of 20–100 IU/mL content of autoantibodies to thyroglobulin (Table 3).

The results presented in the Report 1 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 autoantibodies to thyroglobulin thyroid.

Table 3. The results of identification of autoantibodies to thyroglobulin by developed test system in the RLA

Groups surveyed	Number of sera tested	The range of detection of autoantibodies to thyroglobulin by RLA, IU/mL	Titer RLA
Healthy donors	30	10–30	1:10–1:40
Patients with autoimmune thyroid disease	10	30–100	1:40–1:120
	30	100–400	1:120–1:540
	20	400–1000	1:540–1:1620
	10	1000–1700	1:1620–1:3240

Fig. 1 and Fig. 2 shows the process flow and procedural schemes of test systems for the identification of autoantibodies to thyroglobulin "PM-glycine-Tg".

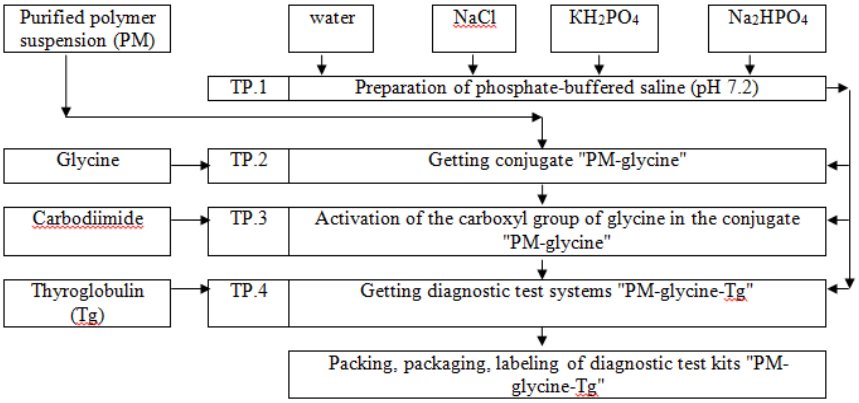

Fig. 1. Process flow scheme for diagnostic test system "PM-glycine-Tg".

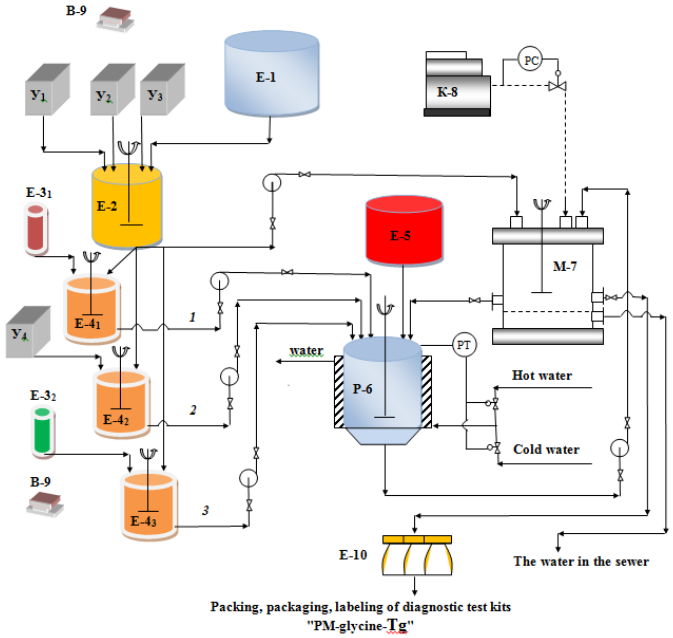

Fig. 2. Procedural scheme for diagnostic test-system "PM-glycine-Tg".

Y_{1-4} – Pack NaCl, KH_2PO_4, Na_2HPO_4, carbodiimide; E-1 – Container of distilled water; E-2 – Tank for preparation of phosphate buffer solution (BR); E-31-2 – Tanks with glycine and thyroglobulin; $E-4_{1-3}$ – Preparation tank with glycine BR, BR with carbodiimide, BR with thyroglobulin; E-5 – Capacity for purified polymer slurry; P-6 – Reactor for the immobilization process; M-7 –Microfiltration cell; K-8 – Compressor; B-9 – Scales; E-10 – Capacity for the finished product (test systems).

Diagnostic test system "PM-glycine-Tg" is produced by immobilizing thyroglobulin on the surface of the polymer particles in the interphase layer containing glycine. The process is proposed to take place in three stages:

The first stage – getting conjugate "PM-glycine".

The reactor (P-6) from the container (E-5) supplied with 0.4% w polymer suspension from the tank, to the same from (E-41) 1% glycine buffer solution is added and mixing turns on. To maintain the required temperature through heat exchange jacket reactor (P-6) water is pumped, the temperature of which is maintained at +37±0,5°C.

Stirring is carried out for 8-10 hours. After 8-10 hours, the reactor contents (R-6) are fed into the microfiltration cell (M-7) equipped with a filter membrane with a pore diameter of 0.45 μm. The contents of the filter cells (M-7), with stirring forced through the membrane, using a compressor (K-8). The filtration is carried out until such time as the cell will remain the initial volume of the polymer suspension (conjugate "PM-glycine"). After that, in the microfiltration cell (M-7) buffer added from tank (E-2), and then the filtration, as described above, 2 more times. In the process of cleaning a concentrate is obtained (buffer particle conjugate "PM-glycine"), which is fed to the reactor (P-6) for the activation of the carboxyl groups of glycine contained in the conjugate "PM-glycine".

Only this method of obtaining particles of conjugate "PM-glycine" allow fully locked aldehyde groups (1.2×10^3 mol/g of polymer), contained on the surface of 0.4% polymer suspension, by amino glycine for 8-10 hours at 37°C.

The second stage – the activation of the carboxyl groups of glycine contained in the conjugate "PM-glycine."

The second stage of the process is similar to the first, except that instead of the concentrate (buffer particle conjugate "PM-glycine") contained in the reactor (P-6), serves 0.1% buffer carbodiimide, taken from the container (E-42), instead of 1% of the glycine buffer. Carbodiimide activation process of the carboxyl groups of glycine contained in the conjugate "PM-glycine" is carried out for 40-50 minutes.

The buffer particles of conjugate "PM-glycine," in which carboxyl group of glycine is activated, is fed to the reactor (P-6) for the immobilization process of thyroglobulin.

The third stage – covalent interaction between the amino groups of thyroglobulin and activated carboxyl groups of glycine. This stage lasts for 12-14 hours.

The third stage of the process is similar to the second, except that the concentrate (buffer particle conjugate "PM-glycine," in which the carboxyl groups are activated glycine) contained in the reactor (P-6), is fed 0.05% buffer thyroglobulin, taken from the tank (E-43), instead of 0.1% of the first buffer carbodiimide.

This method of obtaining diagnostic test system "PM-glycine-Tg" provided content of thyroglobulin on the surface of 0.4% of the polymer suspension in a concentration of 40 μg/mL, and allowed to keep the availability of its determinant sites required for binding to the active sites of autoantibodies. In the resulting concentrate (diagnostic test system "PM-glycine-Tg"), add a preservative (sodium azide) and transfer to the stage of filling, packaging, and labeling of diagnostic test system "PM-glycine-Tg".

Developed diagnostic test system "PM-glycine-Tg" to detect autoantibodies to thyroglobulin by RLA, organoleptic and physical-chemical parameters should meet the requirements and standards of product quality (test-system) Table 4.

Table 4. Valuation parameters as a diagnostic test of the "PM-glycine-Tg" to detect autoantibodies to thyroglobulin by RLA

Indicator	Method	Display
Specificity	The reaction of latex agglutination inhibition (RTLA)	With a positive response RTLA at the bottom of microplate well the spot precipitate is formed; at the negative agglutinates in the form of the inverted "umbrella" is formed.
Sensitivity	The reaction of latex agglutination (RLA)	Titers reaction RLA 1:10–1:80, 1:80–1:640, 1:640 and more mean, respectively, the low – 10–80 IU/mL, average – 80–640 IU/mL, and high – 640 IU/mL or more content of autoantibodies to thyroglobulin.
Expiration date	–	6 months from date of manufacture. In the dark place at a temperature between 2 and 8°C.

The diagnostic value of the developed test system for detection of autoantibodies to thyroglobulin was tested on a panel of healthy human sera (n=30) and patients with autoimmune thyroid disease (Hashimoto's thyroiditis, Graves') (n=70).

Serum titers of the samples 1:10–1:80, 1:80–1:640, 1:640 and > denote, respectively, the low – 10–80 IU/mL (n = 26), the average – 80–640 IU/mL (n=23) and high – 640 IU/mL and> (n=16) content of autoantibodies to thyroglobulin.

Comparative analysis of the detection of autoantibodies to thyroglobulin methods RLA and the traditional method of RIHA (reaction of indirect hemagglutunation) showed that the sensitivity of the developed diagnostic test systems "PM-glycine-Tg" to detect autoantibodies to thyroglobulin thyroid cancer in human serum is not inferior to the method of RIHA, used in clinical practice. Identical units of agglutination titers for RLA is 1:10–1:80, 1:80–1:640,> 1:640 and RIHA – 1:160–1:320, 1:640–1:1280, 1:10240 – > 1:20480.

Agreement of the results to identify autoantibodies to thyroglobulin in methods of RLA and RIHA observed in 96% of cases.

The advantages of the developed diagnostic test systems for the RLA, besides stability, shelf life, technology, is the fact that the range of the analyzed serum titers (detection of autoantibodies) order of magnitude lower in RLA (1:40–1:640) in comparison with RIHA (1:160–1:10240). This advantage makes it possible to reduce the time of posting the analysis 2-3 times.

By the developed technology have been acquired 5 series prototype test kits "PM-glycine-Tg", in the right quantities. Prototypes of diagnostic test systems "PM-glycine-Tg" were presented in sets, each of which was designed for the analysis of 12 donor serum samples (Fig. 3).

Contents of the kit

1. The plate 96-well polystyrene – 1.
2. Bottle number 1: Test-system "PM-glycine-Tg" – 2;
3. Bottle number 2: Buffer solution (BS) – 2;
4. Bottle number 3: Negative control serum (NC) – 1;
5. Bottle number 4: Positive control serum (PC) – 1;
6. Application instruction set – 1.

Fig. 3. Contents of set.

Conclusion

A high-tech technology for highly sensitive test systems was proposed based on conjugates "polymer microsphere–bioligand" for express diagnosis of autoantibodies to the modified protein (proteopathy) on the example of thyroglobulin, using the method of latex agglutination reaction (*in vitro*).

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