# WAYS OF IMPROVING THE SPECIFICITY OF THE LATEX AGGLUTINATION REACTION*

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*The article describes research on the development of new diagnostic test systems operating on the basis of latex agglutination reaction, in which polymer microspheres are used as bioligand carriers instead of erythrocytes. Polymeric microspheres to be used as bioligand carriers must satisfy the following requirements: narrow size distribution, diameter of 5 microns. Besides, they must be characterized by aggregative stability in water and buffer solutions and be contained functional groups in the surface layer for linking with the functional groups of the protein. They are crosslinked particles obtained by copolymerization of styrene and divinylbenzene on polystyrene seed particles 1.5 microns in diameter with a narrow size distribution followed by modification by chloromethylation and amination by ethylenediamine. To increase the hydrophilicity of the surface of the polymer microspheres and to reduce nonspecific adsorption of proteins dextran was immobilized on the surface of the particles by covalent binding with amino groups of particles using Maillard reaction. It was found that diagnostic test systems, where modified dextran particles were used as carriers of the bioligand (Vi-antigen), are characterized by insufficient specificity and require additional modification of the surface of the polymer microspheres to eliminate its non-specific interaction with the surface of the polymer plate used for the latex agglutination reaction. A nonionic surfactant (Tween 80) proposed for the surface modification and used in a certain concentration provides the best reaction specificity.*

***Keywords:*** *polymer microspheres, latex agglutination, Tween 80, Vi-antigen, diagnostic test-systems.*

## Introduction

Polymeric microspheres with narrow distribution of particles by the sizes and by the functional groups on the surface are of great interest for biology and medicine, particularly, for use as carriers of bioligands instead of erythrocytes when creating diagnostic test systems for different types of diseases [1, 2].

In order to replace erythrocytes by polymeric microspheres the latter should be provided with certain properties, which will be discussed below.

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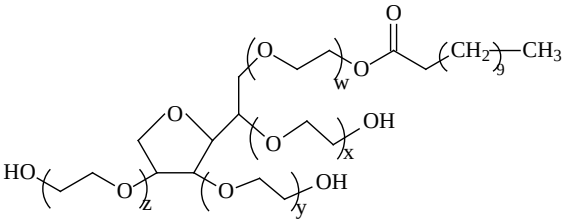
When creating diagnostic test systems, it remains a problem to obtain high specificity of negative test of the latex agglutination reaction.

This work is devoted to the development of a simple, easily reproducible method for carrying out the latex agglutination reaction allowing to determine methods of increasing the specificity of negative control by visual registration of reaction results.

## Experimental

***Starting compounds:***

1) Chloromethylated aminated polystyrene particles with a diameter of 5 μm
2) Tween 20 – polyoxyethylene (20) sorbitanmonolaurate produced by “Ferak” laboratory, Berlin



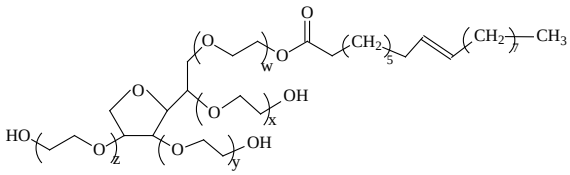
w+y+z = 20

MW = 1226

HLB = 16.7

CCM = 0.055 mmol/l, aggregation number 50–100.

3) Tween 80 – polyoxyethylene (20) sorbitanmonooleate produced by “Ferak” laboratory, Berlin



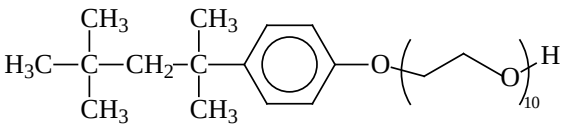
w+y+z = 20

MW = 1308

HLB = 15

CCM = 0.013 mmol/l, aggregation number 50–100

4) Triton X-100 – mono(tetramethylbutanol)phenyl ether of polyethyleneglyclol produced by “Ferak” laboratory, Berlin

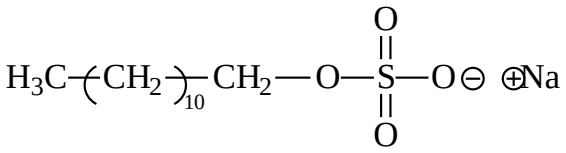


MW = 646

HLB = 13.5

CCM = 0.25 mmol/l, aggregation number 100–150

5) SDS – sodium dodecylsulphate produced by Sigma-Aldrich



MM = 289

HLB = 40

CCM = 8.3 mmol/l, aggregation number 55–62

6) Phosphate-buffered saline (PBS), pH 7.4 produced by Sigma-Aldrich

***Preparation of diagnostic test systems***

In order to obtain diagnostic test systems, polystyrene particles containing amino groups on the surface with an average diameter $d_{av}$ = 5 μm and narrow size distribution were synthesized. The particles were obtained by inoculated copolymerization of styrene and divinylbenzene (4%) on inoculating polystyrene particles with a diameter of 1.5 μm. Then the particles were chloromethylated by monochlorodimethyl ether and aminated by ethylenediamine.

In order to increase the hydrophilicity of the surface of the polymeric microspheres and to decrease the level of nonspecific sorption of proteins, dextran (MM = 60,000) was immobilized on the surface of the particles by its covalent bonding with the amino groups of the particles by means of Maillard reaction. After this Vi-antigen was immobilized on the obtained surface.

***Microphotos of the polystyrene particles***

Microphotos of the polystyrene particles were obtained with the use of an electronic scanning microscope "S-570" (HITACHI, Japan) at an accelerating voltage of 15 kV. The samples were prepared by applying a water suspension of the particles with concentration 0.1% mass onto a little metallic table, drying the samples for 24 h and applying a platinum-palladium layer of 100Å thickness with the use of an Eiko IB-3 device (Japan).

***Determination of the particles sizes***

The sizes of the particles were estimated by dynamic light scattering. For this purpose a 0.1% mass water suspension of the polymeric particles was prepared and studied with the use of a Zetasizer Nano ZS particle analyzer (Malvern, Great Britain) according to the producer technique.

***Determination of the sizes of the polymeric suspension particles by optical microscopy***

In order to create a chart of the size distribution of the particles we used photos of the polymeric suspension obtained with the use of a Motic B Series optical microscope equipped with a KY-F32 color video camera. For this purpose we applied a 0.1% suspension of the particles on the specimen glass of the microscope. The results obtained from the photos were subjected to calculation with the use of ImagePro-Plus 6.0 program. Statistical data processing by the sizes of particles was carried out with the use of Microsoft Excel program.

***Carrying out the agglutination reaction***

The agglutination reaction was carried out in the holes of a polystyrene tablet. For carrying out agglutination in the tablet holes we performed consecutive twofold dilution of Vi-antigen antiserum. The last hole in the row was filled with the phosphate-buffered saline and considered as a negative test. Then a diagnosticum was added into each hole. The reaction titer was taken into account according to the last dilution of the serum, at which the intensity of the agglutination reaction was not less than 3+++. Besides, we studied the sensitivity of the diagnosticum in the phosphate-buffered saline in the presence of a surfactant in various concentrations.

***Carrying out and taking into account the results of the control in the tablet holes***

The control was carried out in U-shaped holes of the polystyrene tablet in the absence and in the presence of a surfactant by resuspending the diagnostic test system in an aqueous solution of the surfactant. The results were taken into account by means of a "Mustek 12000 SP Plus" tablet scanner and ABBYY Fine Reader program.

***Determination of the amount of aggregates in the suspension before and after the detergent addition***

The number of aggregates in the suspension was studied by optical microscopy in the phosphate-buffered saline (PBS) before the detergent addition.

For this purpose 20 μl of a 0.2% suspension of the particles in PBS was mixed with 50 μl of a surfactant solution in PBS at a certain surfactant concentration. Then the polymeric suspensions were kept for 30 and 70 min. The particles concentration was brought to 0.022%. The suspensions were placed into a chamber and photographed by means of a "Motic B Series" optical microscope (COIC, China) equipped with a KY-F32 color video camera. The obtained images were analyzed visually by counting the number of the aggregates.

***Photos of the tablet holes***

The photos were obtained with the use of an MBS-9 optical microscope equipped with a KY-F32 color video camera.

## Results and Discussion

The method based on the agglutination reaction of particles, with the surface of which antigenes or antibodies are bonded, is one of perspective diagnostic methods. Advantages of this method are: the absence of measuring equipment; the quick obtainment of results; the simplicity of carrying out the reaction; the absence of radiation and reactants dangerous for humans; the possibility of long storage of the diagnosticum.

However, erythrocyte diagnosticums have a number of disadvantages:

– Erythrocytes are obtained from the blood of animals, the maintenance of which is labor-consuming and expensive.

– Erythrocytes contain a lot of own antigene determinants, which can react with the components of human and animal serums. This can cause nonspecific reactions and lead to false-positive results.

– The properties of erythrocytes often depend on their source and the method of their isolation, as well as the time of storage, which complicates standardization of the obtained diagnosticums.

These shortcomings can be removed by replacing erythrocytes with polymeric microspheres. The properties of the latter should conform to the following requirements: sedimentation rate 3–8 mm/h; particles diameter about 5 μm; narrow size distribution; aggregative stability in electrolytes in a wide range of pH; reproducible properties and characteristics; the presence of functional groups in the surface layer, the groups being capable of covalent bonding with biological macromolecules without considerable change of their functional activity [3].

Polymeric microspheres with such properties were obtained by inoculated copolymerization of styrene with divinylbenzene followed by their subsequent modification providing high concentration of amino groups on the surface. The microspheres were used for obtaining a diagnosticum for Vi-antigen antibodies (to determine typhoid fever) (Figures 1 and 2).

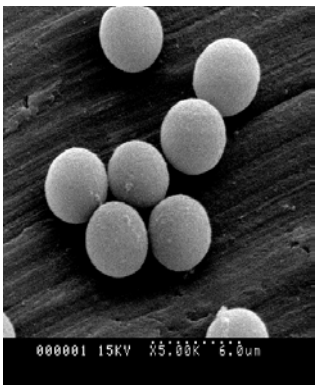



**Figure 1.** Microphotos of chloromethylated aminated polystyrene particles.


**Figure 2.** Size distribution of the chloromethylated aminated polystyrene particles.

The created diagnosticum for Vi-antigen antibodies has sensitivity comparable with that of the erythrocyte-based diagnosticum, which is equal to 1:640 (Figure 3).

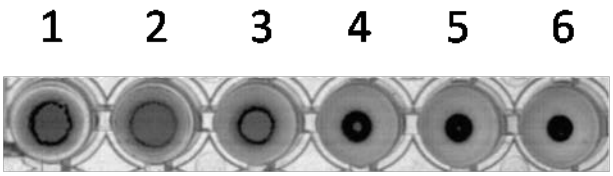



**Figure 3.** Results of determining the sensitivity of the tiphoid fever diagnosticum, serum dilution:
1 – 1:160; 2 – 1:320; 3 – 1:640; 4 – 1:1280; 5 – 1:2560; 6 – test.

We found that the obtained diagnostic test system had a negative "button"-shaped test with indistinct borders ("aura"). This complicates reading the results and gives a false idea about the test system sensitivity (Figure 4A). It was assumed that the particles of the diagnostic test system have high adhesion to the surface of the polystyrene plate, in which the latex agglutination reaction is carried out, due to the high hydrophobicity of their surface.

In order to solve this problem it was suggested to modify the surface of the polymeric particles with surfactants of various nature. For simple and effective registration of the surfactant effect we found conditions, under which the diagnostic test system does not form originally a "button" when carrying out negative control, but forms only an "aura" on the wall of the hole (Figure 4B). In these conditions we found the minimum concentration of the surfactant at which the "button" is formed (Figure 4B). Besides, we determined the concentration of the particles (0.055% mass) at which the "button" is not formed, but the "aura" on the wall of the hole is formed (Figure 5).

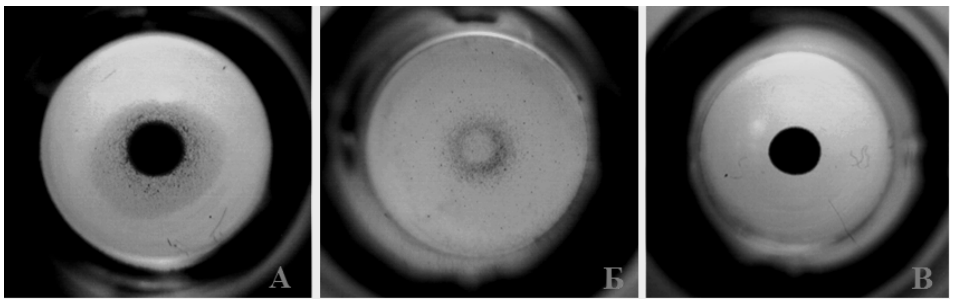



**Figure 4.** Photos of tests obtained by optical microscopy:
А (A) – with indistinct borders (“aura”); Б (B) – with an “aura” and without a “button”;
В (C) – in the presence of Tween-80 surfactant.

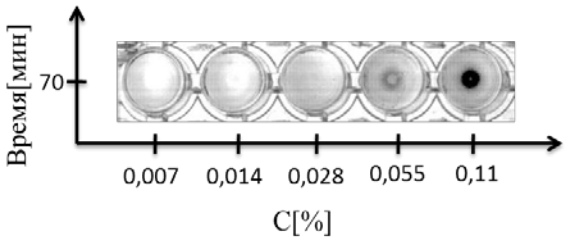



**Figure 5.** Results of a negative test at various concentrations of the particles.
Время [мин] means Time [min]

In order to modify the surface of the particles the following surfactants were chosen: sodium dodecylsulphate, Tween 20 (polyoxyethylene (20) sorbitanmonolaurate) and Tween 80 (polyoxyethylene (20) sorbitanmonooleate), as well as Triton X-100 (mono(tetramethyl-butanol)phenyl ether of polyethyleneglycol). They are widely used in biotechnology [4, 5].

The study results showed that at all the studied sodium dodecylsulphate concentrations no improvement of the negative test occurs (complete absence of “button” formation is observed in the series **d**, Figure 6). When Tween 20 and Tween 80, as well as Triton X-100 are added, a “button” with distinct borders is formed, the improvement of the test in the presence of Triton X-100 occurring at higher concentrations than in the presence of Tween 80 and Tween 20 (Figure 6). The best results were obtained when modifying the surface of the polymeric particles by Tween 80 (a "button" is formed at lower concentrations than in the presence of Tween 20). Therefore, in order to determine the influence of the surfactants on the sensitivity of the agglutination reaction we performed studies in the absence and in the presence of $6.25 \cdot 10^{-5}$ mol/l of Tween 80 (Figure 7).

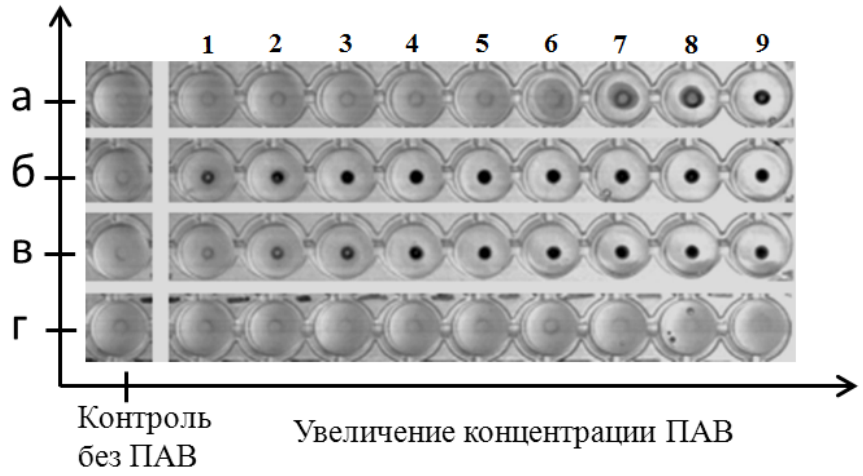



Fig. 6. Results of negative test with a particles concentration of 0.055% mass
in the presence of various surfactants. (The surfactant concentration decreases twice in each next hole starting with the 9th one and to the 1st one.)
а (a) –Triton X-100; б (b) – Tween 80; в (c) – Tween 20; г (d) – SDS
(range of concentrations (0.78–200)·$10^{-5}$ mol/l for a, b, c and (0.013–3.46)·$10^{-3}$ mol/l for d).
[Контроль без ПАВ means Test without surfactant;
Увеличение концентрации ПАВ means Increase of the surfactant concentration]

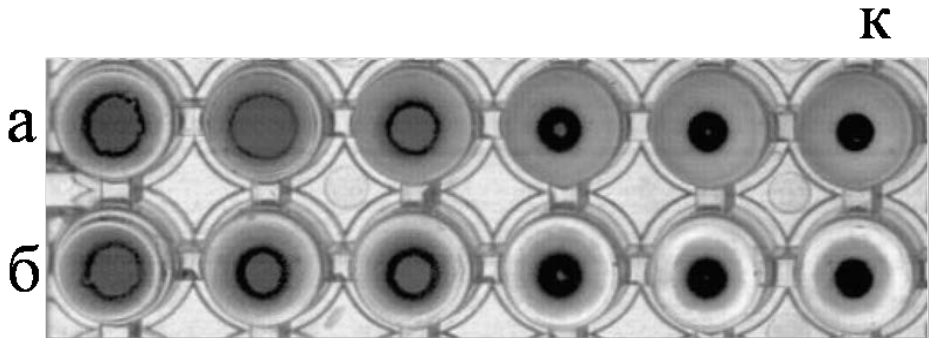



Fig. 7. Results of agglutination reaction of the Vi-antigene diagnosticum
with antiserum in the absence (a) and in the presence (b) of Tween 80 (6.25·$10^{-5}$ mol/l).
[а means a; б means b; К means Test]

It can be seen from the presented data that a black dot on a gray background is observed in the row **a** in the test (T). After the addition of Tween 80 (row **b**) essential reduction of the sizes of the gray background is observed. This indicates an improvement of the negative test of the reaction. Besides, in both cases the agglutination reaction is observed at serum dilution 1/640, which indicates that Tween 80 does not affect the reaction sensitivity.

According to literature data, when modifying the surface of protein-coated particles with Tween 80, aggregation of particles occurs. In order to check these data we carried out experiments with the aim of determining the aggregative stability of the diagnosticum particles in the presence of Tween 80. It was shown by optical microscopy that no aggregation of particles is observed when Tween 80 is present in the concentration chosen for the study (Figure 8).

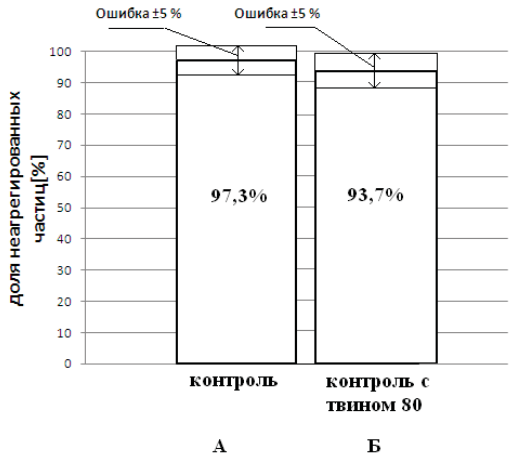



**Figure 8.** Aggregative stability of diagnosticum polymeric particles in the absence (А) and in the presence (Б) of Tween 80.
[доля неагрегированных частиц means fraction of non-aggregated particles; контроль means test; контроль с твином 80 means test with Tween 80; ошибка means mistake]

The obtained data allow recommending Tween 80 and other non-ionic surfactants, which positively affect the stability of polymeric particles, for the modification of the surface of polymeric particles.

## Conclusions

In order to create a diagnostic test system for Vi-antigen antibodies with the use of polymeric microspheres instead of erythrocytes it is necessary:

– to use modified polystyrene microspheres with a diameter about 5 μm, narrow size distribution of the particles, aggregatively stable in electrolytes in a wide range of pH, with a high content of amino groups on the surface;

– to modify the surface of the polymeric microspheres by covalent bonding of dextran with the amino groups located on their surface;

– to increase the hydrophilicity of the surface of the polymeric microspheres by adsorption of a surfactant (Tween 80 is recommended).

**References:**

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