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SYNTHETIC WATER-SOLUBLE POLYMERS IN SELF-ORGANIZING NANOSYSTEMS FOR IMMYNOLOGY

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ABSTRACT
This work presents our results in application of water-soluble polymers in immunology-related areas: design of antigenic erythrocytic diagnosticums and adjuvants for production of hyperimmune serums. We found that long-chain polymeric acids (polymerization degree >6000) can give composite particles with erythrocytes and antigens. The resulting product works as diagnosticum in the reaction of passive haemagglutination. Introduction of hydrophobic or weakly basic imidazole units into polymer chain allows to decrease working concentration of the polymer to 0.01 mg/mL. New test-systems are more sensitive, reproducible and stable than the known analogs.

Tetrazole- and imidazole-containing polymers are active as immunologic adjuvants. Their complexes with antigens stimulate production of antibodies to diphtheria, encephalitis, hepatitis and chicken Marek's disease. The polymeric adjuvants provides 2-10 times higher antibody titers comparing with standard (complete Freund's adjuvant). Low polymer concentration (0.03-0.6 mg/mL) in adjuvant solutions gives rise to cheapness and high usability of the new adjuvants.

The distinguishing feature of the used polymers is their ability to give target products without covalent bonding with antigens. Formation of the polymer-antigen complexes and their self-organization proceeds by multiple cooperative hydrogen/ionic bonds.

KEY WORDS
Functional polymers, antigenic erythrocytic diagnosticums, immunologic adjuvants, self-organization, interpolymeric complexes.

1. Introduction

Synthetic functional polymers were discussed as promising substances for medical applications during several decades [1-3]. Their activities include such important areas as immunopotentiation, correction of haemostasis, blood substitutes, design of prolonged medicines. For instance, iron-containing poly(acrylic acid) is effective haemostatic [4], polycation polyoxidonium is known as commercial immuno-adjuvant [5]. Smart drug-delivery systems are often based on synthetic macromolecules [6-10]. The interesting peculiarity of physiologically active polymers is the absence of strict dependence between chemical structure and activity, comparing with low-molecular precisely targeted medicines. The inherent activity of functional macromolecules is often connected with their ability to interact with biopolymers (proteins, polysaccharides, cell membranes, etc.) by cooperative interactions through ionic and/or hydrogen bonds. So, the effect of polymeric preparations considerably depends on acid-base properties and hydrophobic-hydrophilic balance of the macromolecules.

This work presents our results in application of water-soluble polymers in immunology-related areas: design of antigenic erythrocytic diagnosticums and adjuvants for production of hyperimmune serums.

2. Erythrocytic diagnosticums

The idea to use red blood cells as carriers of immuno-active species, antigen and antibodies had been formulated >50 years ago. They were used as supports of the antigens in test-system for syphilis [11] and this method is still actual. Erythrocytes coated with antigen can interact with the corresponding antibodies resulting in such immunological phenomena as agglutination and haemolysis. The most known realization of these ideas is "the reaction of passive haemagglutination" (RPHA). This reaction is also possible in reverse version: antibodies are immobilized on the erythrocyte surface and the analyzed antigen is in the solution. In spite of the long history of this method, new RPHA procedures are elaborated nowadays [12-15]. The main competitor of RPHA is Enzyme-Linked ImmunoSorbent Assay (ELISA) which is more sensitive method. At the same time, RPHA has some advantages ensuring its survivability:

– a lesser amount of false positive result than in ELISA;
– simplicity and cheapness of the analysis, which do not require skilled personnel and any special equipment excepting 96-well polystyrene plates;

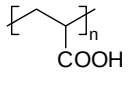
– preparation of the diagnosticums is also simple, the necessary equipment consists of routine laboratory centrifuge only;
– construction of the erythrocytic diagnosticums is an universal procedure, so anybody having the corresponding antigen (antibody) can create the test-system.

RPHA is unmatched method in immunoscreening of large populations, in the control of vaccination efficiency, especially in field conditions or under-developed countries. The main disadvantages of RPHA are:

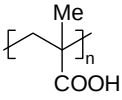
– low sensitivity comparing with ELISA. This sensitivity is sufficiently for control of antibody level but is not enough to detection of antigens from malignant microorganism;
– low stability and reproducibility of the test-systems. Freshly prepared dispersion of the coated erythrocytes in water is stable no more than 2-3 months. Freeze-drying stabilizes the preparations but brings to naught the advantage of simplicity and cheapness.

Design of the erythrocytic diagnosticums consist of two stages: sensitization of the erythrocytes with some binding agent and reaction of the sensitized cells with antigen (antibody). Various binding agents (rivanol, amidol, hydroquinone, tannin, $CrCl_3$) were tested and the most used at present time is chromium chloride.

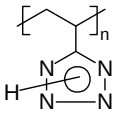
We tried to use hydrophilic polymers as binding agents in construction of erythrocytic diagnosticums. The studied polymers contain acidic (carboxy and tetrazole), basic (imidazole) and hydrophobic moieties:



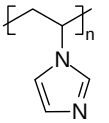
Poly(acrylic acid) (**PAA**)



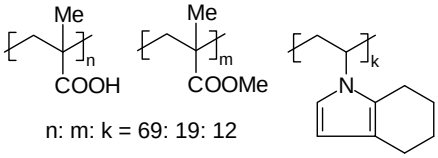
Poly(methacrylic acid) (**PMAA**)



Poly(5-vinyltetrazole) (**PVT**)



Poly(1-vinylimidazole) (**PVI**)



n: m: k = 69: 19: 12

Co(methacrylic acid – methylmethacrylate – 1-vinyl-4,5,6,7-tetrahydroindole) (**MAAIn**)

These polymers were selected because of the known ability of polymeric acids and PVI to give complexes with proteins [16-18]. MAAIn was obtained by radical copolymerization of methylmethacrylate and 1-vinyl-4,5,6,7-tetrahydroindole following with alkaline hydrolysis. Tetrahydroindole groups are very hydrophobic and can give additional aggregation effects in water [19, 20].

Erythrocytic diagnosticums were prepared by incubation with water solution of polymer, washing off unconjugated polymer and reaction with solution of antigen from cell walls of pathogenic *Corynebacterium diphtheriae* and symbiotic *Bifidum longum*. The treatment with synthetic polymer results in polymeric film on the surface of erythrocytes (Figure 2, *B*). Antigen looks like 50-200 nm round or elongated particles (Figure 2, *C, D*).

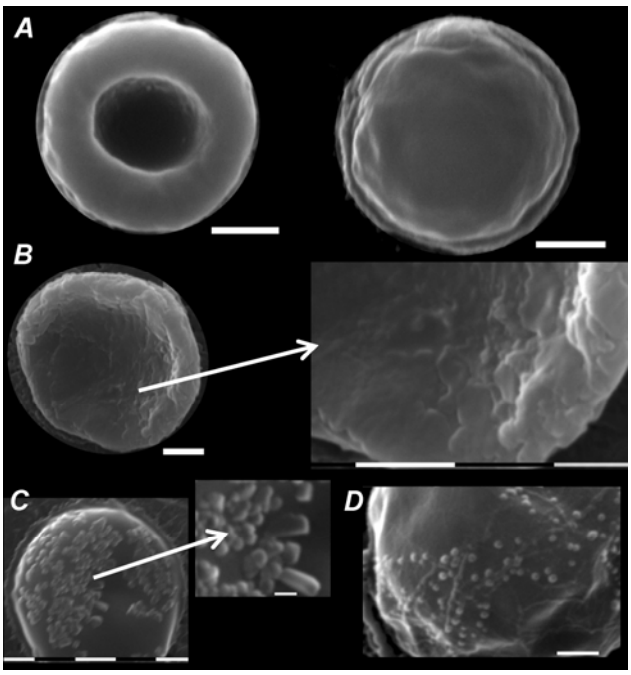


Figure 2 Scanning electron microscopy images of initial sheep erythrocytes (***A***), erythrocyte activated with PVI (***B***) and erythrocytes with immobilized antigen of *C. diphtheriae* (***C***) and *B. longum* (***D***). Scale bar: 1 μm (***A-C***), 500 nm (***D***) and 200 nm (***C***, insertion).

Erythrocytic diagnosticums prepared with various binding agents were tested in analysis of antibody-containing serums from groups of 10-15 patients. The optimal concentration of PMAA was 0.8 mg/mL (Figure 3). Polymer-containing diagnosticums are more sensitive than test-systems based on $CrCl_3$ (Figure 4). PMAA is the most active among studied polymeric acids (Figure 5). Decrease of polymerization degree of synthetic polymers considerably decrease their ability to work as binding agents. Probably this is connected with the necessity to have a relatively long chain enough to interact concurrently with antigen and surface of erythrocyte.

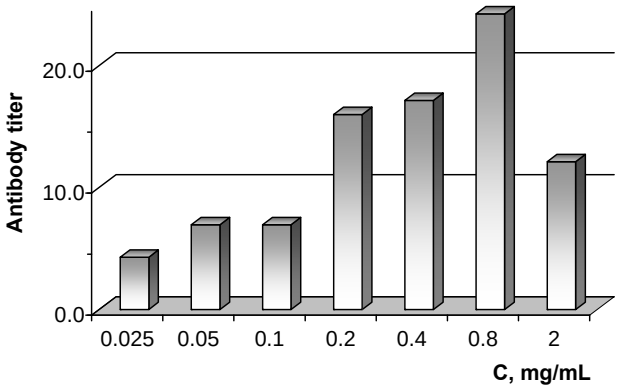


Figure 3 Dependence of average antibody titers (inverse ratio) on concentration of PMAA-25000 using as binding agent

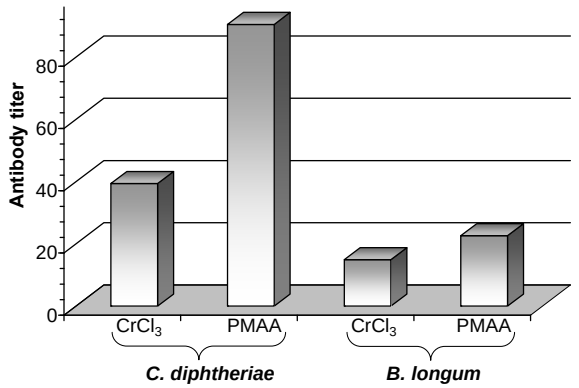


Figure 4 Average antibody titers (inverse ratio) obtained with diagnosticums based on $CrCl_3$ and PMAA (polymerization degree 25000, 0.8 mg/mL).

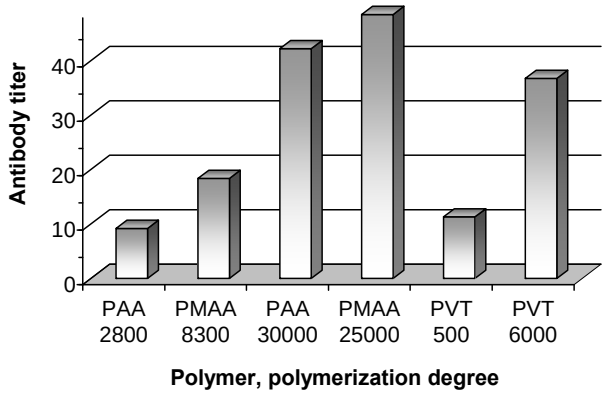


Figure 5 Dependence of average antibody titers (inverse ratio) on the nature and polymerization degree of polymeric acids.

Insertion of tetrahydroindole units into PMAA chain allows to decrease working concentration of the binding agent (Table 1) apparently due to additional hydrophobic interactions with erythrocyte surface and hydrophobic moieties in antigen macromolecules. PVI is very active in interactions with proteins in physiological conditions [18]. It is a peculiarity of PVI that imidazole units are unprotonated near pH 7 but rather active in hydrogen bonding which explain high reactivity of PVI in interpolymeric reactions [21]. So, it was not unexpected to find high sensitivity of PVI-based diagnosticum at very low concentration of polymer in the binding composition (0.01 mg/mL, Table 1).

Table 1
Testing of erythrocytic diagnosticums with immobilized bifidobacterium and diphtheria antigens

Binding agent	Antibody titers (inverse ratio)						
	4	8	16	32	64	128	256
Bifidobacterium							
MAAIn, 0.8 mg/mL	+	+	+	+	+	+	+
MAAIn, 0.04 mg/mL	+	+	+	+	+	+	+/–
PVI, 0.01 mg/mL	+	+	+	+	+	+	+/–
PVI, 0.005 mg/mL	+	+	+	–	–	–	–
Hydroquinone, 4.3 mg/mL	+	+/–	–	–	–	–	–
Diphtheria							
MAAIn, 0.8 mg/mL	+	+	+	+	+	–	–
MAAIn, 0.04 mg/mL	+	+	+	+	+	–	–
PVI, 0.01 mg/mL	+	+	+	+	+	–	–
PVI, 0.005 mg/mL	+	+	–	–	–	–	–
Hydroquinone, 4.3 mg/mL	+	+/–	–	–	–	–	–

"+" – full agglutination, '–" – negative reaction, "+/–" – ambiguous result. The results were obtained from an averaged serum.

Erythrocytic diagnosticums based on polymeric binders were used in scientific and medical practice of our laboratories more than 5 years. On the whole, they showed the following advantages comparing with the known test-systems with chromium chloride or hydroquinone:

- >10 times higher sensitivity;
- good reproducibility between various batches;
- long shelf life (>3 years at +4°C).

Thus, the using of functional polymers allows to produce new generations of erythrocytic diagnosticums competitive with the other immunologic test-systems.

3. Immunologic adjuvants

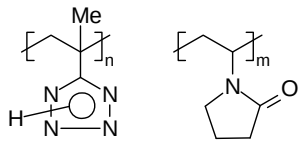
Vaccination remains the main active-prophylaxis method for the prevention of infectious diseases. Different strategies have been developed to induce cellular and/or humoral immune responses against microorganisms or their product in order to limit microbial pathogenicity and to prevent actual disease development. Synthesis of antigen-specific antibodies is the basis for the neutralization and/or opsonization effect of the majority of vaccines. On the other hand, the expansion of antigen specific Th1 type helper and cytotoxic T lymphocytes is the main target of vaccinations aimed at protection from

pathogens with intracellular growth. It is known that administration of pure antigens to humans as well as animals does not result in a highly efficient immune response. Much better results can be achieved with the administration of antigens in association with special compounds: adjuvants. Several diverse adjuvants have been tested in laboratory animals, to induce preferential cellular or humoral responses, such as oil-based mixtures, killed mycobacteria, colloidal solutions of Au and Ag, some natural polymers, unmethylated CpG sequences, but aluminum hydroxides (alum) is currently the only adjuvant really used for humans. Adjuvants behaves as "danger signals" [22] for the cells of the natural immune system that trigger the initiation of an adaptive immune response. Mechanisms of adjuvant action also include prolongation of the antigen half-life in tissues.

Several decades ago it was found that water-soluble synthetic polymers can work as effective adjuvants [23]. They show high activity with the absence of production of irrelevant antibodies which can be formed in the case of adjuvants based on natural materials. The important advantages of polymeric adjuvants are low price, high storage life of solid polymers, low viscosity of the working solution. There are two principal routes for construction of antigen-polymers conjugates:

– covalent binding of antigen or hapten with water-soluble polymer [24]. These conjugates are characterized by high stability in the body but it is necessary to develop the binding procedures for each type of antigen taking into account that some chemical agent can dislocate structure of antigen.
– solution of antigen can be mixed with adjuvant such as in the case of the well-known natural oil-based complete Freund's adjuvant (**CFA**). Interaction between polymer and antigen is realized by hydrogen and ionic bonds [25-27]. It is more easy adjuvanting method but the requirements for the structure of synthetic polymer are more extensive: the polymer has to be able to form stable complex with antigen, the obtained complex has to be soluble and to have free active units for interaction with immunocompetent cells. The reversibility of non-covalent binding is promising for design of "intelligent" immunogenic systems capable of release of antigen at desired pH, ionic strength or in the presence of competing biopolymers.

We have synthesized and studied several polymers as adjuvants: PVI, PVT, poly(5-isopropenyltetrazole) (**PIPT**) and co(5-isopropenyltetrazole – 1-vinyl-2-pyrrolidone) (**IPTVP**):



n: m = 66.6: 33.3

Polymers were used as water solutions which were mixed with antigen solutions in 1: 1 ratio for one hour before injection to experimental animals. CFA was applied as standard. PIPT and high-molecular PVT (560 kDa) did not show adjuvant activity. Introducing of pyrrolidone units into isopropenyltetrazole chain resulted in high adjuvant activity: antibody titers with IPTVP (80 kDa) exceeded values for CFA in 2-10 times (Table 2). Low-molecular PVT (47 kDa) shows some activity and PVI (30 kDa) is the most active adjuvant with cell walls of *C. Diphtheriae*.

Protective efficiency of IPTVP and PVI was studied against chicken Marek's disease. Survivability in acute experiments increased from 70 to 100% when standard vaccine diluent was changed with IPTVP and to 97.8% with PVI.

The polymers are non-toxic (LD50 > 1200 mg/kg) and do not give skin irritation after injection. Their optimal concentration is 0.03-0.6 mg/mL and depends on the antigen nature. In addition to high activity, new polymeric adjuvants have more advantages:

– cheapness taking into account low concentration of the active component;
– low viscosity which is important for injection, especially in works with small animals;
– absence of any proteins or other bipolymers which can induce allergic reactions.

Table 2
Antibody titers (inverse values) under use of synthetic immunologic adjuvant.

Antigen	Adjuvant			
	IPTVP	PVT	PVI	CFA (standard)
Cell walls of *C. Diphtheriae*	64	8	100	6
Surface antigen of virus B hepatitis	128*	ndt	ndt	64
Surface antigen of California encephalitis complex virus (Irkutsk №1796)	160	ndt	ndt	40
Surface antigen of Batai virus № 2513	80	ndt	ndt	40

4. Conclusion

We have found several polymeric structures capable to interact with immuno-active particles, in particular with antigens. The resulting aggregates can be used as erythrocytic diagnosticums of new generation and as basis of new vaccine preparations. The distinguishing feature of the used polymers is their ability to give target products without covalent bonding with antigens. Formation of the polymer-antigen complexes and their self-organization proceeds by multiple cooperative hydrogen/ionic bonds.

Further study of these interesting and promising systems requires thorough interdisciplinary work with collaboration of specialists in chemistry, nanomaterials, biochemistry and medicine.

Acknowledgement

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