# Synthesis of Polymer Suspensions for Immunochemical Studies

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SYNOPSIS

New methods of preparing polymer suspensions of narrow particle size distribution with amino and carboxylic groups on the particle surface have been suggested. The effects of various factors on the diameter and size distribution of polymer particles, their stability, and concentration of functional groups on the particle surface were investigated. The application of such polymer suspensions for immunochemical studies was shown. © 1996 John Wiley & Sons, Inc.

## INTRODUCTION

Functional polymer suspensions are widely used in medicine, biology, and biotechnology. The application of polymer suspensions in these areas is limited by the structure of microspheres and by the methods of their synthesis.

The functional polymer suspension must

- have narrow distribution of particles by size,
- have reproducible colloid-chemical properties,
- be stable under storage, and
- preserve their chemical properties in physiological solution.

Polymer particles must also contain surface functional groups of definite concentration able to bind covalently with bioligands.[1]

Of great interest is the use of functional suspensions whose particles contain amino groups on their surface. Such groups are highly reactive and easily activated by available difunctional compounds (such as glutaric aldehyde).

Methods of synthesizing functional polymer suspension with amino groups on the surface of the microspheres, presented in scientific literature, presume either the use of multistep reactions of polymer–analogue transformations or a polymerization of different monomers, such as aminostyrene.[2,3] In this connection the research on the development of simple and effective methods of suspension preparation is of great scientific interest and is carried on by a number of investigators. The most attractive is the application of compounds containing reactive amino groups, i.e., of sulfur-containing amino acids.

The scientific literature gives data on the modifications of the artificially received polyisoprene dispersion by sulfur-containing cystine or cysteine.[4] Due to the presence of reactive disulfide and thiol groups in the molecules of these compounds, they are capable of direct interaction with partially polarized double bonds of 1,4-cis-polyisoprene. This reaction goes by ionic mechanism and results in amino acid residue of following structure: $-S-CH_2-CH(NH_2)COOH$. The application of such modified polyisoprene dispersion for the immunodiagnostic test-systems development is highly problematic because of the low stability of suspensions during their cleaning or storage and of wide distribution of suspension particles by size. The application of polyisoprene–styrene suspensions with higher stability than polyisoprene ones is more promising in this respect.

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Cystine and cysteine may be introduced into the starting mixture of monomers either immediately at the beginning of polymerization or modification of the preliminary synthesized polystyrene–izoprene suspensions. Both of these approaches to the preparation of polymer suspension with amino groups of controlled concentration on the surface of microspheres for immunodiagnostic researches were closely analyzed in this paper.

## EXPERIMENTAL

### Materials

Styrene (purity 99.6%) was purified before use by distillation under reduced pressure (36°C, $2.6 \times 10^3$ Pa). Isoprene (purity 99.0%) was double distilled under atmospheric pressure. Distilled water was used in all experiments. All polymerizations were conducted in a nitrogen atmosphere. Other commercial reagents (potassium peroxidisulfate, sodium salt of EDTA, ferrous sulfate, rongalite, cumene hydroperoxide, cysteine, hydroxylamine hydrochloride, glutaric dialdehyde, formalin) were used without further purification.

### Preparation Procedure

Emulsion, emulsifier-free, and seed polymerization were carried out in a jacketed, glass, round-bottomed reactor with a volume of 250 mL thermostated to an accuracy of ±0.1°C. The reaction mixture was stirred with an anchor stirrer; the distance between the shoulders of the anchor stirrer and the reactor wall was 0.5 cm. Oxygen was removed from the monomer phase by purging the purified nitrogen for 30 min; boiling water used in the preparation of the aqueous phase was cooled in the stream of nitrogen. After the required quantities of monomers, water, and seed latex particles (in the case of seed polymerization) were fed into the reactor, an inert gas was introduced to remove oxygen. The reactor was thermostated to required temperature, the initiator was added to the mixture, and stirring was begun. In the case of seed polymerization the monomer (isoprene) and water phases were stirred at 2–5°C to swell particles with monomer, followed by initiator addition.

For the emulsifier-free polymerization the following components were used: 100 wt part of styrene, 500 wt part of water, and 1 wt part of potassium peroxydisulfate.

To introduce amino groups on the surface of microspheres, polymer suspensions were modified with cysteine at different condition.

To obtain aldehyde groups, the modified microspheres were treated with glutaric dialdehyde.[5]

### Characterization

The latex particles were cleaned by means of ultrafiltration techniques using an Amicon filtration device.

The size and polydispersity of polymer particles were determined with a Coulter Model 4MD apparatus. Accuracy of the method was checked by evaluation of scanning electron microscope micrographs.

The morphology of particles was investigated using microtome cutting of stained particles. Thin microtomed sections (ca. 100 nm) of the polystyrene/polyisoprene composite particles were obtained under frozen conditions using an LKB Cryomicrotome equipped with a diamond knife. The sections were stained with osmium tetroxide and examined in a Phillips 400 TEM.

The definition of amino and carboxylic groups concentration was carried out by conductometric methods on Mera-Elwro (Poland) conductometer Model N5721 under 25°C and 3500 Hz frequency.[6]

## RESULTS AND DISCUSSION

Copolymerization of isoprene with styrene was carried out at mass ratio 70/30, respectively, in the presence of a redox initiation system at 5 ± 0.1°C.[6] Cystine and cysteine of different concentrations were introduced into the emulsion of monomers; potassium oleate was used as emulsifier, at a concentration of 6 wt % per monomer.

Figure 1 gives the conversion–time curves, obtained in the process of isoprene–styrene copolymerization in the presence of cystine and cysteine (curves b and c, respectively) and in their absence (curve a). It is seen that copolymerizations of monomers reach high conversion with practically equal rates both in the presence and absence of cystine. At the same time, in the presence of cysteine the rate of polymerization falls remarkably after 30–40% conversion.

It was supposed that the fall in polymerization rate is due to the depletion of the initiator due to the side reaction between hydroperoxide and cysteine, which was proved by spectroscopy and conductometric research of polymerized systems. The introduction of additional initiator into the reaction

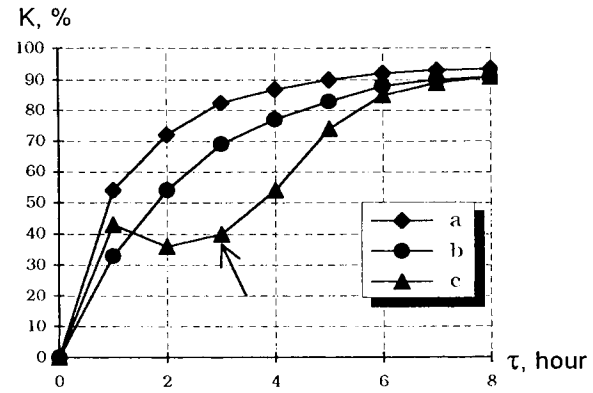


**Figure 1** Conversion ($K$)-time ($t$) curves of isoprene and styrene copolymerization. Monomer mass rotation equals 70 : 30 correspondingly: Curve a, without amino acid; curves b and c, in presence of cysteine (1.5 w. mass part/polymer) and cystine (3.0 mass part/polymer), respectively.

system, in the form of styrene solution, allowed polymerization to continue to deep conversion—90–95% (curve c).

Infrared-spectrum analyses of polymer films received from polymer suspensions show that they contain the absorbing stripes specific for valence oscillation of carboxylic (3300–3000 $cm^{-1}$) and amino (3100–3000 $cm^{-1}$) groups and for deformation oscillation of amino groups (1650–1450 $cm^{-1}$). This indicates that spontaneous connection of amino acids with polymer takes place. Poly(isoprene–styrene) suspensions, received in the absence of amino acid, were modified by cystine and cysteine in a glass reactor at 50°C and agitation speed 100–150 turns/min. Spectrum analyses of polymers showed they contain the same absorption stripes.

The definition of the copolymers' element composition showed that when cysteine is used the quantity of amino acid groups in the copolymer is much higher than when cystine is used, both taken in equimolar concentration. It was supposed that this results from the process of transmitting the polymer chain onto the amino acid, and confirmed by the data of characteristic viscosity of toluene solutions of copolymers obtained from modified suspensions.

For quantitative estimation of amino acid groups' concentration on the surface of microspheres, the modified method of conductometric titration was used. Typical conductometric titration curves are shown in Figure 2. It is clear that the conductometric titration curves of nonmodified polymer suspension have two characteristic parts: the first corresponds to titration of carboxylic groups of oleic acid and the second to collection of free alkaline (curve a). A third part, corresponding to the titration of amino acid's carboxyl group (curves b and c), appears on the curves of titration of modified polymer suspensions.

Table I clearly shows that in the case of cysteine modification, the concentration of functional groups on the surface of particles is the greatest. It is worth noting that the method of polymer-suspension modification does not influence the amino group concentration on the surface of the particles.

One of the most important demands on functional suspensions is high stability in physiologic media. The research shows that the modified suspensions are characterized by high stability of the electrolyte solution (0.2$M$ NaCl) due to the increase in concentration of ionogenic groups on the surface of suspension particles.

According to scientific literature, the sensitivity of immunochemical reaction is defined not by maximum but by some optimal concentration of functional groups on the surface of microspheres. *The optimal concentration of amino groups defines the concentration of albumin connected with these groups, which is necessary for high sensitivity of immunochemical reaction.*

First it is necessary to estimate what quantity of amino groups on the surface of microspheres may be achieved as the result of polymer suspension modification with amino acids and to choose the methods for definition of the contents of amino and carboxylic groups on the surface of microspheres, provided the reliable results.

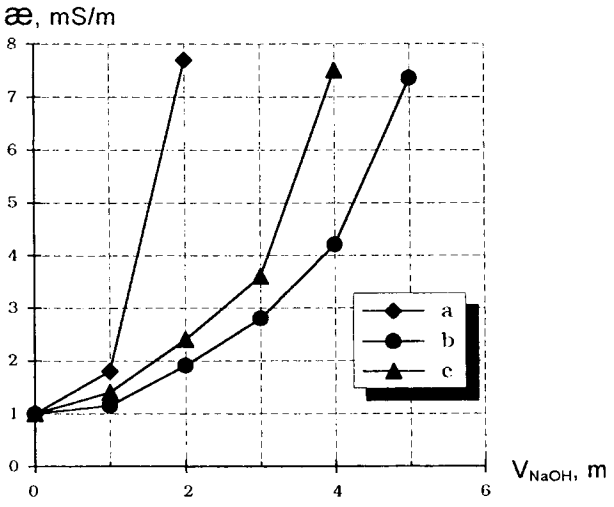


**Figure 2** Conductometric titration curves: Curve a, nonmodification polymer suspension; curves b and c, modification polymer suspension by cysteine and cystine, respectively.

**Table I Results of Consuctometric Titration of Isoprene Styrene Suspensions Modified with Cystin and Cystein**

| Type of Suspension | Concentration of Carboxylic Group (mmol/g) | | Degree of Connection of Amino Acid (%) |
|---|---|---|---|
| | Oleine Acid | Amino Acid | |
| Nonmodification | 17.7 | — | — |
| Modification by: | | | |
| Cysteine (3 mass part) in process at synthesis | 18.0 | 40.9 | 16.5 |
| Cystine (1.5 mass part) in process of synthesis | 16.5 | 37.9 | 15.3 |
| Cysteine (3 mass part) preliminary received suspension | 17.9 | 69.4 | 28.0 |
| Cystine (1.5 mass part) preliminary received suspension | 17.0 | 66.6 | 26.9 |

To solve the first task, the styrene–isoprene copolymerization at different concentrations of cysteine (in the range 1–10 wt % per monomer) was studied. It turned out that under increased cysteine concentration the concentration of amino groups on the surface of microspheres grows unequally, reaching a limited meaning equal to 120 mmol/g under concentration of cysteine equal to 7–8% mass per polymer (see Table II).

Synthesized suspensions in the process of ultrafiltration purification from the remains of unreacted components of reaction partially coagulated and had wide distribution of particles by size. These drawbacks excluded the possibility of using polymer suspensions synthesized by the above-mentioned methods for immunochemical research.

As expected, the stability of polymer suspension grew noticeably with the increase of styrene units in copolymer chains. Copolymerization of isoprene with styrene in this case was under mass monomers ratio of 50/50, with other conditions equal. Nevertheless, the concentration of amino acid groups on the surface of microspheres decreased, possibly due to the lessening of the concentration of the isoprene in the microsphere surface layer zone.

The analyses of the data received have shown the necessity of synthesizing polymer suspensions with "core-shell" type particles. In this case the method of seed polymerization, guaranteeing the reception of polymer suspension with narrow distribution of particles by size and with high concentration of double-linked polyisoprene molecules forming the shell of polystyrene microspheres, was suggested.

The process of receiving modified suspensions is divided into three independent stages (Fig. 3). The first stage produces the synthesis of seed polystyrene particles with given diameters and narrow distribution of particles by size. The second stage produces the seed polymerization of isoprene chains on the particles' surface, and the third stage produces the modification of previously received of isoprene–styrene polymer suspensions with cysteine.

The seed polystyrene microspheres were received by heterophase polymerization of styrene in presence of potassium persulfate as initiator without emulsifier. The hystogram of distribution of the

**Table II Concentration of Amino and Carboxyl Groups on the Surface of Suspension Partical, Received in the Presence of Different Concentrations of Cystein Introduced During the Polymerization Process**

| Concentration of Cystein Mass Part | Concentration of Groups | | Degree of Concentration (%) | Surface per One Amino Group (Å²/groups) |
|---|---|---|---|---|
| | Carboxylic | Amino | | |
| 1.0 | 20.3 | 21.5 | 28.5 | 1037.0 |
| 3.0 | 58.7 | 64.0 | 28.0 | 260.0 |
| 5.0 | 100.1 | 109.5 | 29.0 | 109.0 |
| 7.0 | 100.5 | 120.2 | 20.0 | 103.0 |

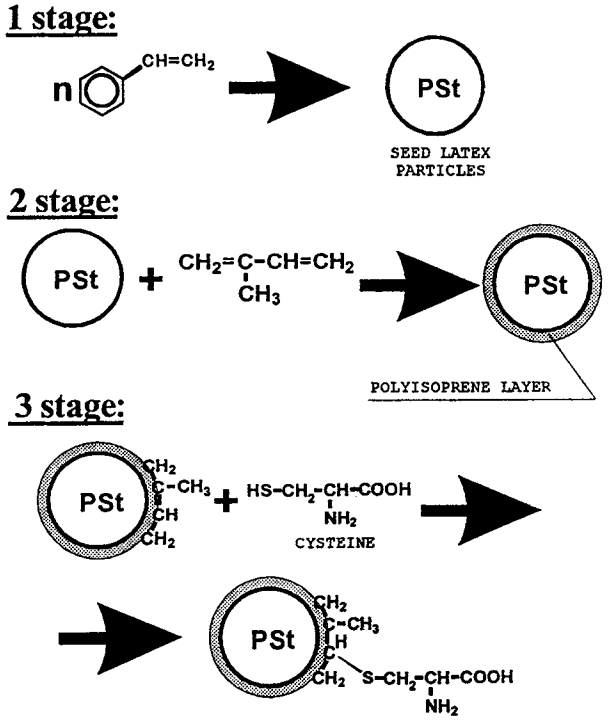


**Figure 3** The scheme of the synthesis of functional polymer suspensions.

particles by size is shown in Figure 4(a). It is clear that the median diameter of particles is about 1 mkm and the particles have narrow distribution by size. During the second stage—seed polymerization of isoprene—it is necessary to exclude the appearance of new monomer–polymer particles.

The conditions of seed polymerization were chosen on the bases of data on the dependence of time and the degree of swelling of polystyrene particles from isoprene concentration: the time of swelling of polystyrene suspension is 10–12 h under temperature of 5–7°C, and concentration of isoprene is not higher than 10 mass % per polymer.

The hystograms of the distribution of polymer suspensions by size are shown in Figure 4(b), which shows that the particles grow in size under storage of narrow distribution by size.

Electron microscopy research of ultrathin cuts of polymer particles treated with $O_sO_4$ showed that the particles have a core–shell type structure.

Because the modification of polystyrene suspensions with cysteine is the key factor from the point of view of the creation of necessary concentration of functional groups on the particles' surface for immobilization of albumin, we studied the influence of suspension modification time, pH medium, and the concentration of amino acid in the reaction system on the concentration of the functional groups on the surface particles.

Figures 5 and 6 show the dependence of the ratio of amino group of cysteine on temperature and modification time, and pH medium, respectively. It is clear that optimal conditions for modification are: pH medium 10.0–10.5; time of modification, 2 h; and temperature, 50°C. Such conditions provide for maintenance of narrow distribution of particles by size [Fig. 4(c)].

The investigation of amino-group concentration dependence on cysteine concentration showed that

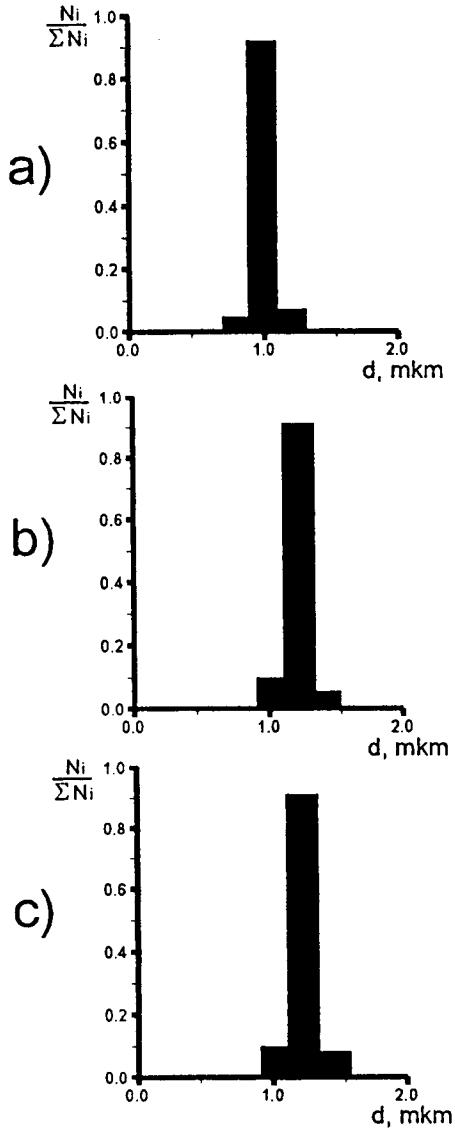


**Figure 4** The hystogram at the distribution of polymer particles by size: (A) after the stage of the emulsifier-free heterophase polymerization; (B) after the stage of the seed polymerization with isoprene; (C) after the stage of the modification of prepared particles by cysteine.

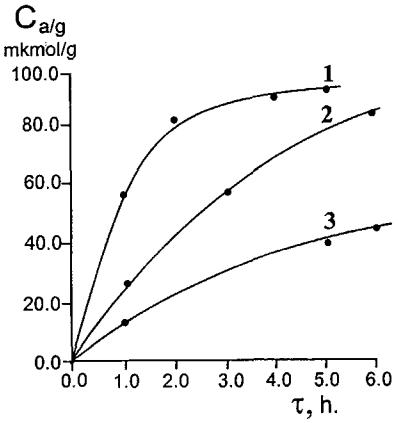


**Figure 5** The dependence of surface amino group concentration ($C_{a/g}$) vs. the temperature and time of modification: (1) 50°C, (2) 40°C, (3) 30°C.

the limited concentration of amino groups is about 150 mkmol/g. This concentration limit is determined by the contents of double links of polyisoprene on the surface of particles (Fig. 7). The particles of polymer suspension contain on the surface both sulfate and carboxyl groups.

The sulfate groups on the surface of polymer particles are formed as the result of initiating reaction by potassium persulfate, but the formation of carboxyl groups is determined by hydrolysis of sulfate groups to hydroxyl, with their following oxidation according to the Kolthoff reaction.[7]

High concentration of ionogenic groups on the surface of the particles provides great stability of suspension to electrolytes [Fig. 8(b)]. Significant decrease in the concentration of ionogenic groups on the surface of the particles received during seed

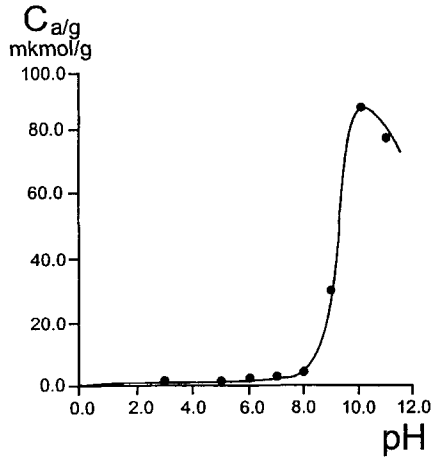


**Figure 6** The dependence of surface amino group concentration ($C_{a/g}$) vs. the pH of reaction medium.

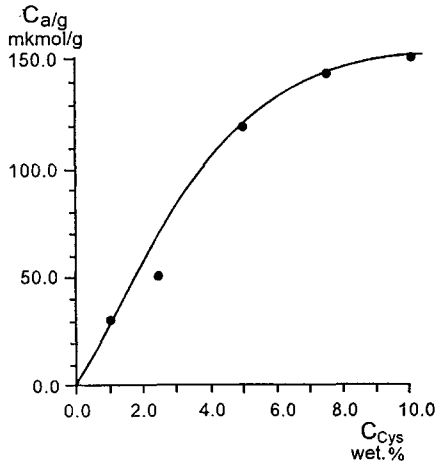


**Figure 7** Surface amino group concentration ($C_{a/g}$) as a function of a concentration of cysteine in reaction medium ($C_{cys}$).

polymerization of isoprene takes place due to their partial screening with polyisoprene molecules, formed on the particles' surface. The suspension stability in electrolyte solution falls as much as 0.05

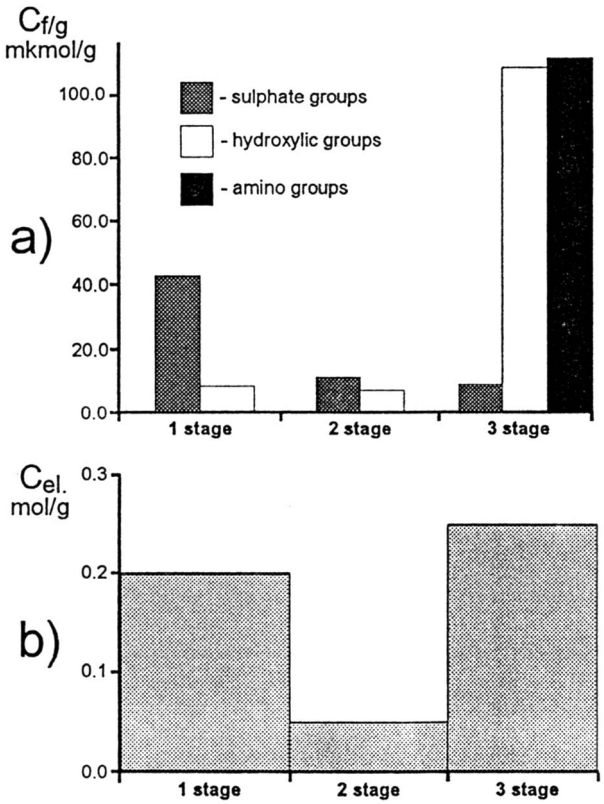


**Figure 8** Modes of surface functional group concentration $C_{f/g}$ (A) and stability of polymer suspensions under different electrolyte (NaCl) concentrations $C_{el}$ (B) after various stages of preparation process.

moles per liter. The modification of the polymer suspension with cysteine restores stability to the previously shown level.

On the basis of these polymer suspensions, highly sensitive diagnostic test systems were created, i.e., for air-pollution control at factories producing forage additions of a type of paprin.

## CONCLUSIONS

The data presented above indicate that the proposed modification technique makes it possible to obtain a series of polymer microspheres with properties (uniform particle size and size distribution, different surface chemical compositions, etc.) suitable for their use in medicine, biology, and biotechnology.

In particular, such modified latex particles were applied in developing a diagnostic kit for environmental pollution control. It was found that latex particles with amino group surface concentrations in the range 40–100 mmol/g displayed the optimum properties. Investigation of sensitivity changes in obtained immunomicrospheres during a one-year storage showed no remarkable decrease in sensitivity.

The clinical tests show that in comparison with commercial diagnostic kits on the basis of erythrocytes, the obtained immunomicrospheres display the same sensitivity but have much better reproducible properties, colloidal stability to electrolites, and pH changing. The combination of these properties indicates that modified latex obtained could be widely used in immunochemical investigations.

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