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# Synthesis of monodisperse functional polymeric microspheres for immunoassay

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## Contents

I. Introduction 167
II. Synthesis of polymeric suspensions with functional groups on the particle surface 168
III. Activation of functional groups on the surface of particles of a polymeric suspension 172

**Abstract. Methods for the synthesis of functional polymeric suspensions with narrow particle size distribution are analysed. Attention is concentrated on the conditions of the preparation of polymeric microspheres with aldehyde, carboxyl, epoxy, or amino groups on the surface, which possess a set of properties enabling their use for biochemical studies. Methods of modification of functional groups of the polymeric suspensions that make it possible to accomplish covalent binding of the surface groups to bioligands are considered. The bibliography includes 218 references.**

## I. Introduction

Aqueous suspensions containing strictly spherical polymer particles of identical diameters are usually referred to as polymeric suspensions with narrow particle size distribution (PSD).[1] Typical magnitudes of the variation coefficient (a measure of the deviation of the particle diameters from the mean value) are 1% – 3% (for particles 0.1 – 10 μm in diameter)[2–4] or 10% – 30% (for particles with diameters smaller than 0.06 or greater than 10 μm).[2,5]

Polymeric microspheres with a narrow PSD are used in various fields of science and engineering, for example, as calibration standards in the electron and optical microscopy and light-scattering, for counting aerosol particles, in small-angle X-ray diffraction, for counting viral particles, for determining pore sizes in filters or biological membranes, for stimulating cellular production of antibodies and their purification,[6–9] as model colloid systems for studying their rheology,[10,11] stability, or sedimentation[11] etc. In recent years, monodisperse functional suspension particles have found wide application as protein carriers in development of immunodiagnostic assays.[2]

The function of these assays is based on the immunochemical reaction between an antigen (a substance carrying signs of genetic information alien for a particular type of organism) and antibodies (proteins belonging to the class of immunoglobulins and produced by the cells of the organism's immune system as a response to the appearance of the antigen) yielding an 'antigen – antibody' complex.[12] The reaction in which this complex is formed is highly immunospecific, i.e. in response to the appearance of a particular antigen, the immune system generates antibodies of a strictly specified structure capable of interacting only with this antigen. Since both an antigen and an antibody can react simultaneously with several molecules, spatial networks are produced with the antigen molecules as nodes.

The formation of network agglomerates and, therefore, the appearance of one or another type of antigens can be detected by various methods, for example, by spectrophotometry, turbidimetry, nephelometry, laser self-correlation spectroscopy, etc. The need to use special expensive equipment for the detection of 'antigen – antibody' complexes hampers wide employment of the above methods in the diagnosis of diseases. However, when antigens (or antibodies) are bound to a polymeric carrier serving only as an indicator, the complexes can be detected even by the naked eye as crowding of agglomerates of the carrier particles.

The first communication on successful use of polyvinyltoluene and polystyrene microspheres with a narrow PSD in the diagnosis of rheumatoid arthritis was published in 1956.[13] It was reported that the interaction of antigens present in the blood serum of a patient with gamma-globulin adsorbed on the microsphere surface of a suspension resulted in the agglutination of the polymeric particles affording large agglomerates, easily discernible by the naked eye. Later, publications appeared[14–24] dealing with the use of polystyrene and poly(methyl methacrylate) suspension particles as protein carriers for preparing diagnostic test-systems for other types of diseases.

However, the use of particles of polymeric suspensions in the development of immunodiagnostic tests has been limited by irreproducibility of the results of analysis, which is caused, as has been suggested by Yen et al.,[24] by the fact that on slight variations of the conditions (pH, ionic strength of the medium, temperature, etc.), the protein adsorbed on the surface of particles is desorbed to the aqueous phase; and this lowers the sensitivity of the tests and leads to nonspecific reactions with other biological objects.

Polymeric suspensions containing hydrophilic particles with surface functional groups capable of forming covalent bonds with bioligands have proved to be free of these drawbacks. Polymeric suspensions based on copolymers of methyl methacrylate (MMA), 2-hydroxyethyl methacrylate, acrylic and methacrylic (MAA) acids, acrylamide, vinylpyridine, etc. have been synthesised and used successfully for developing immunodiagnostic tests.[24–29]

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The advantages of these suspensions over hydrophobic ones are the following:

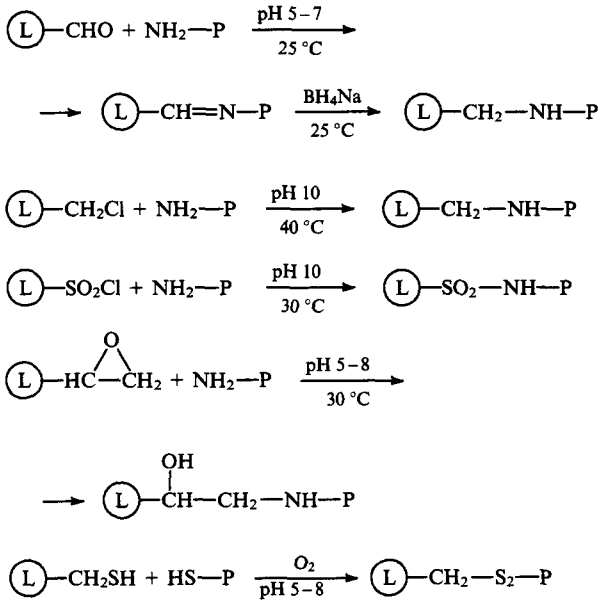
— the absence of nonspecific adsorption of bioligands due to the high hydrophilicity of the surface of particles;

— the absence of desorption of protein molecules from the surface of microsphere due to the covalent bonding between a bioligand and functional groups of a polymeric particle;

— the possibility of covalent bonding of antigens and antibodies that are poorly sorbed on hydrophobic suspension surfaces;

— the possibility of the appearance of conditions ensuring the optimal orientation of biomolecules on microsphere surfaces, which would provide the maximum sensitivity and high specificity of the diagnostica synthesised.

The polymeric suspensions used in immunodiagnostics are classified in terms of the type of functional groups present on the surface. According to this criterion, they can be divided into two types. Suspensions, whose particles contain surface groups capable of direct interaction with amino, carboxyl, or mercapto groups of biomolecules, belong to the first type. These are, for example, chloromethyl,[30-34] chlorosulfonyl,[31] aldehyde,[35-38] epoxy,[39] and mercapto[40,41] groups, which react with biomolecules in an aqueous medium at moderate temperatures.




(L) — polymeric particle; P — protein

Suspensions with functional groups, which are not able to interact directly with proteins, but can form chemical bonds with them after a fairly simple activation reaction, belong to the second group. These are, for example, polymeric suspensions with carboxyl,[2,26,27,42-45] hydroxyl,[24,46] amino,[24,27] amide,[27,47-50] glycol,[51,52] and other groups. Methods of activation of these groups are considered below.

At present, numerous methods of preparation of polymeric microspheres with various functional groups on the surface have been reported (these are emulsion, suspension, dispersion, precipitation, emulsifier-free, seed, and other types of polymerisations). The choice of the method of synthesis is determined by the necessity of obtaining suspensions with a specified set of properties: particle diameter and structure, narrow size distribution, stability in physiological solutions and during storage, the presence of functional groups of a particular nature at a specified concentration on the surface of the particles. Below we survey the methods of the synthesis of functional polymeric suspensions with definite functional groups on the particle surface.

## II. Synthesis of polymeric suspensions with functional groups on the particle surface

### 1. Synthesis of polymeric suspensions with aldehyde groups on the particle surface

Polymeric suspensions with aldehyde groups on the particle surface are obtained by homopolymerisation of unsaturated aldehydes (acrolein, formylstyrene) or by their copolymerisation with monomers of various natures.

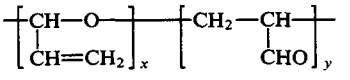
Polymerisation of acrolein has been studied most comprehensively. Two routes of preparing polyacrolein microspheres with narrow PSD's have been reported, namely, the anionic precipitation polymerisation in an alkaline medium[39] and the radical emulsion polymerisation induced by γ-radiation or a redox system.[53]

The radical emulsion polymerisation of acrolein has been carried out in an aqueous solution at a monomer concentration of 10 vol.% in the presence of poly(ethylene oxide) as an emulsifier. The process was initiated by the potassium persulfate – silver nitrate redox system and conducted in the dark at room temperature and varying acrolein concentrations. Polyacrolein microspheres 0.01–0.2 μm in diameter (depending on the acrolein concentration in the system) with a variation coefficient of less than 10% were obtained in this way. It was noted that kinetic features of the acrolein polymerisation differ substantially from those observed for hydrophobic monomers, which was accounted for[53] both by the high solubility of the monomer in water (~20%) and by intermolecular cross-linking processes occurring in polymer-monomeric particles (PMP).

The γ-radiation induced radical polymerisation was carried out in an aqueous solution containing a mixture of ionogenic (sodium dodecyl sulfate) and nonionic [poly(ethylene oxide) with a molecular weight of 100,000] surfactants at concentrations of 0%–20% and 0%–15% relative to the monomer concentration, respectively and a radiation dose of 0.4 Mrad at room temperature for 4 h. It was noted that the initial polymerisation rate obeys the first-order kinetics with respect to the monomer. The size of the particles and their PSD depend considerably on the monomer and surfactant concentrations: the particle size increases with the increase in the monomer concentration and with the decrease in the surfactant concentration. By varying the above-mentioned parameters, the authors obtained polyacrolein microspheres of a broad range of sizes (from 0.01–0.02 to 5.0 μm). Stable monodisperse microspheres were formed only when the monomer concentration did not exceed 15%.

Schlund et al.[39] managed to obtain polyacrolein suspensions with particle sizes ranging from 0.04 to 8.0 μm and variation coefficients of less than 10% by the precipitation homopolymerisation of acrolein in alkaline medium (pH > 11.0) with solution of an alkali being gradually added to an aqueous solution of acrolein, only in the presence of a specially synthesised emulsifier produced by the interaction of polyglutaraldehyde oligomers with sodium hydrogensulfite. The polymerisation was carried out for 10 h at room temperature.

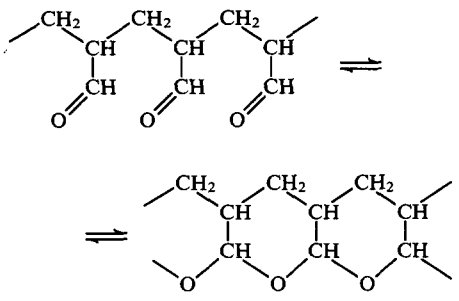
It has been shown that the structure of polyacrolein depends significantly on the conditions in which the microspheres are synthesised. For example, it has been reported[40] that polymerisation of acrolein can involve both the double bond and the aldehyde group. Therefore, polyacrolein incorporates two types of repeating units:




The $x:y$ ratio depends on the method and conditions of preparation of the microspheres and varies from 4:1 for 'anionic' microspheres to 1:7 for 'emulsion' microspheres. It has been noted that the conditions in which polyacrolein suspensions are synthesised determine the concentration of functional aldehyde

groups on the surface of particles and, hence, the number of covalently bound protein molecules.

Based on spectrophotometric studies, Rembaum et al.[40] have suggested that the polyacrolein molecules synthesised by γ-radiation induced polymerisation incorporate the following structural fragments:



Due to the bifunctional character of acrolein, the polymeric chains are formed by a fairly complex mechanism and their structure is a combination of the above-presented fragments. Thus, the structure of the macromolecules and, consequently, the properties of the polymeric suspensions (particle size, PSD, concentration of aldehyde groups on the particle surface, etc.) vary over a wide range depending on the polymerisation conditions, and, hence, the process is poorly reproducible. This is especially pronounced in the case of precipitation polymerisation. Radiation-induced polymerisation has a number of advantages over the precipitation polymerisation, namely, it is better controlled and reproducible, and it yields particles with higher concentrations of reactive groups on the surface.

Poor reproducibility of the precipitation polymerisation of acrolein has also been reported in other papers.[39,53] In addition, it was reported that particles of polyacrolein suspensions always contain a certain amount of an oligomeric product, associated with characteristic features of the precipitation polymerisation mechanism. In the course of time, these oligomeric molecules can diffuse from the particle bulk to the surface; therefore, the properties of the microspheres can change noticeably during storage.

A number of methods have been suggested to eliminate the above drawbacks which make it possible to increase considerably the chemical stability of polyacrolein microspheres, to decrease the physical adsorption of protein molecules on their surface, and to increase the yield of the target product.[54–57] For example, the synthesis of polyacrolein suspensions in an aqueous alkaline solution in the presence of 3%–6% radical initiators with respect to the monomer, has been reported.[56] The polymerisation was carried out in two temperature regimes: at 5–35 °C for 1.5–2.5 h or at 40–90 °C for 2–3 h. The resulting polyacrolein microspheres had diameters of 0.2–6.0 μm and a narrow PSD (variation coefficient ~10%); they were chemically more stable than the microspheres obtained by the conventional anionic precipitation polymerisation, apparently due to additional cross-linking of the polymer by radical initiators.

A similar effect has been achieved when the anionic precipitation polymerisation of acrolein has been carried out in an aqueous alkaline medium in the presence of water-soluble crown-ethers[57] at 2–38 °C and the reaction system has been kept in the presence of radical initiators [potassium persulfate, benzoyl peroxide, or azobis(isobutyronitrile) (AIBN)] at 40–90 °C. The polyacrolein suspension particles had a diameter of 0.04–6.0 μm and a variation coefficient of 14%–18%.

Polystyrene-acrolein suspensions obtained by the emulsifier-free copolymerisation of acrolein and styrene are more stable.[35,58] The copolymerisation was carried out at various proportions of comonomers in the presence of potassium persulfate at 55 °C for 8 h in an atmosphere of nitrogen.

The copolymerisation rate has been shown to depend on the molar concentration of acrolein and is optimal at 1 : 1 molar ratio between the comonomers. The copolymeric polystyrene-acrolein particles had an average diameter of 0.25 to 0.30 μm and a variation coefficient of less than 3%. The concentration of the aldehyde groups on the surface of these microspheres exhibited linear dependence on the molar concentration of acrolein and varied from 0.08 (at 20 mol.% acrolein) to 0.17 μmol $g^{-1}$ (at 90 mol.% acrolein).

A similar picture has been observed in the case of the γ-radiation-induced copolymerisation of acrolein with 2-hydroxyethyl methacrylate (HEMA).[36] A mixture of acrolein and HEMA was dispersed in a 1% aqueous solution of polyvinyl alcohol, then the emulsion of monomers was rapidly frozen, and the radiation-induced copolymerisation was carried out at −78 °C applying a radiation dose of 1 Mrad over a period of 1 h. The resulting microspheres were thawed and purified from the remaining monomer. Depending on the copolymerisation conditions, the concentration of the comonomers, and the ratio between them, the copolymeric polyacrolein–2-hydroxyethyl methacrylate microspheres had a diameter ranging from 0.6 to 4.0 μm and a narrow PSD.

A method for preparing polymeric microspheres with aldehyde groups on the surface by the post-radiation graft polymerisation of acrolein from the gaseous phase onto stable polystyrene or poly(methyl methacrylate) particles of identical sizes serving as matrices has been described.[59]

Since the conditions of the synthesis of a polymeric suspension (PS) influence essentially the properties of the modification product, the suspension particles obtained both in the presence and in the absence of an emulsifier have been used as matrices for the radiation-chemical graft polymerisation of acrolein.

Water-soluble hydroxyethylated poly(propylene glycol) [PS(F-68)] and di-*p*-tolyl-*o*-alkoxycarbonylphenylmethanol soluble in the monomer phase [PS(DTC)] were used as surfactants. In all cases, the polymerisation was initiated by potassium persulfate and conducted until the complete conversion of the monomers was achieved, which took 24 h. The particles of these polymeric suspensions proved to be stable in physiological salt solutions or during storage and had a narrow size distribution and diameters of 0.2 and 0.45 μm, respectively. The resulting polymeric suspensions were freeze-dried, and the polymer powders were used as matrices.

To determine the irradiation dose necessary for the graft copolymerisation of acrolein, the accumulation of radicals in the supporting polymer was studied by EPR. An irradiation dose of 15 Mrad was chosen at which the radical growth curves flatten out, i.e. a steady-state concentration of radicals is established.

A study of the sorption of acrolein vapour by the support particles at various acrolein vapour pressures showed that the concentration of acrolein in the particles depends appreciably on the nature of the polymer serving as the support and the surfactant used.

From the kinetic curves of acrolein sorption on the support particles, the rate constants for this process have been determined, and it has been shown that the yield of grafted polyacrolein as a function of time depends on the nature of the polymer.

An analysis of the IR spectra of the initial polymers and of those modified by polyacrolein showed that they all exhibit an absorption band in the region of 1725 $cm^{-1}$ corresponding to vibrations of the carbonyl groups in aliphatic aldehydes. An increase in the degree of polyacrolein grafting on the polymeric matrix leads to an increase in the intensity of absorption in this region of the spectrum.

The spectra of polyacrolein-modified polymers contain an absorption band at 1100 $cm^{-1}$ corresponding to vibrations of C–O bonds in acetals. These bonds can be formed either via polymerisation of acrolein with involvement of the carbonyl group or due to the interaction of the aldehyde groups existing in the hydrated form in the polymer produced. The latter is possible,

because free aldehyde groups exist in equilibrium with the corresponding hydrated groups, since acrolein polymers, even being dried in a high vacuum, contain certain amount of water.

To elucidate the manner in which the C–O bonds in the synthesised polymers are produced, the polymers were subjected to reaction with hydroxylamine hydrochloride. It was suggested that if the polymer incorporated free or hydrated aldehyde groups, this reaction would yield the corresponding polymeric oxime. Otherwise, if the C–O bonds are incorporated in the backbone, this reaction will not occur.

An analysis of the IR spectra showed that the reaction resulted in a decrease in the absorption intensity not only in the region of 1725 $cm^{-1}$, but also at 1100 $cm^{-1}$. The latter indicates that the C–O bonds result from the formation of cyclic acetals, which can be opened to give free aldehyde groups.

Based on the results obtained by IR spectroscopy, Bastos et al.[59] concluded that the radical polymerisation of acrolein involves mostly the vinyl group.

The amounts of free and bound CHO groups in polyacrolein are mainly determined by the conditions in which it is synthesised.

For example, the microspheres prepared by the ionic polymerisation of acrolein contain 2.9 mmol of aldehyde per g of microspheres, whereas those obtained by the radical polymerisation, for example, under the action of a radical redox system contain 12.0 mmol of aldehyde groups per g.

Calculations have shown that polyacrolein-modified microspheres obtained by the emulsifier-free polymerisation, PS(DTC) microspheres, and microspheres of the MMA–MAA copolymer contain on their surface 5.39, 4.80, and 9.34 and 8.07 mmol of aldehyde per g of grafted polyacrolein, respectively. Comparison of these results with the published data indicates that the content of aldehyde groups in the microspheres prepared by postradiation polymerisation of acrolein is twice as high as that in the microspheres prepared under conditions of basic catalysis, even in the sample with the lowest acrolein content.

Polymeric suspensions with aldehyde groups on the surface can also be synthesised by dispersion polymerisation of formylstyrene in aqueous ethanol using AIBN as the initiator and polyvinylbenzoic acid as the stabiliser.[31,60] The polymerisation was conducted for 24 h at 70 °C. Depending on the monomer concentration, the resulting particles had a diameter of 0.5 to 2.0 μm, the variation coefficient was 8%–10%, and the concentration of aldehyde groups on the particle surface was 0.1–0.2 μmol $g^{-1}$.

A method of preparing particles with aldehyde groups on the surface involving polymerisation of glutaraldehyde has been reported.[38,61,62] Polyglutaraldehyde suspensions are unstable (especially in strongly alkaline media) and have no apparent advantages over the suspensions considered above. Therefore, they have found only a limited application for marking cellular receptors.

## 2. Synthesis of polymeric suspensions with chloromethyl groups on the particle surface

Polymeric suspensions with chloromethyl groups on the particle surface can be obtained by precipitation[30,31,63] or seeded[33] polymerisation of chloromethylstyrene and chloromethyl methacrylate. A serious drawback of this method is the high toxicity of the monomers.

The precipitation polymerisation is induced by AIBN and conducted in ethanol in the presence of a stabiliser (polyvinylbenzoic acid) at 70 °C. Polymeric particles were from 0.5 to 2.0 μm in diameter (depending on the monomer concentration), with the variation coefficient being about 10%. During storage of polychloromethyl suspensions, reactive chloromethyl groups are partially hydrolysed, and, hence, properties of the suspensions changed.

The polymeric suspensions obtained by the seeded copolymerisation of chloromethylstyrene with styrene were more stable on storage.[33] Seed polystyrene particles were obtained by the dispersion polymerisation of styrene initiated by AIBN (1 mol $l^{-1}$) in aqueous ethanol, 1:4 by volume, in the presence of polyacrylic acid (1 g $l^{-1}$) as a stabiliser. The reaction yielded seed particles with a diameter of 1.9 μm and a variation coefficient of less than 3%. The polymerisation was conducted for ~10 h at 70 °C. After that, the seed latex, styrene, chloromethylstyrene, and AIBN were mixed in a specified ratio and stirred for 24 h at 0 °C, to allow the particles of seed suspension to swell, and then the polymerisation was carried out for 24 h at 70 °C.

The resulting suspension particles had a diameter of 2.1 μm, a variation coefficient of less than 3%, and a 'core–shell' structure. Virtually all the chloromethyl groups were located on the surface of the suspension particles.

Data concerning the use of styrene–chloromethylstyrene copolymeric suspensions in immunodiagnostics have been reported.[64,65]

## 3. Synthesis of polymeric suspensions with epoxy groups on the particle surface

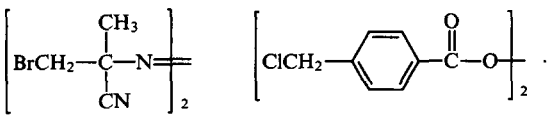
Polymeric suspensions, whose particles contain epoxy groups on the surface, are mostly prepared by the emulsifier-free copolymerisation of styrene with glycidyl methacrylate. According to published data,[66–77] the optimal conditions of the styrene copolymerisation with glycidyl methacrylate are as follows: ionic strength of the aqueous phase 0–0.02 mol $l^{-1}$, concentration of potassium persulfate 0.011–0.02 mol $l^{-1}$, and overall concentration of monomers $3.7 \times 10^{-3}$ mol $l^{-1}$. The process is carried out for 5–10 h at 65 °C. Under these conditions, microspheres having an average diameter of 220–400 nm and a variation coefficient of 2%–6% are produced. During the synthesis of styrene–glycidyl methacrylate copolymeric suspensions some of the epoxy groups located on the surface of particles are lost due to hydrolysis. Nevertheless, the number of these groups on the particle surface remains sufficient for ensuring a required concentration of a bioligand by its covalent bonding.

To suppress the hydrolysis of epoxy groups during the synthesis of the suspension, it has been advised that the polymerisation be carried out with gradual introduction of the functional monomer into the polymerising system.[39,76] This procedure makes it possible to obtain particles with a structure of the 'core–shell' type. This is exemplified by the synthesis of particles containing a copolymer of n-butyl acrylate with butyl methacrylate as the 'core'. These particles were obtained by the emulsion copolymerisation of the monomers in the presence of sodium dodecyl sulfate and a redox system consisting of potassium persulfate and sodium thiosulfate. The process was carried out at 20 °C for 20 min, after which a mixture of monomers with glycidyl methacrylate was introduced.

The resulting microspheres had diameters of 150–300 nm and a variation coefficient of < 10%. According to the data obtained by Schlund et al.,[39] under these conditions, up to 90% of the epoxy groups are preserved and virtually all of them are distributed within a thin layer (about 150 Å) on the surface of the particles.

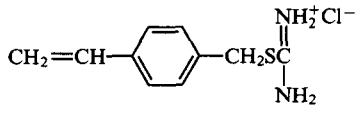
## 4. Synthesis of polymeric suspensions in the presence of surface active functional initiators and surfactants

The use of polyfunctional initiators and surfactants in the synthesis of polymeric suspensions with narrow PSD to be employed in immunodiagnostics has been reported. However, the use of these substances is restricted by their high prices and by the absence of a technology for their production. Azo-bis(bromoisocapronitrile) **1** and di(4-chloromethylbenzoyl) peroxide **2** can be mentioned among the polyfunctional initiators which make it possible to obtain reactive functional groups, capable of forming covalent bonds with biomolecules, on the surface of suspension particles:[78]



1 2

Isothiouronium salts, in particular (4-vinylphenyl)methylisothiourea hydrochloride, are used as polyfunctional emulsifiers.[41]



The latter compound is a cation-active emulsifier, able to copolymerise with vinylic monomers. For example, in the presence of this compound, polystyrene microspheres 0.15–0.19 μm in diameter with a variation coefficient of 2.7%, containing isothiouronium groups on the surface, have been prepared. Hydrolysis of these groups under mild conditions (pH 10–11, 25 °C, 10 min) gave mercaptogroups on the particle surface.[41] It was found that the average diameter of particles or their PSD are almost constant throughout the hydrolysis.

Polymeric suspensions, whose particles contain aldehyde groups on the surface, can be prepared by modifying other functional groups. For example, the particles of polystyrene suspensions obtained in the presence of the polysaccharide dextran or ammonium glycyrrhizate as a stabiliser contain carbohydrate groups on the surface.[79] The periodate oxidation under mild conditions leads to the cleavage of the α-glycol groups and formation of reactive groups that can directly interact with the amino groups of proteins.

In both cases, the conditions for the synthesis are chosen in which polymeric suspensions with narrow PSD stable in physiological salt solutions and during storage are formed. The concentration of aldehyde groups on the surface of suspension particles is in turn determined by the concentration of the stabilising agent and the oxidation conditions.

## 5. Synthesis of polymeric suspensions with carboxyl groups on the particle surface

The main and most studied method for synthesis of polymeric suspensions with carboxyl groups on the surface of particles is the copolymerisation of hydrophobic monomers with various unsaturated acids (acrylic, methacrylic, itaconic, etc.) in the presence or in the absence of an emulsifier. The presence of readily dissociating carboxyl groups imparts an additional stability to the suspension particles, therefore, these microspheres are resistant to mechanical action or low temperatures (freezing).[11,80–83]

The distribution of the carboxyl groups between the aqueous phase, the surface, and the bulk of polymeric particles is a rather important question in the synthesis of carboxyl-containing polymeric suspensions. In a study of the emulsifier-free copolymerisation of styrene with three unsaturated acids possessing different hydrophilicities, namely, acrylic (AA), methacrylic (MAA), and itaconic (IA) acid, it has been found[84,85] that AA concentrates in the surface area of the particles, the more hydrophobic MAA is distributed between the bulk of microspheres and their surface, and the most hydrophilic IA mainly homopolymerises in the aqueous phase. These features of the distribution of the acrylic monomers among the phases of an emulsion system determine the properties of the suspensions obtained.

Similar information has been presented in other papers[86,87] devoted to the study of the distribution of carboxyl groups in the suspension particles obtained by the copolymerisation of acrylic monomers with styrene or butadiene. It was found that the functional groups are distributed between the aqueous phase, the microsphere surface, and the microsphere bulk in a ratio of 2 : 3 : 1 in the case of acrylic acid or 0.1 : 1 : 1 when methacrylic acid is used.

Thus, it is obvious that the conventional way of conducting copolymerisation of hydrophobic monomers with unsaturated acids affords polymeric suspensions in which functional groups are distributed among various phases of the polymerisation system.

The distribution of the carboxyl groups between the surface and the bulk of the particles can be changed by gradual introduction of the functional monomer into the reaction system. It has been shown[88,89] that in the case where itaconic acid is added in the initial stages of the polymerisation of styrene, only 12% of itaconic acid units are located on the surface of copolymer particles, while introducing the acid at the stage where the degree of styrene conversion has already reached 80% or 95% leads to an increase in the concentration of the functional monomer on the surface to 60% or 80%, respectively.

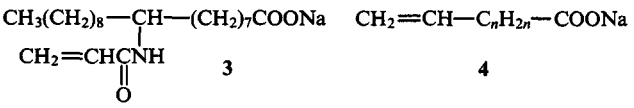
Another method of preparing a polymeric suspension with a high concentration of a carboxyl-containing comonomer on the surface of particles consists in the seeded emulsifier-free copolymerisation of styrene and butadiene with unsaturated acids (AA, MAA, or IA),[90] with pH of the medium being varied in the course of the synthesis. In the first stage of the process carried out at low pH, all the components (including the acid), but only a portion of the main monomers, are added to the reaction system. Then the pH of the seed latex is increased, and, consequently, the carboxyl groups located near the surface of particles are ionised. In the second stage, seeded copolymerisation of the rest of the monomers is carried out.

Some authors believe that the concentration of carboxyl groups on the surface of polymeric microspheres can be increased by heating the suspension at high pH values, which ensures the migration of the ionised carboxyl groups from the particle bulk to the interface with the aqueous phase (see, for example, the paper by Okubo et al.[91]).

The presence of MAA or AA in the monomeric mixture leads to the formation of emulsions characterised by a higher dispersity than emulsions of hydrophobic monomers, due to a lower interfacial tension ($\sigma_{12}$). A study carried out by Okubo et al.[92] has shown that MAA, present at a concentration of 2% with respect to styrene, diminishes the surface tension at the interface between a styrene solution of MAA and water to 22 mN $m^{-1}$. A dispersion analysis of styrene-methacrylate emulsions obtained with MAA concentrations being 0.2% or 3.0% with respect to styrene showed that in the former case, the emulsion of the monomers consists of drops, whose size is 1–5 μm, and in the latter case, the size of the drops varies from 0.2 to 1.0 μm.

The dispersity of the initial monomer emulsion influences the size distribution of the polymer-monomeric particles (PMP). In fact, the copolymerisation of styrene with MAA, induced by potassium persulfate and carried out at an MAA concentration in an emulsion of 0.2% with respect to styrene gives a polymeric suspension, in which 90% of the particles have an average diameter of 0.6 μm, while in the case where the MAA concentration is 3% with respect to styrene, 85% of the particles have an average diameter of 0.3 μm. When copolymerisation of styrene with MAA is carried out under the same conditions, but in the presence di-*p*-tolylalkoxycarbonylphenylmethanol (DTC), which is a water-insoluble stabiliser, polymeric suspensions with a narrower distribution of the particles of the same size were formed. These suspensions were stable in physiological salt solutions or on six-months storage.

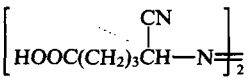
Functional microspheres with carboxyl groups on the surface can also be synthesised by the polymerisation or copolymerisation of surface active monomers,[93–103] for example, sodium acrylamidostearate **3**[93] or salts of vinylalkylcarboxylic acids **4**,[78] or surface active initiators, such as 5,5′-azobis-5-cyanopentanoic acid **5**.[78]



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However, these compounds have not found wide application owing to their high costs.

A new interesting method of synthesis of polymeric suspensions with carboxyl groups on the particle surface is the polymerisation of styrene in the presence of water-insoluble surfactants, whose molecules incorporate carboxyl groups. These are, for example, DTC, oligomeric peroxy esters,[94] and α-(ethoxycarbonyl)-ω-(trimethylsilyloxy)polydimethylsiloxane (PDS).[95]

In the papers considered above,[94,95] a hypothesis was put forward that the PMP are formed from drops of the monomer and that their structure is similar to that observed in the 'core–shell' type particles, whose shell is a surfactant displaced by the polymer to the interface. It was shown that the strength of the interfacial layer depends on the molecular mass of the resulting polymer, the concentration of the initiator (in this particular case, potassium persulfate), and the concentration of the surfactant.[95]

The particle size distribution of particles in polymeric suspensions obtained in the presence of water-insoluble surfactants are much narrower than those observed in the presence of water-soluble surfactants. First of all, this precludes the possibility of the formation of PMP from emulsifier micelles or globules of surfactant molecules in the aqueous phase of the emulsion.

Polystyrene, poly(methyl methacrylate), polychloroprene, and polystyrene–methacrylate suspensions with narrow particle size distributions have been obtained in the presence of water-insoluble surfactants. For example, polystyrene microspheres with diameters varying from 0.2 to 0.9 μm and the content of dry substance from 8% to 33% were prepared at surfactant and potassium persulfate concentrations of 1%–4% and 0.5%–1.0% with respect to styrene, respectively. These polymeric suspensions exhibited high stabilities in physiological salt solutions during storage and have found wide application in immunochemical studies.[96]

### 6. Synthesis of polymeric suspensions with amide groups on the surface of particles

Amide-containing polymeric suspensions are synthesised most frequently by emulsifier-free copolymerisation of styrene with amides of acrylic or methacrylic acid.[2,25,104–106]

The emulsifier-free copolymerisation of styrene with acrylamide (AAm, 0.1%–0.5% with respect to styrene) at pH 9 has been studied most comprehensively.[106] Three stages can be distinguished in the copolymerisation process: (1) copolymerisation of the monomers in the aqueous phase yielding oligomers; (2) polymerisation of styrene within the particles formed by these oligomers; (3) copolymerisation of AAm with styrene dissolved in the aqueous phase.

Special attention in the studies of styrene copolymerisation with AAm is paid to the distribution of amide groups between the aqueous and polymeric phases (owing to the high hydrophilicity of AAm, the aqueous phase always contains a substantial quantity of the water-soluble homopolymer).[107,108] The particle diameters in the resulting polymeric suspensions vary from 0.2 to 0.5 μm, and the variation coefficient is less than 3%.

### 7. Synthesis of polymeric suspensions with hydroxyl groups on the particle surface

Polymeric suspensions with hydroxyl groups on the particle surface are synthesised by the emulsifier-free copolymerisation of styrene with HEMA or 2-hydroxyethyl acrylate.[106–108] An essential feature of these monomers is the absence of ionising groups in their molecules, which allows one to change hydrophilic properties of the surface of the polymeric microspheres without changing its charge. This is particularly convenient for selecting the optimal conditions for binding biomolecules.[109–111]

Polymeric microspheres with high concentrations of surface functional groups are obtained by seeded copolymerisation with gradual introduction of a mixture of styrene and HEMA taken in a certain ratio.[112–114] The polystyrene particles with diameters of 0.3–0.9 μm and a narrow PSD are used as the seed suspension. Polymeric suspensions with a particle size of 0.6–1.1 μm and a polydispersity coefficient of 1.0003–1.0006 are obtained in this way.

### 8. Synthesis of polymeric suspensions with amino groups on the particle surface

Polymerisation of functional monomers such as aminostyrene,[115] 2-aminoethyl acrylate, 2-dimethylaminoethyl methacrylate, or allylamine[66] is a rather uncommon method for the synthesis of amine-containing polymeric suspensions. This is caused by the high cost of these monomers and by the low stability of the polymeric microspheres based on them as well as by the fact that a narrow particle size distribution is difficult to achieve due to the high hydrophilicity of the monomers.[116–124]

Amine-containing polymeric suspensions are much more readily prepared by modifying available suspensions containing hydroxyl, chloromethyl, amide, or other groups. As an example, let us consider the synthesis of polymeric suspensions with surface amino groups by the dispersion catalytic polymerisation of *p*-aminostyrene. The polymerisation of *p*-aminostyrene is carried out in the presence of a mineral or organic acid (with $pK_a$ below 4.76) and an organic solvent (10%–80% with respect to water), which is miscible with water and whose dielectric permeability is lower than that of water.[115] This process yields polyaminostyrene microspheres with diameters varying from 0.6 to 10.0 μm and a narrow particle size distribution.

Polymeric microspheres with diameters of 0.2–3.5 μm and polydispersity coefficients of no more than 1.08 have also been obtained by dispersion cationic polymerisation of *p*-aminostyrene in aqueous methanol at 60 °C with a monomer : water ratio of 1 : 9, a concentration of cetylpyridinium bromide of 1% with respect to the monomer, and a catalyst (hydrogen chloride) concentration of 10% with respect to the monomer.

## III. Activation of functional groups on the surface of particles of a polymeric suspension

The methods for activation of functional groups on the surface of particles of a polymeric suspension considered in this Section are widely applied in biochemistry and biology, in particular, to prepare sorbents for affinity chromatography.[89,90] However, to be suitable for preparing a highly sensitive diagnostic, these methods should comply with the following requirements:[2]

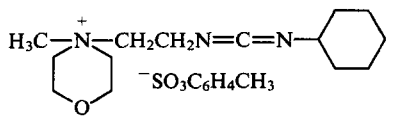
— to proceed quantitatively and irreversibly under as mild conditions as possible;

— not to decrease the stability of suspensions and not to influence noticeably the particle diameter and PSD;

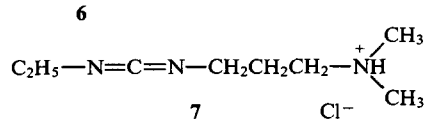
— to have no influence on the biochemical activity of the bioligand molecules covalently bound to the functional groups on the surface of the suspension particles.

### 1. Methods for the activation of carboxyl groups on the particle surface

The carboxyl groups on the surface of suspension particles can react with amino groups of a biopolymer in the presence of water-soluble carbodiimides (WSC).[80] The most frequently used WSC are 1-cyclohexyl-3-[2-(*N*-methylmorpholinio)ethyl]carbodiimide toluenesulfonate **6** and 3-ethyl-1-(3-dimethylaminopropyl)carbodiimide hydrochloride **7**.

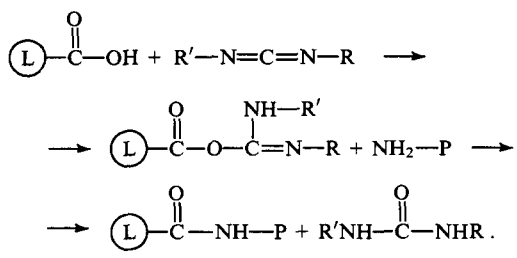


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The activation reaction occurs via the following scheme:

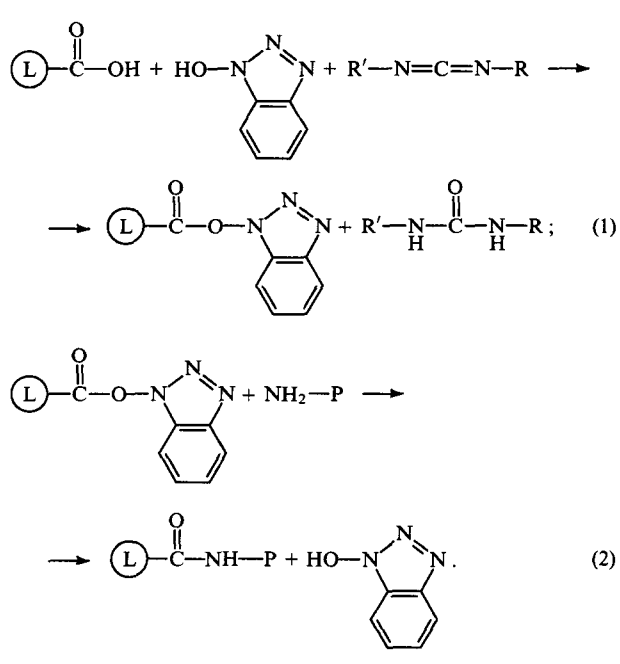


It is carried out at a temperature of 4–6 °C and pH 6–7 for 2–4 h in the presence or absence of an inert buffer like 4-(2-hydroxyethyl)piperazin-1-yl(ethane-2-sulfonic) acid.

One of the advantages of this method for activating functional groups is its simplicity. However, since protein molecules contain both carboxyl and amino groups, inter- or intramolecular cross-linking is possible, which reduces the immunochemical activity of the bioligand.[26, 104, 125–128]

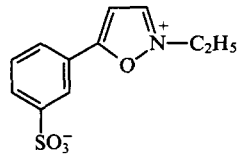
This problem can be solved to a substantial extent by using two-stage activation: in the first stage, the carboxyl groups are converted into an active (but stable) form and the microspheres are separated from the dispersion medium containing the unchanged components, and in the second stage, the covalent binding to a bioligand is carried out.

The so-called method of 'activated esters' is among the most generally used two-stage methods for the activation of functional groups.[2, 48, 129] In this case, the activation occurs according to the following scheme:



Apart from *N*-hydroxybenzotriazole, *N*,*N*-dialkylhydroxylamines and some other compounds can be used. The conditions in which the first stage of the process is conducted are similar to those described above for the 'carbodiimide' method. Following the first stage, the suspension is purified from side products. The second step proceeds at 4 °C and pH 7–7.5 for 2–3 days, and after that, the obtained diagnostic is completely freed from impurities.

Yet another possible version of two-stage activation is the use of the Woodward reagent, namely, *N*-ethyl-5-phenylisoxazoline-3′-sulfonate.[130]



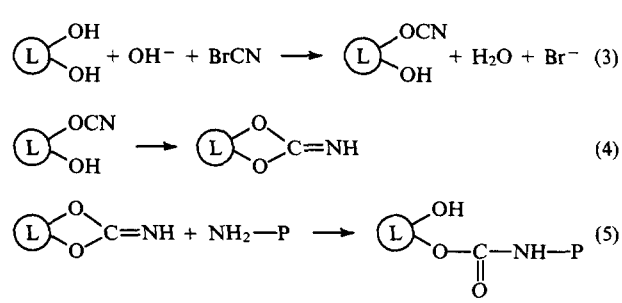
In this case, the first stage is carried out at pH 8.5 and a temperature of 3 °C for 3 h, the resulting suspensions with the activated carboxyl groups on the particle surface are purified from side products and then bound to bioligands at pH 7 and a temperature of 5 °C over a period of 3 h.

Common drawbacks of the above methods are their high costs and the low stabilities during storage of the reagents used, which makes the process of binding biomolecules to the functional groups of microspheres poorly reproducible. Moreover, the biomolecules bound by these methods are arranged near the surface of polymeric particles, which may result in changes in their conformations and may decrease their biochemical activity.

Therefore, the method involving the introduction of a spacer by the reaction of carboxyl groups on suspension particles with various diamines (1,6-diaminohexane, 1,7-diaminoheptane) has gained acceptance.[47, 131–133] In the first stage, the carboxyl groups on the microsphere surface are bound to diamine in the presence of WSC, and after that the resulting amino groups are activated by one of the methods described below.

## 2. Methods for the activation of hydroxyl groups

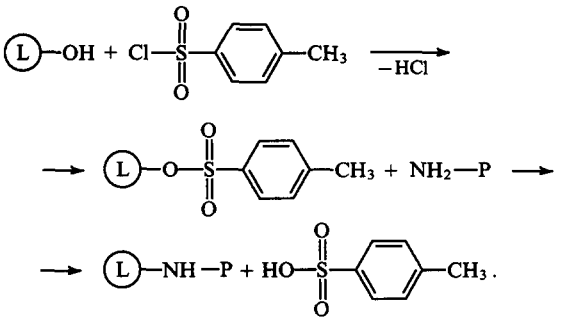
The 'cyanogen-bromide' method for the activation of hydroxyl groups consists in their interaction with cyanogen bromide in strongly alkaline media (pH 10–11) followed by the formation of bonds with amino groups of protein molecules.[134]



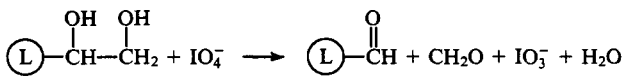
The first two stages, (3) and (4), proceed at 25 °C over a period of 15 min, alkaline pH being maintained by the periodical addition of 2 M sodium hydroxide. Then the suspension is purified from side products, and stage (5) is carried out in a 0.1 M borate buffer at pH 8.0–8.5 and a temperature of 4 °C for 4 h. The unaffected activated hydroxyl groups are bound by adding 0.1 M glycine buffer with pH 8.5, and then the suspension is extensively purified.

This activation method is applicable only to the covalent binding of biomolecules stable in strongly alkaline media. The high toxicity of cyanogen bromide and the noticeable aggregation of polymeric microspheres during binding bioligands to the functional groups are among the drawbacks of this method.

Activation of hydroxyl groups can be carried out by using some other reagents such as tosyl chloride,[2] 2,2,2-trifluoroethanesulfonyl chloride[135, 136] and carbonyldiimidazole.[137, 138] Overleaf we present the scheme of the activation of hydroxyl groups by tosyl chloride as an example:



A fairly convenient method for the activation of hydroxyl groups is the oxidation of glycol groups, which can be readily obtained by the hydrolysis of epoxy-groups or oxidation of double bonds in polydienes (see below). Microspheres with glycol groups on the surface are obtained, as has been mentioned above, by the polymerisation of styrene in the presence of dextrins as functional surfactants.[139] The oxidation is carried out by periodic acid or periodates at room temperature at neutral pH for 30–60 min in the dark.[50, 140, 141] Only glycols with primary or secondary hydroxyl groups enter into this reaction, which involves the cleavage of the C–C bond yielding two aldehyde groups.

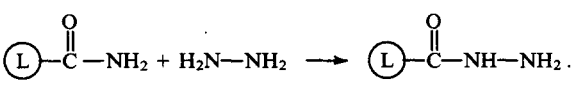


The side products are removed during purification of the polymeric microspheres.

### 3. Methods for the activation of amide groups

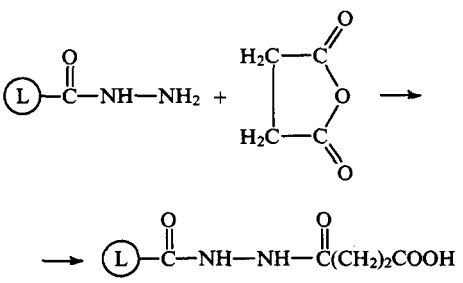
Although amide groups do not interact directly with protein molecules, there are a number of methods involving polymer-analogous transformations that make it possible to convert amide-containing polymeric suspensions into microspheres with some other groups that can be easily activated.

For example, the modification of amide groups to hydrazide groups is widely used.[27, 46] The reaction occurs at 50–80 °C in 7–10 h according to the following scheme:

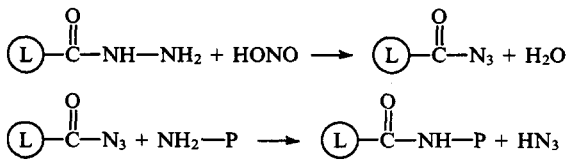


The hydrazide groups formed in this reaction can be transformed either into the carboxyl groups, which need to be further activated, or into azide groups, which can directly interact with biomolecules.

The former process can be accomplished by treating the hydrazide groups with succinic anhydride.[142, 143]



Azide-containing polymeric suspensions are obtained by diazotisation in the presence of nitrous acid at a low temperature (2–5 °C);[27] after that, the direct interaction of biomolecules with azide groups on the microsphere surface is possible (pH 7.0 to 9.5, duration 4 h).



It should be noted that hydrazide groups can be activated by virtue of the same methods as amino groups.

Another possible way of modification of amide-containing polymeric suspensions is the transformation of amide groups into primary amino groups under the action of sodium hypochlorite in an alkaline medium (the Hoffmann reaction[139, 140]). The factors influencing the course of this process have been studied.[143,144] It was shown that, apart from the transformation of amide groups into amino groups, hydrolysis yielding carboxyl groups occurs to a substantial extent, the degree of hydrolysis increasing with increase in the temperature and decrease in the NaOCl : polyamide ratio. The following optimal conditions under which the maximum quantity of amino groups is obtained on the surface of the suspension particles were found: a ratio between the concentrations of hypochlorite and amide groups of 0.6 to 0.9, a temperature of 5–10 °C, and a duration of the process of 1–2 h. It was shown that under these conditions, the diameter of suspension particles or their PSD are virtually unchanged during modification. This method has been used for the covalent binding of enzymes to the amide groups located on the particle surface in acrylamide-styrene copolymeric suspensions (glutaraldehyde was used to activate the resulting amino groups).[104]

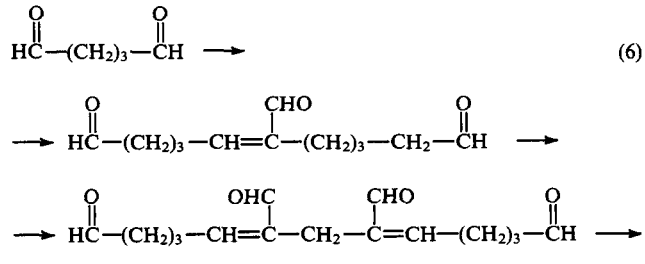
The transformation of amide groups into carboxyl groups, which is frequently required for increasing the stability of microspheres, can be performed by alkaline hydrolysis under mild conditions (30 °C, 3–4 h).[144] The degree of hydrolysis and, consequently, the concentration of carboxyl groups on the surface of suspension particles, can vary depending on the duration of the process, and this allows one to obtain particles with various concentrations of carboxyl groups on the surface.

Finally, polymeric suspensions with hydroxyl groups on the surface can be obtained from amide-containing suspensions by hydroxymethylation. The reaction proceeds for 1 h at 50 °C and pH $\geqslant$ 12.0. In relation to the emulsifier-free copolymerisation of styrene and AAm taken in a ratio of 9 : 1 followed by the modification of the amide groups to the hydroxyl groups, it has been shown that this process yields stable monodisperse microspheres 220 nm in diameter containing 4.58 OH groups per nm$^2$ of the surface of particles.[144]

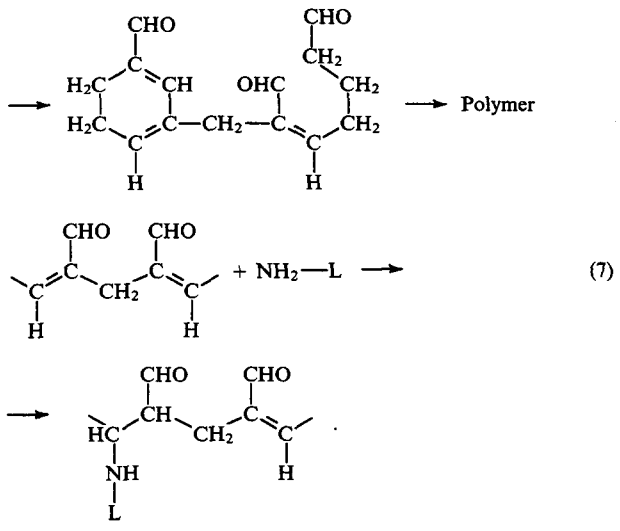
### 4. Methods for the activation of amino groups

Since primary amino groups are highly reactive, polymeric suspensions containing these groups on the surface of microspheres are readily activated via reactions with various bifunctional compounds.

The most commonly used activation method involves reaction with glutaraldehyde, which is due to both the ease of conducting this reaction and the availability of the reagent itself. The primary amino groups react with glutaraldehyde by the following scheme:[145, 146]



(6)




The activation can be accomplished in one or in two stages.[147] According to the one-stage procedure, linking a bioligand to the amino groups on the particles is carried out in the presence of an excess of glutaraldehyde. Although this method is rather simple, appreciable aggregation of polymeric microspheres due to their cross-linking is observed in the course of activation.

Preference is given to the two-stage activation. The first stage (6) involves oligomerisation of glutaric aldehyde and the reaction of the resulting oligomer with the amino groups on the surface of polymeric microspheres (7). After liberation of the suspension from side reaction products, covalent binding to bioligands is performed. The first stage is carried out at 20–25 °C in 0.01 M phosphate buffer (pH 7.0) for 1 h.[24] After removal of the excess of glutaraldehyde by dialysis (24 h, against a system consisting of 0.01 M phosphate buffer and 0.01 M salt buffer, pH 7.0), the molecules of a bioligand are bound to the microsphere functional groups for 5 h at 25 °C. Then the unaffected aldehyde groups on the microsphere surface are blocked with a 0.1 M solution of glycine at pH 7.0, and the resulting test-system is purified. This activation method makes it possible to avoid inter- or intramolecular cross-linking of bioligands and to prevent the functional microspheres from aggregation.[24]

Other bifunctional compounds, for example, 1,5-difluoro-2,4-dinitrobenzene, 4,4′-difluoro-3,3′-dinitrophenyl sulfone, or 2,4-dichloro-6-methoxycarbonylmethyl sulfone, can also be used in place of glutaraldehyde.[148, 149] Drawbacks of these methods are the high costs of the reagents and the prolonged period (4–7 days) required for covalent binding of functional groups to biomolecules.[2] However, the properties of the test-systems obtained by these methods are more reproducible than those of the test-systems prepared using glutaraldehyde; in the latter case, the properties of the test-systems are determined by the conditions and duration of the storage of glutaraldehyde (polymerisation is possible).

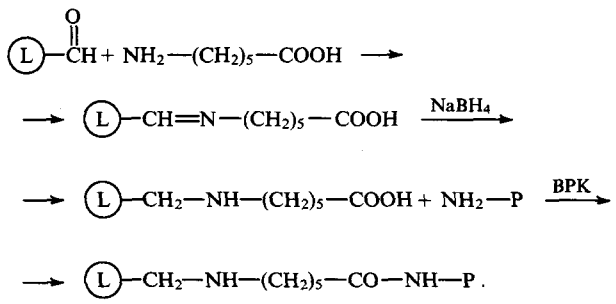
It is noteworthy that one of the possible ways of binding antibodies and enzymes incorporating polysaccharide residues to amino groups on the surface of a polymeric suspension is the treatment of the bioligand itself with periodic acid or periodates, rather than the activation of the amino groups.[104] The oxidation of the glycol groups of the polysaccharides incorporated in biomolecules affords reactive aldehyde groups, which can react with the amino groups on the surface of polymeric microspheres. The use of this method for binding glucose oxidase and glucose peroxidase makes it possible to retain up to 40% of their activities (compared with 10% of the activity retained when other methods, in particular the carbodiimide method, are used).[104]

## 5. Methods for the modification of chloromethyl, epoxy, and aldehyde groups

In this section we consider polymer-analogous transformations involving functional groups capable of direct interaction with bioligands. These reactions are often required either to introduce an additional spacer group between a polymeric particle and a biomolecule in order to retain the biological activity of the latter, or to generate chemical groups on the surface of the suspension particles, whose reactivity is retained during the preparation and storage of microspheres (the transformation of epoxy or chloromethyl groups into aldehyde groups is an example).

Aldehyde groups are most frequently modified in order to introduce a spacer. This is achieved by conducting the reaction with diamines and the subsequent activation of the $NH_2$ groups with glutaraldehyde. It should be noted that the Schiff's base resulting from the interaction of a primary amino group with an aldehyde group and containing a CH=N fragment is unstable at low pH values. Therefore, it is often reduced to $CH_2NH$ with mild reducing agents like sodium tetrahydroborate (25 °C, 30 min).[37, 38]

Aldehyde-containing suspensions can also be modified using the reaction of aldehyde groups with amino acids (for example, with 6-amino-n-caproic acid).[35, 150] This reaction affords carboxyl groups on the surface of the polymeric microspheres:




Chloromethyl groups are modified both in order to introduce a spacer and to suppress hydrolysis, which occurs to a substantial degree at high pH values.[31] In the former case, the same methods as for aldehyde groups can be applied.[28, 29, 34] The reaction is carried out in neutral or weakly alkaline media at 30–40 °C for 1–2 h.

Chloromethyl groups are rather reactive and possess pronounced electrophilic properties,[32] which makes it possible to perform various polymer-analogous transformations to impart additional valuable properties to the functional microspheres, such as storage stability, hydrophilicity of the surface, etc.[151] For this purpose, chloromethyl groups are most frequently converted into aldehyde groups. The functional groups located on the surface of the particles of a polymeric suspension are modified by treatment with iodates in an aqueous solution of chromic acid,[117, 151–153] or by the oxidative alkylation with the sodium salt of 2-nitropropane.[154–156]

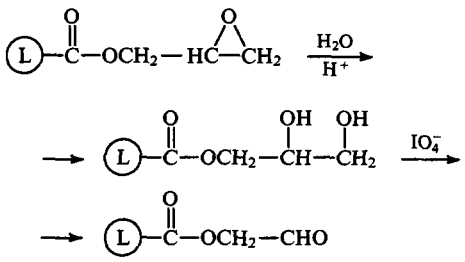
In the latter case, sodium methoxide and 2-nitropropane are gradually added to a poly(styrene-chloromethyl) copolymeric suspension. The reaction proceeds at room temperature over a period of 2 h, and after that, the modified copolymeric microspheres are purified from the unchanged components and side products. It was shown that under these conditions almost all the chloromethyl groups are oxidised to aldehyde groups, the modification having virtually no effect on the diameter or PSD of the resulting polymeric particles. A comparison of the reactivities of the initial chloromethyl groups and the resulting aldehyde groups toward human immunoglobulin G has shown that the latter are better in binding this antibody and provide higher sensitivity of the diagnostic.

As has already been mentioned, epoxy groups can be hydrolysed, especially at low pH values. Therefore, epoxy groups are subjected to further modification with the aim of obtaining more

stable groups on the particle surface or to introduce an additional spacer group.

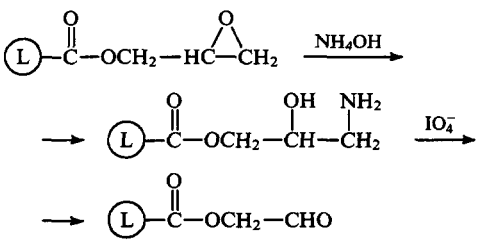
Owing to the high reactivity of epoxy groups, they participate in various polymer-analogous transformations.[40]

For example, the hydrolysis of surface epoxy groups at 50 °C and pH 2 gives particles with glycol groups, which are readily converted thereafter into aldehyde groups by treatment with periodic acid.

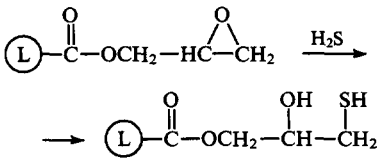


It is noteworthy that up to 60% of the theoretically possible (based on the concentration of the epoxy groups on the surface of the suspension particles) number of glycol groups can be obtained by the hydrolysis, and the particle, diameter, or PSD, virtually do not change during the process: these are 440 nm and a variation coefficient of 3.9% for the starting suspension and 475 nm and a variation coefficient of 5.8% for the modified particles.

The reaction of epoxy groups with ammonium hydroxide (ammonolysis) occurs even more readily at 20 °C.



Epoxy groups on the surface of suspension particles can be converted into thiol groups by the reaction with an aqueous solution of hydrogen sulfide, which proceeds under mild conditions (20 °C, 2–4 h).



However, in this case, it was noted that the modified particles undergo substantial agglomeration (variation coefficient 15.5%).

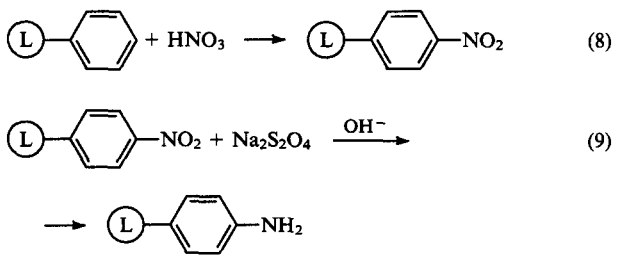
Epoxy groups on a microsphere surface can also be modified by introducing a spacer via the reaction with diamines or amino-acids (see above).

## 6. Methods for the preparation of polystyrene suspensions with a narrow particle-size distribution containing functional groups on the particle surface

Polystyrene suspensions have found the most extensive application in immunodiagnostic studies. This is caused by the fact that polymerisation of styrene has been most thoroughly studied and it is easy to obtain microspheres with the required diameter and a narrow PSD.[3-5,157-175] Here, it is expedient to consider methods for the synthesis of functional polystyrene suspensions consisting of particles with a specified diameter and a narrow PSD.

Polystyrene microspheres with hydroxyl groups on the surface are obtained in two stages: the first stage involves emulsifier-free polymerisation of styrene initiated by potassium persulfate giving monodisperse suspensions, whose particles contain sulfate groups on the surface and which are stable in physiological salt solutions. In the second stage, the sulfate groups are converted into hydroxyl groups by the hydrolysis in alkaline media (pH 9–10, 50–70 °C, 2–5 h).[176,177] The resulting hydroxyl groups can be either modified further by virtue of the cyanogen-bromide method or oxidised in the presence of $Ag^+$ ions (50 °C, 5 h)[127] to carboxyl groups, which are then activated. It should be noted that the hydrolysis of ionogenic sulfate groups to yield non-dissociating hydroxyl groups is accompanied by a sharp reduction in the stability of the modified microspheres due to the decrease in the electrostatic repulsion between the particles. Therefore, to prevent coagulation, non-hydrolysing sulfonate groups should be introduced.[32,175,178]

The preparation of polystyrene particles with amino groups on the surface is an even more complex and cumbersome procedure.[179,180]



The first stage [reaction (8)] is carried out in glacial acetic acid at room temperature for 4 h, concentrated nitric acid being gradually added to the reaction mixture. The polymeric particles are then separated from the nitrating reagent by decantation and washed. The second stage (9) is carried out in ethanol; it involves the treatment with sodium dithionite at 40 °C for 2 h. The resulting polystyrene microspheres with amino groups on the surface can be activated thereafter by any of the procedures considered above. It is obvious that to be subjected to all the stages of the process, polystyrene suspensions should meet very stringent requirements for their stability, therefore, the above way of modification is applicable only to particles with cross-linked structures (copolymer of styrene with 2%–5% divinylbenzene) and diameters of no less than 0.8 μm (they can be redispersed fairly easily).[2]

An original method for binding bioligands to polystyrene microspheres has been suggested by Berge et al.[138] This method makes it possible to avoid any chemical effect on the surface of polystyrene particles and thus to maintain their stability. The first step of this process involves the adsorption of bovine serum albumin (BSA) onto polystyrene particles of a specified diameter with a narrow PSD; then the suspension is treated with a bifunctional compound (glutaraldehyde, diethyl adipinimidate, etc.) for the cross-linking of protein molecules and their fixation on the microsphere surface. After that, further binding of biomolecules is possible via amino or carboxyl groups of BSA.

## 7. Methods for the activation of polydiene microspheres

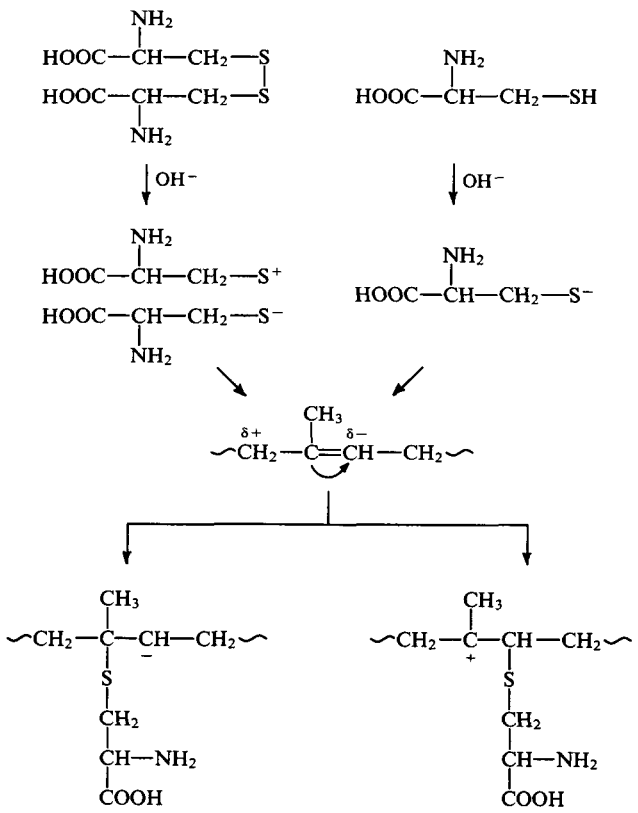
The main characteristic feature of polydiene microspheres is the presence of double bonds, which can undergo further modification.[139,140] However, good film-forming ability of diene polymers is responsible for the low stability of microspheres during their purification by ultrafiltration, microfiltration, or centrifugation.[2] Therefore, the functional polymeric suspensions are synthesised by copolymerisation of dienes (butadiene, isoprene, etc.) with various proportions of styrene.

A possible method of introducing hydroxyl groups into polydiene-styrene microspheres (for example, polyisoprene-styrene microspheres) is the oxidation of the double bonds of the polydiene by such oxidising reagents as potassium permanganate

or hydrogen peroxide.[181,187] The oxidation proceeds under mild conditions (pH 3–7, 5–10 °C, 30 min). The resulting glycol groups can be subjected to further activation.

The oxidation of the polybutadiene or polyisoprene double bonds to epoxy groups by their interaction with peracetic acid at reduced temperatures is of great interest.[188,189] It was shown that the rate and degree of epoxidation depend to a substantial extent on the polymer content in the suspension and on the concentration of peracetic acid, and does not depend on the nature of the surfactant present in the reaction system, the size of polymeric particles, or the rate of stirring. The following optimal conditions of the oxidation were found: a concentration of the polymer in the suspension of 15%–30%, a concentration of peracetic acid of 2%–3%, and a duration of the reaction of 2–3 h. Data on epoxidation of unsaturated polymers with a mixture of hydrogen peroxide and formic acid has also been reported.[189]

Regarding the modification of polydiene microspheres, the use of thio-compounds containing mercapto groups which readily react with double bonds of polydiene microspheres, is of considerable interest. Thioglycols or thioamines are known [190] to be used for enhancing plastic properties of polybutadiene rubbers. In this respect, attention is attracted by the possibility of modifying polydiene microspheres by thiol compounds containing various functional groups, for example, by sulfur-containing amino acids. The modification of an artificial dispersion of SKI-3 polyisoprene by sulfur-containing amino acids has been reported.[191] It was shown that amino acids such as cystine or cysteine, due to the presence of reactive disulfide and mercapto groups in their molecules, are capable of direct binding to partially polarised double bonds of 1,4-*cis*-polyisoprene, which occurs by an ionic mechanism.



The preparation of polymeric suspensions with amino groups on the particle surface with the aid of modification with sulfur-containing amino acids has been reported by Gritskova et al.[192] They suggested a method for synthesising modified suspensions that consisted of three independent stages: the first stage involved the synthesis of seed polystyrene particles with a specified diameter and a narrow PSD, the second stage was the seed polymerisation of isoprene, ensuring a high concentration of polyisoprene units on the surface of the particles, and in the third stage, the resulting isoprene-styrene polymeric suspensions having the 'core–shell' structure were modified by cysteine.

If all the stages of this process are conducted under controlled conditions, it is possible to obtain polymeric suspensions with diameter of particles varying over a wide range (0.1–2.0 μm), a narrow PSD (1%–5%), and a high concentration of amino groups on the particle surface.

It was shown that by varying conditions in which modification is carried out, one can control the properties of the suspensions obtained, which is particularly significant for choosing the optimal conditions for the immobilisation of protein molecules and for further application of modified suspensions in immunodiagnostics.

* * *

Thus, the data considered above indicate that the main stage in the synthesis of functional polymeric suspensions suitable for immunodiagnostic studies is the heterophase polymerisation. The choice of polymerisation conditions determines the stability of the suspensions, the particle diameter, and their size distribution. Functional groups on the surface of microspheres can be obtained either during the synthesis or by modifying the resulting polymeric suspensions.

Despite the fact that numerous methods for the synthesis of functional suspensions have been suggested, studies in this field are being intensely carried out.[193–218] The purpose of the studies is to find conditions of a technologically facile method for preparing suspensions with the use of available reagents.

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