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Synthesis and Reactions of Hydrophilic Functional Microspheres for Immunological Studies

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ABSTRACT

There is a need for hydrophilic polymeric microspheres with functional groups on their surface which can be reacted efficiently with proteins. These microspheres with antibodies (immunoglobulins) covalently bound to their surfaces constitute valuable immunoreagents capable of marking specific receptors (antigens) on cell surface membranes. The main requirements of the microspheres for the above applications are: insolubility in aqueous or organic media, absence of aggregation and of nonspecific interaction with cells and presence of suitable functional groups for covalent binding

with antibodies. Hydrophobic polystyrene or poly(methyl methacrylate) latices do not meet these requirements. Copolymerization of hydrophilic monomers under suitable experimental conditions yielded microspheres with the required characteristics. Emulsion polymerization and ionizing radiation were found to constitute convenient techniques for the synthesis of hydrophilic and crosslinked (and therefore insoluble) functional microspheres ranging in diameter from 0.01 to 8 μm. By choosing suitable comonomers, it was possible to incorporate hydroxyl, carboxyl, amido, and dimethylamino functional groups into the particles. Copolymerization with isomeric vinylpyridines or dimethylamino methacrylate yielded weakly or strongly basic groups, respectively, capable of binding with acids. The experimental conditions suitable for obtaining desired particle sizes, in a relatively narrow distribution, were determined. It was found that the particle size depended to a large extent on the water solubility of the monomers, the presence or absence of stabilizer, the concentration of a surfactant, and the monomer concentration. The preferred technique to bind antibodies to the microsphere surface consisted of reacting amino groups with glutaraldehyde followed by the reaction with proteins. The use of polyglutaraldehyde instead of glutaraldehyde was also investigated. For this purpose the rate of polymerization of glutaraldehyde as a function of concentration and pH was first studied, followed by a study of the reactivity of polyglutaraldehyde microspheres with immunoglobulins. A recent new development of importance for cell separation is the synthesis of functional microspheres containing magnetic iron oxide. Preliminary investigations show that red blood cells and lymphocytes labeled with magnetic immunomicrospheres can be efficiently separated by means of permanent magnet. Separation of labeled from unlabeled human red blood cells was also achieved by means of a free-flow electrophoretic instrument.

INTRODUCTION

Progress in biology and medicine depends to a large extent on our knowledge of the role played by specific cells in normal and pathological processes. Most of these studies require the identification and separation of specific populations of cells. In recent years this has been accomplished using immunological labeling techniques based on differences in antigens, most often membrane proteins, displayed on cell surfaces. In this technique, antibodies against these cell surface antigens are coupled to either radioactive isotopes, fluorescent

dyes, biological macromolecules or synthetic particles [1-6]. These immunological reagents are then used to label the specific population of cells displaying the particular antigenic site. Labeled cells can be detected, quantitated and characterized by radioactive counting, light or electron microscopy or related methods. Immunological cell labeling techniques have been successfully applied to the identification of various blood cells and, in particular, subpopulations of lymphocytes i. e., B and T cells [7-9]. Similar methods are currently being used to analyze specific types of neural cells [10] as well as to distinguish normal from tumor cells. The practical applications of these studies are diagnostic tests of a large number of diseases and their therapy.

Immunological cell labeling also has applications in the isolation of specific types of cells. The biophysical and biochemical properties of cells can be drastically altered by labeling them with certain immunological reagents, thereby facilitating their separation from unlabeled cells. Such separations have been accomplished by means of fluorescence cell sorting, affinity chromatography, differential partitioning, electrophoresis, and magnetophoresis [11-16].

The present paper summarizes our recent efforts to synthesize and characterize new reagents [16-27] in form of polymeric functional microspheres for the visualization of antigens in the scanning electron microscope (SEM) or light microscope and also for identification and separation of cells and their surface membranes. The purpose of the functional groups is to form a covalent bond between the antibody molecule and the microsphere by means of a chemical reaction. Due to the exceptional specificity of antibodies, the microsphere, hitherto referred to as an immunomicrosphere, will seek out the corresponding antigen on a cell membrane and bind to it as illustrated schematically in Fig. 1.

Differences in cell surface antigens may be used not only as a means for their identification and quantitation but also as a means for their separation. Immunomicrospheres containing iron or magnetite offer a promising method of cell separation by means of a magnetic field. Preliminary results show that cell subpopulations, e. g., B and T lymphocytes and other cell types can be separated magnetically [16]. Therefore, this technique may lead to exciting applications, including the possibility of separation of specific cells present in small numbers only, the isolation of which is extremely difficult at present. Similar techniques can also be used to isolate specific types of cellular membranes, e. g., plasma membranes, and specific antigens for biochemical and immunochemical studies. Advantages of immunomicrospheres are as follows.

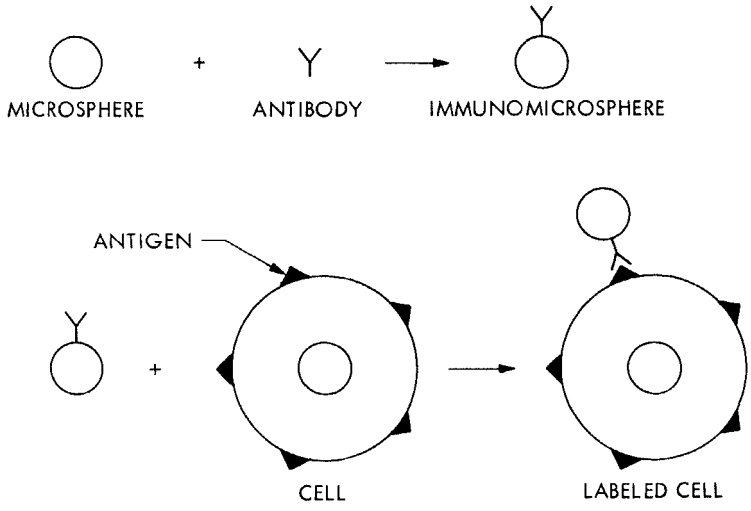

FIG. 1. Schematic representation of direct immunological labeling of living cells by means of microspheres covalently coupled to antibodies.

Choice of Size

Microspheres can be prepared in a wide range of uniform sizes. Large microspheres greater than 3000 Å in diameter can be used to identify cells by light microscopy in the presence of biological stains for simple routine analysis [19]. Small microspheres of the order of 300-1000 Å in diameter serve as visual markers to study the distribution of antigens on cell surfaces by high resolution SEM [4].

Amplification of Fluorescence Intensity

A large number of fluorochrome molecules can be bound to microspheres prior to reaction with antibodies. This results in an increase in fluorescent signal intensity without adversely affecting the binding activity of the antibody. This has practical applications in fluorescent light microscopy and fluorescent cell sorting analysis [12] when a high sensitivity is needed, i. e., when there are few antigenic sites per cell.

Chemotherapy

In addition to antibodies, other desirable molecules, e. g., drugs, enzymes, or radioactive elements can be bound to immunomicrospheres, which are likely to guide the multifunctional particles to a desired site or body organ.

Phagocytosis

The new reagents are also suitable for studies of phagocytosis as a function of size and composition of the particles. Preliminary studies revealed an inhibitory effect of amide groups on the phagocytic uptake [28].

This review of the work carried out since 1973 includes: the general and structural requirements for the preparation of microspheres for a variety of biomedical applications; the synthetic methods used, and the characterization of the products; the recent studies of polyvinylpyridine (PVP), polyglutaraldehyde (PGL), and magnetic microspheres.

REQUIREMENTS

An essential requirement is the absence of nonspecific interaction between the microsphere and cells. Occurrence of this interaction defeats the objective of cell identification and quantitation based on the binding of immunomicrospheres to specific cell surface antigens. It is dependent on the type of cell under investigation, e. g., red blood cells (rbc) in general exhibit less "nonspecific stickiness" than lymphocytes. Applications of polystyrene microspheres are limited because the hydrophobic polystyrene particles stick nonspecifically to the surfaces of many living cells, including rbc. Interaction of polystyrene latex with blood platelets [29] for example, results in their activation, release of enzymes and eventual clotting. In contrast, the highly hydrophilic polyacrylamide microspheres under identical experimental conditions do not produce observable platelet changes. A hydrophilic character is therefore one of the essential conditions for successful applications of immunomicrospheres.

Presence of functional groups capable of relatively simple derivatization, i. e., of covalent binding, constitutes an equally important requirement. Hydroxyl groups may be activated with cyanogen bromide and reacted with amino groups on protein molecules [30]. Carboxyl groups which provide negative charges at physiological pH

and thus prevent particle aggregation can be coupled to amino groups on proteins using the carbodiimide [31] reaction. The hydrophilic nature of the amide group is an important factor in particle stabilization and in partial or complete elimination of nonspecific interaction with cell surfaces. In addition, these chemical groups can be converted to the hydrazide derivative and subsequently coupled to proteins via the acyl azide [32]. Alternately, amide groups, as well as amino groups, can be used for chemical binding of antibodies by means of glutaraldehyde [4, 18]. Microspheres with aldehyde functional groups on their surfaces are particularly desirable since the chemical derivatization procedures may be greatly simplified.

The lack of solubility of microspheres in aqueous media is obviously another requirement. In the cases where derivatization must be carried out in organic solvents, the insolubility in the latter is necessary. For this reason, a crosslinking agent is normally added to the hydrophilic functional monomers selected for the synthesis of immunomicrospheres.

SYNTHESIS AND CHARACTERIZATION OF COPOLYMER MICROSPHERES

Emulsion, ionizing radiation and suspension polymerization of hydrophilic monomers were utilized in order to produce microspheres of the desired properties. Six main classes of hydrophilic crosslinked microspheres recently synthesized by either the emulsion or the Co γ-radiation technique are: (a) EM class: methyl methacrylate (MMA), 2-hydroxyethylmethacrylate (HEMA), methacrylic acid (MA), and ethylene glycol dimethacrylate (EGDMA); (b) EM AM class: MMA, HEMA, acrylamide (AA), MA, and EGDMA; (c) L class: HEMA, MA, and bisacrylamide (BAM); (d) BAH class: HEMA, acrylamide (AA), MA, and BAM; (e) DMA class: HEMA, 2-dimethylaminoethyl methacrylate (DMA), and BAM; (f) PVP class: 4-vinylpyridine alone or with HEMA and/or AA. Of these, classes (a) and (b) were prepared by emulsion polymerization [18]; classes (c)-(f) were prepared by cobalt γ-radiation [19].

The main advantage of the ionizing radiation system is that microspheres practically free of impurities are obtained, since the polymerization takes place in absence of a redox initiator (e. g., ammonium persulfate) or emulsifier (e. g., sodium dodecyl sulfate). On the other hand, microspheres can be synthesized more readily in most laboratories by emulsion polymerization since a cobalt γ-radiation source is not required.

Copolymer Acrylic Microspheres

Synthesis. Em microspheres have been synthesized from the hydrophilic monomers HEMA and MA and the more hydrophobic monomer MMA in a variety of uniform sizes. Experimental conditions for these spheres containing hydroxyl and carboxyl functional groups have been already described in detail [18, 20]. For microspheres 300-3500 Å in diameter the size was observed to vary linearly with the total monomer concentration. More recently, a related class of microspheres, EMAM, has been prepared by polymerization of 25% acrylamide, 25% HEMA, 10% MA, and 33% MMA. The reaction was carried out with a total monomer concentration of 3% (w/w) in the presence of 0.1% sodium dodecyl sulfate, and 0.01% ammonium persulfate for 1 hr at 90°C. The addition of acrylamide and reduction in MMA content give these microspheres a more hydrophilic character and adds the amide functional group. The L class of microspheres and the BAH microspheres containing hydrophilic monomers, HEMA, MA, and AA and crosslinked with bisacrylamide have been prepared by cobalt γ-irradiation in the presence or absence of poly(ethylene oxide) as previously reported [19]. These microspheres have a number of functional groups, i. e., carboxyl, hydroxyl, and amide groups. Of particular interest are microspheres based on 4-vinylpyridine (PVP class), which offer the possibility of reaction with the pyridine ring in addition to the reactions of OH and $CONH_2$ functional groups.

Properties. Copolymer microspheres have been observed to aggregate at acidic pH where most of the carboxylate groups are neutralized. This is illustrated in Fig. 2 by the sharp increase in turbidity of the suspension in the pH range of 3 to 5. The pH at which half the maximal change in turbidity is observed varies for each class of microspheres (Table 1). In general, there is a shift to a lower pH for microspheres having a more hydrophilic monomer composition, i. e., BAH and L microspheres. BAH microspheres which have been synthesized in the presence of poly(ethylene oxide) (PEO) do not aggregate even at low pH. This is presumably due to grafting of PEO onto the microsphere surface. No flocculation or change in turbidity of any of the classes of microspheres was observed at alkaline pH values (Fig. 2). The hydrogen ion titration curves for various classes of microspheres were also determined. As illustrated in Fig. 3, the carboxyl groups on the microspheres titrate over a relatively wide pH range indicative of some electrostatic interaction between carboxyl groups on the same microspheres [33]. The apparent pK values as summarized in Table 1 are also influenced by the composition of the microspheres. For example, EMAM microspheres containing the hydrophilic monomer acrylamide have an apparent pK 0.8 units lower than for the EM microspheres

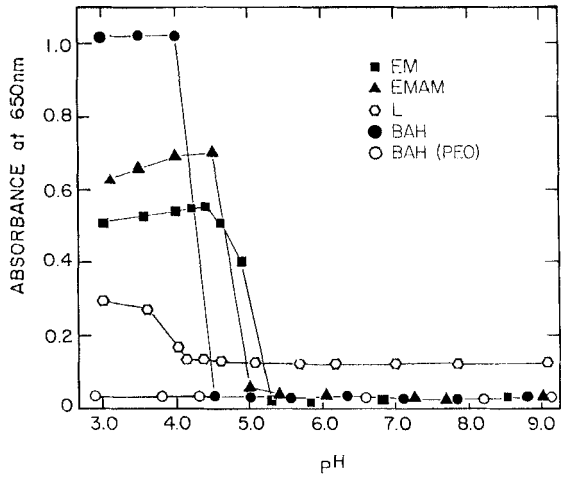

FIG. 2. Effect of pH on the aggregation of microspheres. Aggregation was determined by measuring the turbidity (absorption at 650 nm) of suspensions of microspheres (1 mg/ml) in an acetate-borate buffer at various pH values.

containing a high content of the hydrophobic monomer methyl methacrylate. From the titration data, one can estimate that there are approximately 38,000 titratable carboxyl groups per EM microsphere having an average diameter of 40 nm and a density of 1.23 g/cc [26].

Earlier studies using EM microspheres of either 40 nm or 100 nm indicated that these reagents show a low degree of nonspecific binding to red blood cells, Dictyostelium discoideum, bovine photoreceptor cells, thymocytes, and glutaraldehyde-fixed mouse spleen lymphocytes [4, 16-18, 26, 34, 35]. Recent studies, however, indicate that this class of microspheres shows a significant degree of nonspecific binding to adrenal tumor cells as well as unfixed mouse B-lymphocytes. This nonspecific binding appears to be reduced for the more hydrophilic microspheres, i. e., BAH microspheres.

PVP Microspheres

The application of Co γ-rays to poly(ethylene oxide) (PEO, 0.1 to 4% w/v) containing aqueous methanol and aqueous acetone solutions of isomeric vinylpyridine (4-vinyl, 2-vinyl, and 5-vinyl-2-methylpyridine) in presence of N,N'-methylene bisacrylamide (BAM)

TABLE 1. Properties of Acrylic Copolymer Microspheres

Polymer microsphere	Monomer		Diameter (Å)	pK_{app}	pK_{agg}	NaOH (μmole/mg microsphere)
	Component	%				
EM series	MMA	53	400	7.0	5.10	1.54
	HEMA	30				
	MA	10				
	EGDMA	7				
EM AM series	MMA	33	800	6.2	4.75	1.53
	HEMA	25				
	MA	10				
	AA	25				
	EGDMA	7				
L series	HEMA	70	1500	6.5	3.80	2.50
	MA	20				
	BAM	10				
BAH series	HEMA	30	600	5.8	4.25	1.52
	AA	30				
	BAM	30				
	MA	10				

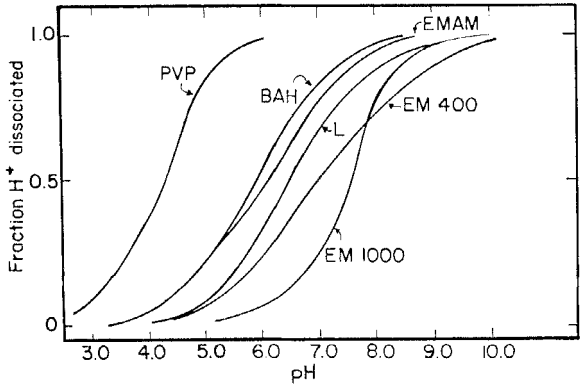

FIG. 3. Hydrogen-ion titration curves of various classes of microspheres at 25°C in the presence of 0.1 M NaCl.

yielded microspheres varying in size between a few hundred angstroms and 10 μm [24]. PEO in water in concentrations of 1% or higher, yields crosslinked gels when exposed to ionizing radiation and this suggests that the stabilization of microspheres may be due partly to grafting and partly to physical entanglement of PEO chains.

Parameters Affecting Particle Size. In presence of the crosslinking agent (BAM, 5-10%) all three isomers yielded well-defined microspheres with a relatively uniform particle distribution and a coefficient of variation of 5-10%. The effect of monomer concentration (4-vinylpyridine) on particle diameter in the presence of BAM is shown in Fig. 4. In the presence of methanol, a relatively large initial monomer concentration [M] can be used while keeping the system homogeneous, and the increase in size is readily apparent from Fig. 5. Similarly, at constant monomer concentration but increased amounts of methanol, the particle diameter undergoes a drastic increase (Fig. 6).

The fact that the size depends to a considerable extent on the solubility of particles in the polymerization medium is indicated by the results shown in Fig. 7.

The polymerization was carried out in acetone-water and methanol-water mixtures in which the concentrations of acetone and methanol were adjusted to obtain the same solubility parameter. In this case, the chemical nature of the solvent does not seem to affect the particle diameter (Fig. 7).

Copolymerization of 4-vinylpyridine with acrylamide in presence of the crosslinking agent in aqueous solutions containing 10 and 20% acetone yielded particle of various sizes as shown in Figs. 8 and 9, respectively.

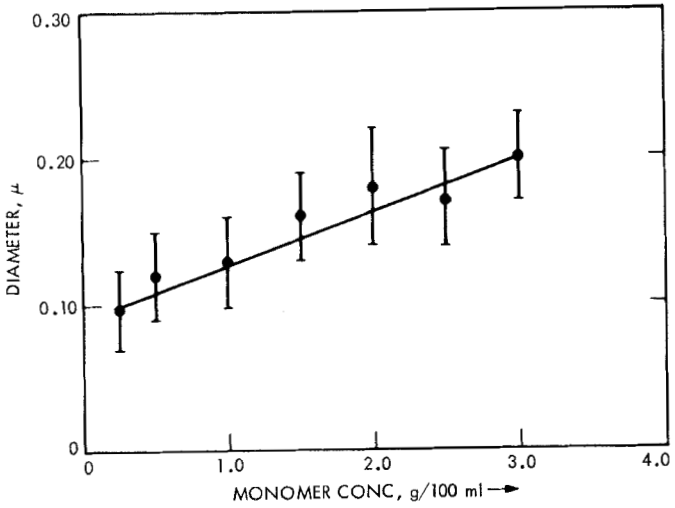

FIG. 4. Effect of monomer concentration on particle size. Monomer composition [M]; 4-VP = 2.7 g (90%); BAM = 0.3 g (10%) in polymerization medium, H_2O.

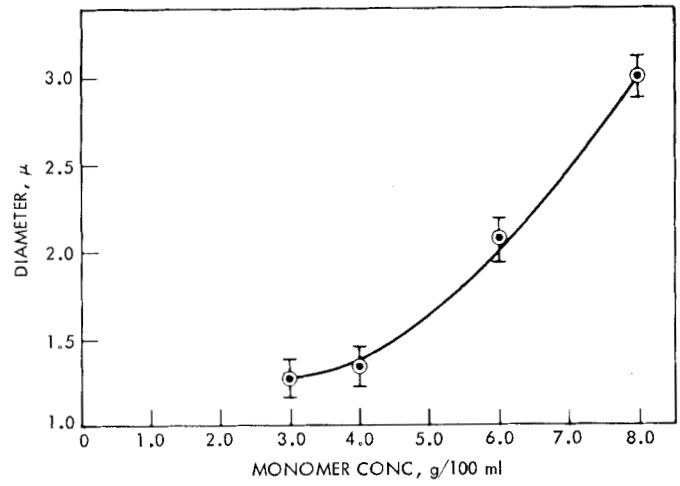

FIG. 5. Effect of monomer concentration on particle size. [M]; 4-VP = 2.7 g (90%), BAM = 0.3 g (10%). Polymerization medium, 30% methanol/water; 0.4% PEO.

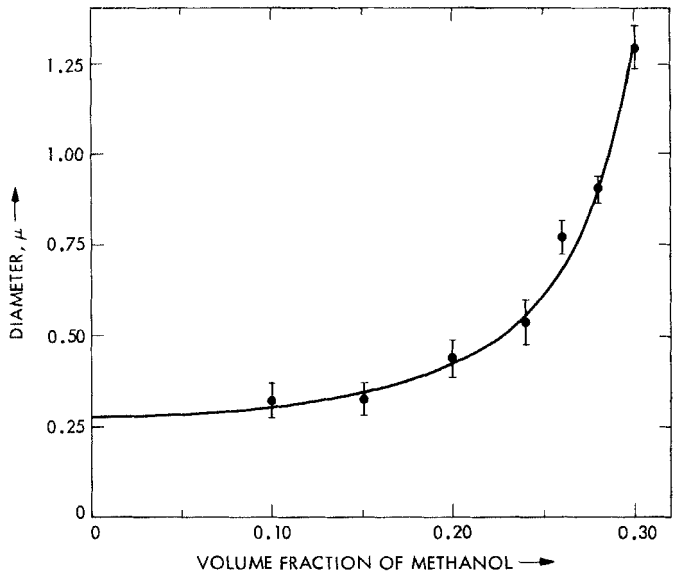

FIG. 6. Effect of methanol concentration on particle size. [M] = 2.7 g 4-VP 1100 ml; 0.3 g BAM/100 ml; 0.4% PEO.

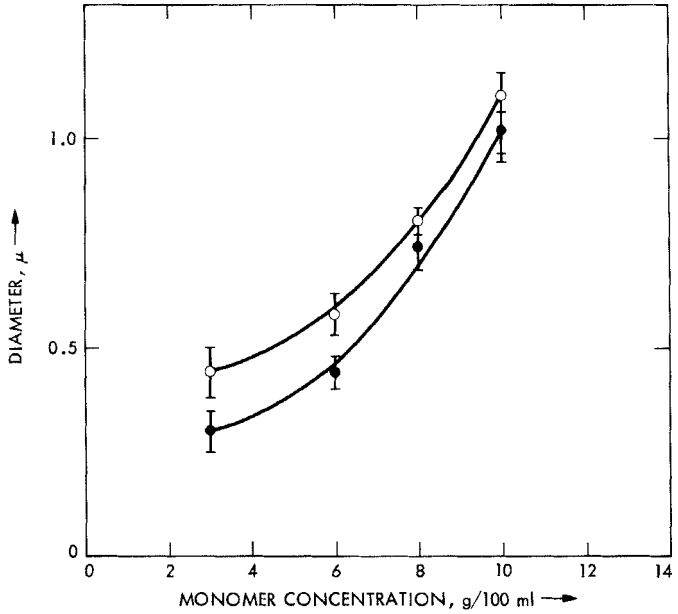

FIG. 7. Effect of mixture of solvents of the same solubility parameter on particle size: (○) acetone, δ = 21.6, 13.2%; (●) methanol, δ = 21.6, 20.0%. Calculated solubility parameter = 21.6.

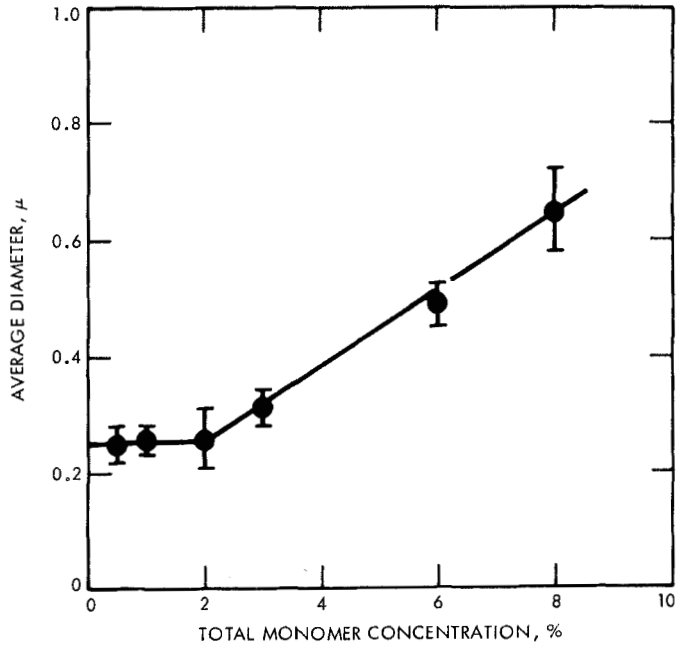

FIG. 8. Effect of monomer concentration on particle size. Polymerization medium: H_2O = 90%, acetone = 10%. [M] = 4-VP, 70%; acrylamide, 20%, BAM, 10%.

The SEM photomicrographs of PVP microspheres produced in aqueous acetone solutions of 4-vinylpyridine (70%), acrylamide (20%), and BAM (10%) after an irradiation dose of 4 Mrad are shown in Fig. 10, which illustrates the increase in size and polydispersity with increased concentration of the monomer mixture. At low concentrations of monomer mixtures and in absence of cosolvent, little variation of size was observed (Table 2).

Rate of Polymerization. The observation that the rate of formation of PVP microspheres in presence of a cosolvent was drastically decreased and required a radiation dose of several Mrad for completion, prompted the study of the rate of polymerization of 4-VP in presence of variable amounts of methanol and in absence of a crosslinking agent, i. e., in a homogeneous phase. The conversion as a function of time, (Fig. 11) was obtained by using a radiation dose of 2.4×10^5 rad/hr and freshly distilled 4-VP concentration of a 2% (w/v) in distilled water. The amount of polymer formed with

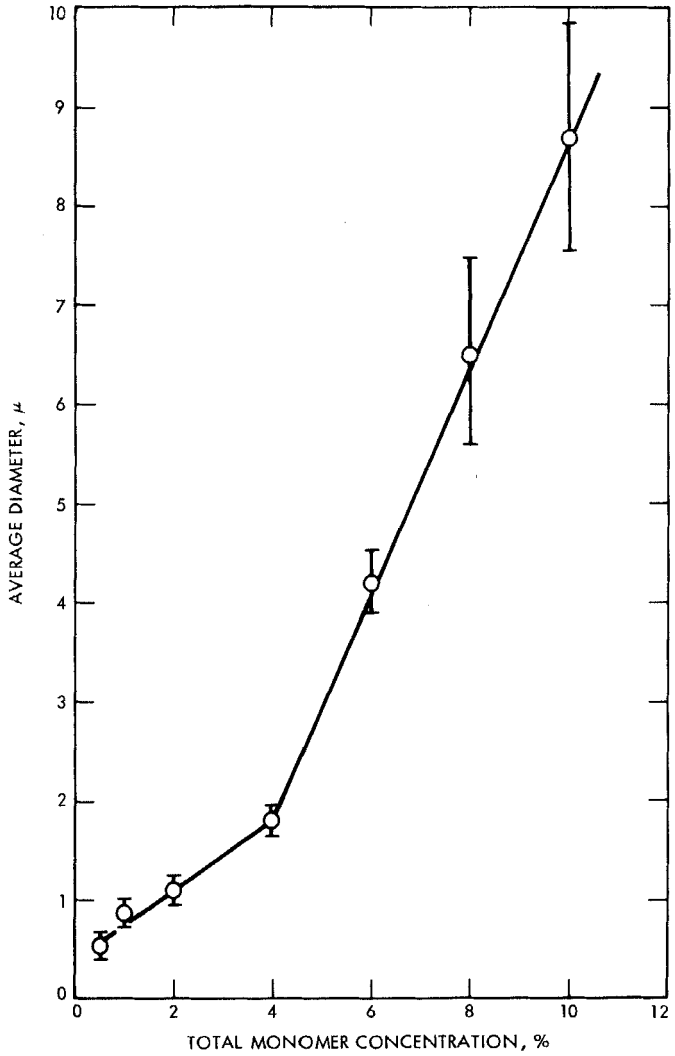

FIG. 9. Effect of monomer concentration on particle size. Polymerization medium; H_2O, 80%; acetone, 20% [M] as in Fig. 8.

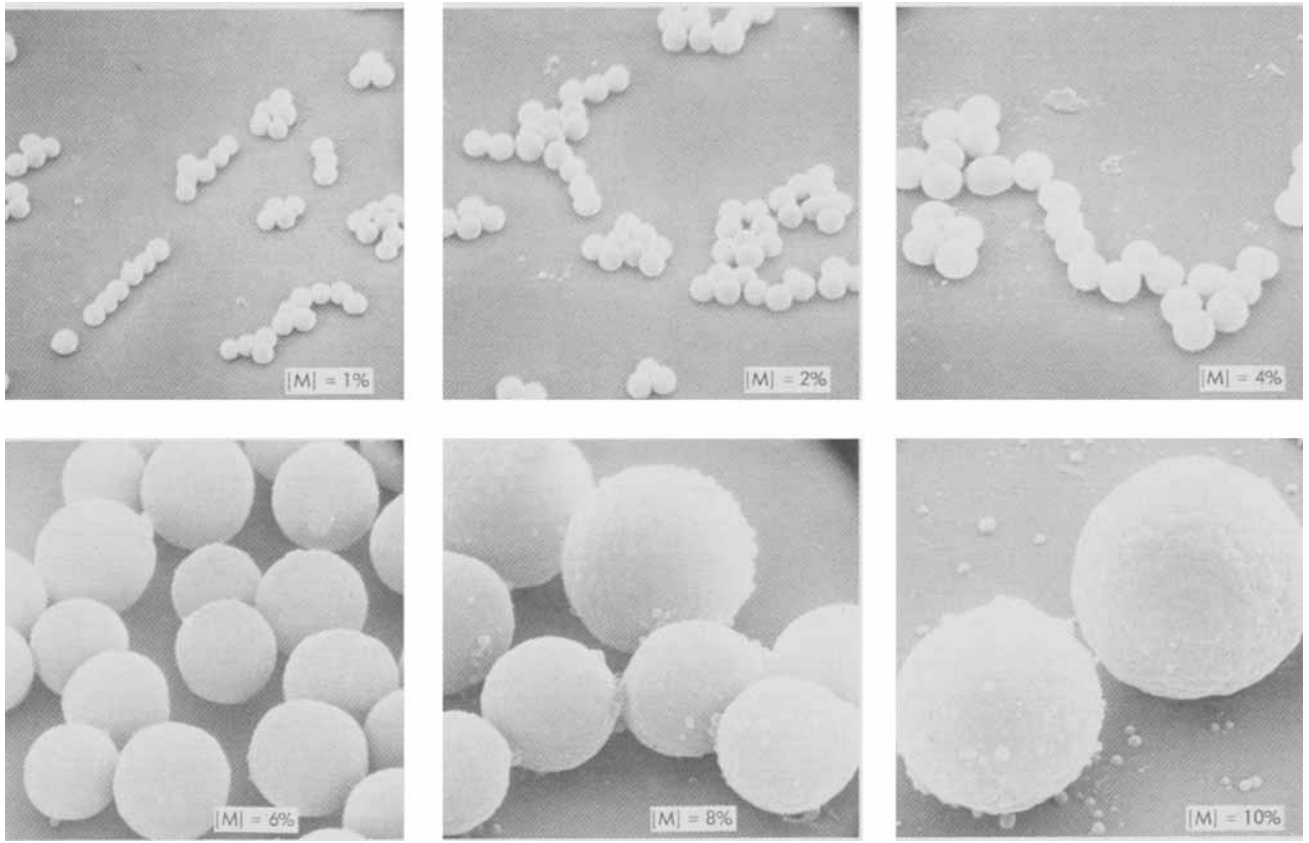

FIG. 10. SEM photomicrographs of PVP microspheres synthesized at various [M]; experimental conditions as in Fig. 9.

TABLE 2. Formation of PVP Microspheres by Copolymerization of 4-Vinylpyridine (4-VP) with HEMA (2-Hydroxyethyl Methacrylate), AM (Acrylamide), or MAM (Methacrylamide) in Water[a]

4-VP (wt %)	HEMA (wt %)	AM (wt %)	MAM (wt %)	BAM (wt %)	Diameter (Å)[b]
80.0	10.0	-	-	10.0	1800 ± 100
70.0	20.0	-	-	10.0	1500 ± 90
60.0	30.0	-	-	10.0	1800 ± 95
70.0	-		20.0	10.0	1800 ± 90
60.0	-		30.0	10.0	1900 ± 100
40.0	-		50.0	10.0	2500 ± 120
80.0	-	10.0	-	10.0	1600 ± 80
70.0	-	20.0	-	10.0	1700 ± 85
60.0	-	30.0	-	10.0	1800 ± 80

[a]Crosslinking agent, BAM (10% of total monomer weight); total dose rate, 0.8 Mrad; total monomer concentration, 2.0 g/100 ml H_2O; PEO concentration; 0.4% (w/v).

[b]Mean of twenty measurements on SEM photomicrographs.

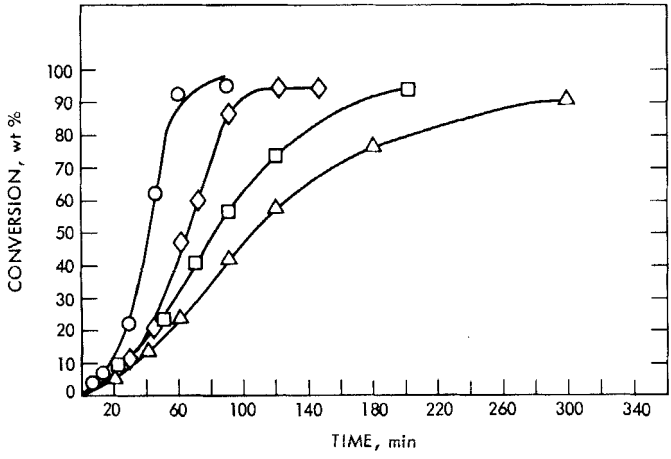

FIG. 11. Effect of methanol on the polymerization rate R_p: (○) 100% H_2O; (◇) 10% MeOH; (□) 20% MeOH; (△) 30% MeOH. Radiation dose, 2.4×10^5 rad/hr; [M] = 1.0 g/50 ml.

TABLE 3. Effect of Methanol Concentration on the rate of Polymerization (R_p) of Homogeneous Aqueous Solutions of 4-VP (2% w/v) at a Radiation Dose of 2.4×10^5 rad/hr

Methanol (volume fraction)	R_p (mole/liter-sec $\times 10^5$)	$\overline{M}_v \times 10^{-4}$ [a]
0	8.0	8.70
0.1	4.9	4.95
0.2	2.7	2.25
0.3	1.9	1.70

[a] From intrinsic viscosities $[\eta]$ on samples of 65% conversion measured in ethanol and calculated [36] from $[\eta] = 2.5 \times 10^{-4} \overline{M}_v^{0.69}$.

time was established gravimetrically on degassed samples irradiated in the Co gamma source at different times. The rate of polymerization calculated from the linear portions of the curves in Fig. 11 (between 20 and 60% conversion) is recorded in Table 3, which also shows that the decrease of rate due to the presence of methanol is associated with a considerable decrease of viscosity-average molecular weight ($\overline{M}_v$). It should also be noted that in pure water the molecular weight of PVP increased relatively fast up to about 62% conversion as shown in Fig. 12. The species formed during irradiation of water or methanol are believed to be as shown in Eqs. (1) and (2).

$$H_2O \longrightarrow H_3O^+_{aq} + \cdot OH + e^-_{aq} + H\cdot + H_2O_2 + H_2 \quad (1)$$

$$CH_3OH \longrightarrow \cdot CH_2OH + CH_3O\cdot + H\cdot + H_2 + CH_2O + CH_2OH\text{–}CH_2OH \quad (2)$$

The initiation of polymerization may be caused by reaction of ·OH radicals with the monomers. In the presence of methanol destructive interference with radicals originating from methanol radiolysis is a distinct possibility. The expected event of partial destruction of radical concentration is a decrease in initiation rate and increase in molecular weight. However, the observed decrease in the molecular weight of the polymer with increased volume fraction of methanol rules

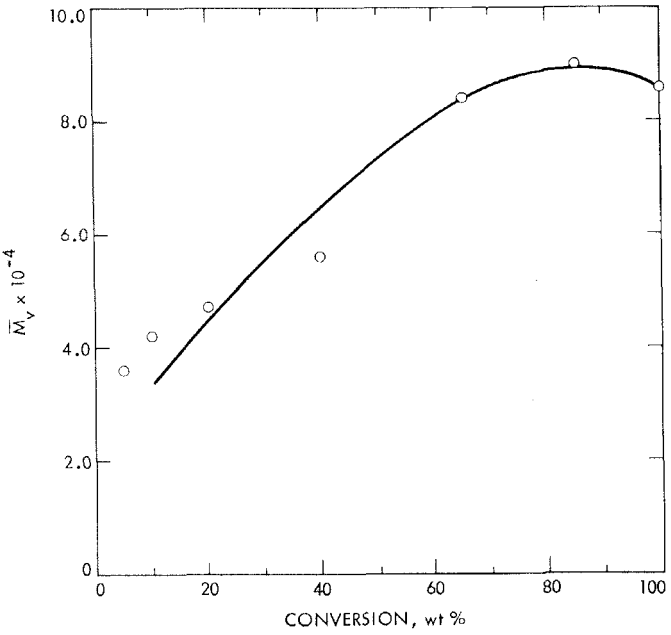

FIG. 12. Viscosity average molecular weight of PVP as a function of % conversion. [M] = 2.0 g 4-VP/100 ml; solvent, H_2O; radiation dose, 2.4×10^5 rad/hr.

out this mechanism. Therefore, an increase in termination rate by chain transfer to methanol is likely to be responsible for the decrease of R_p and of molecular weight.

Properties. The hydrophilicity of PVP microspheres as well as their surface area increases with increased concentration of hydrophilic crosslinking agent (BAM) or of the comonomer (acrylamide). The increase of hydrophilicity with increased BAM content was proven by measuring the amount of water absorbed (Fig. 13). PVP microspheres did not flocculate over a pH range of 2-11. This may be due either to the pyridine groups on the spheres or the grafting of PEO to their surface. The hydrogen ion titration curve confirmed the presence of pyridine groups having an apparent pK value of 4.4 (Fig. 3).

Electrophoretic Mobility. The availability of microspheres of relatively narrow size distribution offers an exceptional opportunity to study the electrophoretic mobility of spheres which

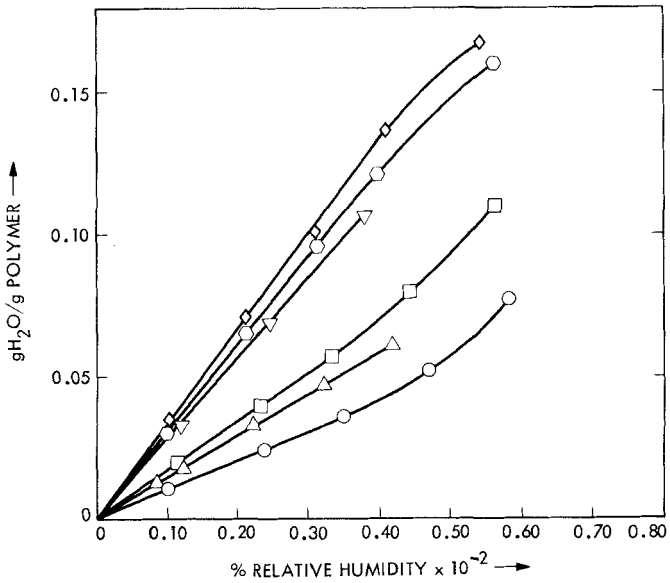

FIG. 13. Water adsorption isotherms at 25°C: (○) homopolymer; (△) 5% BAM; (□) 15% BAM; (▽) 30% BAM; (○) 40% BAM; (◇) 50% BAM.

acquire formal positive charges and therefore a polyelectrolyte nature. Studies now in progress [37] have yielded some information on their electrokinetic behavior. Figure 14 shows the pH profile of the electrophoretic mobility of PVP microspheres the diameter of which was found to vary from 3.6 to 7.6 μm as a function of pH and ionic strength. The electrophoretic mobility in presence of salts of the same molarity (0.15 M) undergoes reversible changes (Fig. 15).

The positive electrophoretic mobility at low pH decreases in presence of counterion in the order $SCN^- < I^- < Br^- < Cl^-$. At high pH's an increase in the negative electrophoretic mobility occurs in the reverse order, i. e., $SCN^- > I^- > Br^- > Cl^-$. The interpretation of these observations is as follows. At low pH and at equilibrium, ions of higher polarizability such as SCN^- form tighter ion pairs and therefore screen the positive charges on the PVP surface more efficiently than the counterions of lower polarizability (e. g., Cl^-). Consequently, the electrical double layer is compressed resulting in a decrease of electrophoretic mobility.

Examination of diameter changes as a function of salt concentration and pH confirms this interpretation (Figs. 16 and 17). In contrast

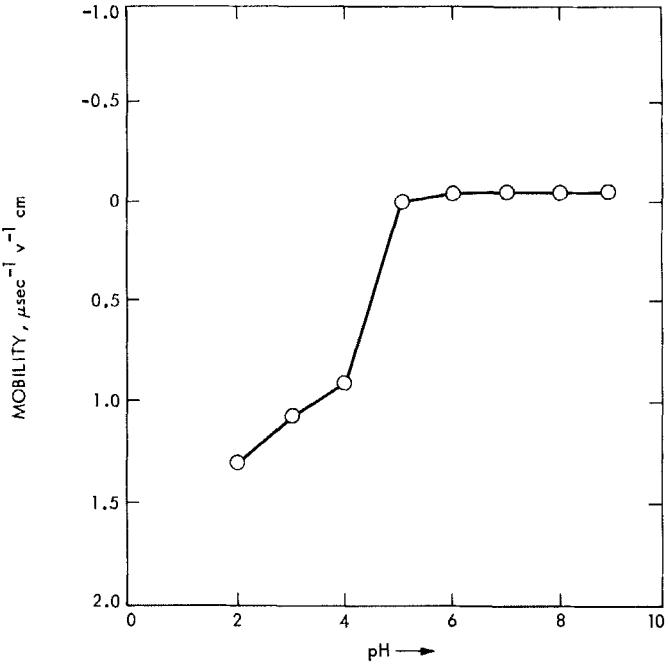

FIG. 14. Electrophoretic mobility of PVP microspheres synthesized in 20% aqueous acetone; [M] 4-VP = 95%, BAM = 5%; diameter at pH 7 = 3.6 μm.

to the reversible size changes at low pH, no variation of size is observed at pH 5 to 9 (Fig. 16), in which case the increases in the negative mobility are probably due to a counterion adsorption to electrically neutral PVP particles without formation of ion pairs.

The electrophoretic mobility of human rbc unlabeled and labeled with PVP microspheres determined under identical experimental conditions was sufficiently different (the electrophoretic mobility of unlabeled and labeled human rbc was found to be -1.08 ± 0.04 and $+0.65 \pm 0.03$ cm/μsec-V, respectively) to achieve separation in a free flow electrophoretic instrument [38]. Studies of electrophoretic separation of cell subpopulations labeled with several types of immunomicrosphere are in progress.

Polyglutaraldehyde Microspheres

Glutaraldehyde polymerizes in aqueous solution and its polymerization rate depends on the pH, concentration and temperature [39-42].

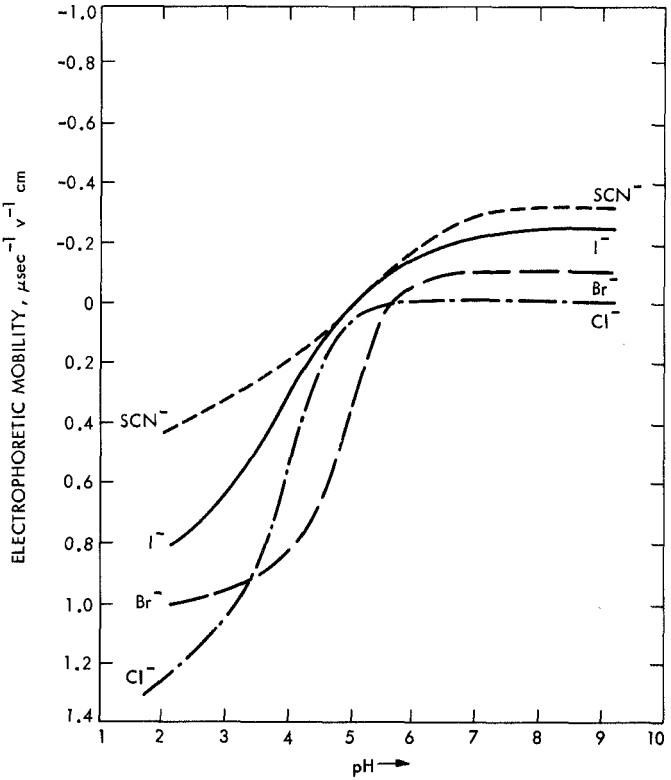

FIG. 15. Electrophoretic mobility of PVP microsphere in presence of salts at a constant ionic strength of 0.15.

The fact that conjugated aldehyde groups present in the polymer, yield stable reaction products with amines and that polyglutaraldehyde (PGL) may bind proteins by means of a simple one-step reaction prompted us to investigate the feasibility of formation of PGL microspheres. PGL was in fact found to react with proteins and when bound to microspheres of the PVP class successfully labeled a subpopulation of lymphocytes. The suspension polymerization of glutaraldehyde in presence of a surfactant yielded microspheres of various sizes depending on the reaction conditions [43]. As anticipated, a one-step reaction with antibodies yielded immunomicrospheres which successfully labeled human red blood cells (Fig. 18).

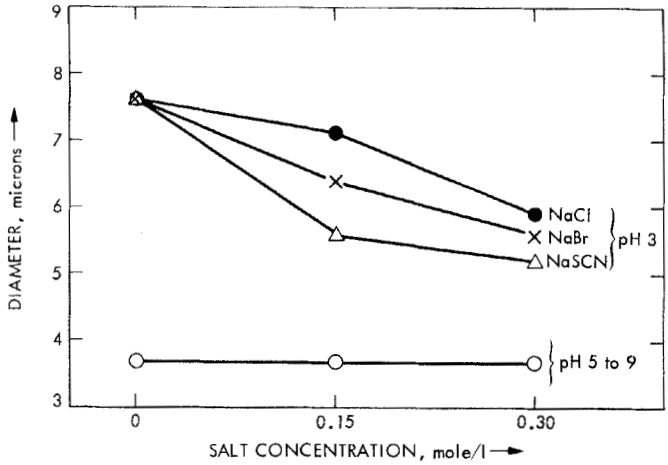

FIG. 16. Size of PVP microspheres as a function of ionic strength and pH.

Polymeric Magnetic Microspheres and Cell Separation

Polymeric magnetic microspheres were prepared by encapsulation of Fe_3O_4 particles with a crosslinked functional copolymer, obtained by radiation or redox polymerization of a variety of monomers.

The Fe_3O_4 particles used in our research were either from a "commercial" source (Ferrofluidics, Burlington, Mass., AOI, 5% w/w) or prepared in our laboratory. The method of preparation of particles 100 to 400 Å in diameter was based on a modified IBM procedure [44] by reacting the mixture of ferrous and ferric chloride with sodium hydroxide in the presence of a surfactant (Genamine 0-80, American Hoechst, Sommerville, N. J.). Radiation and redox polymerization techniques were used.

Radiation Polymerization. Preparation of magnetic polymeric microspheres by radiation polymerization was reported before [16]. The polymeric magnetic microspheres were synthesized from iron oxide (Fe_3O_4) particles and from methyl methacrylate (MMA), hydroxethyl methacrylate (HEMA), methacrylic acid (MAA), and ethylene glycol dimethacrylate (EGDA) purified as described previously [18]. Briefly, a mixture of 1.6 g MMA, 0.9 g HEMA, 0.3 g MAA, 0.2 g EGDA, 1 g iron oxide suspension, and 1 g Triton X-405 was deaerated and polymerized by means of Co γ-irradiation

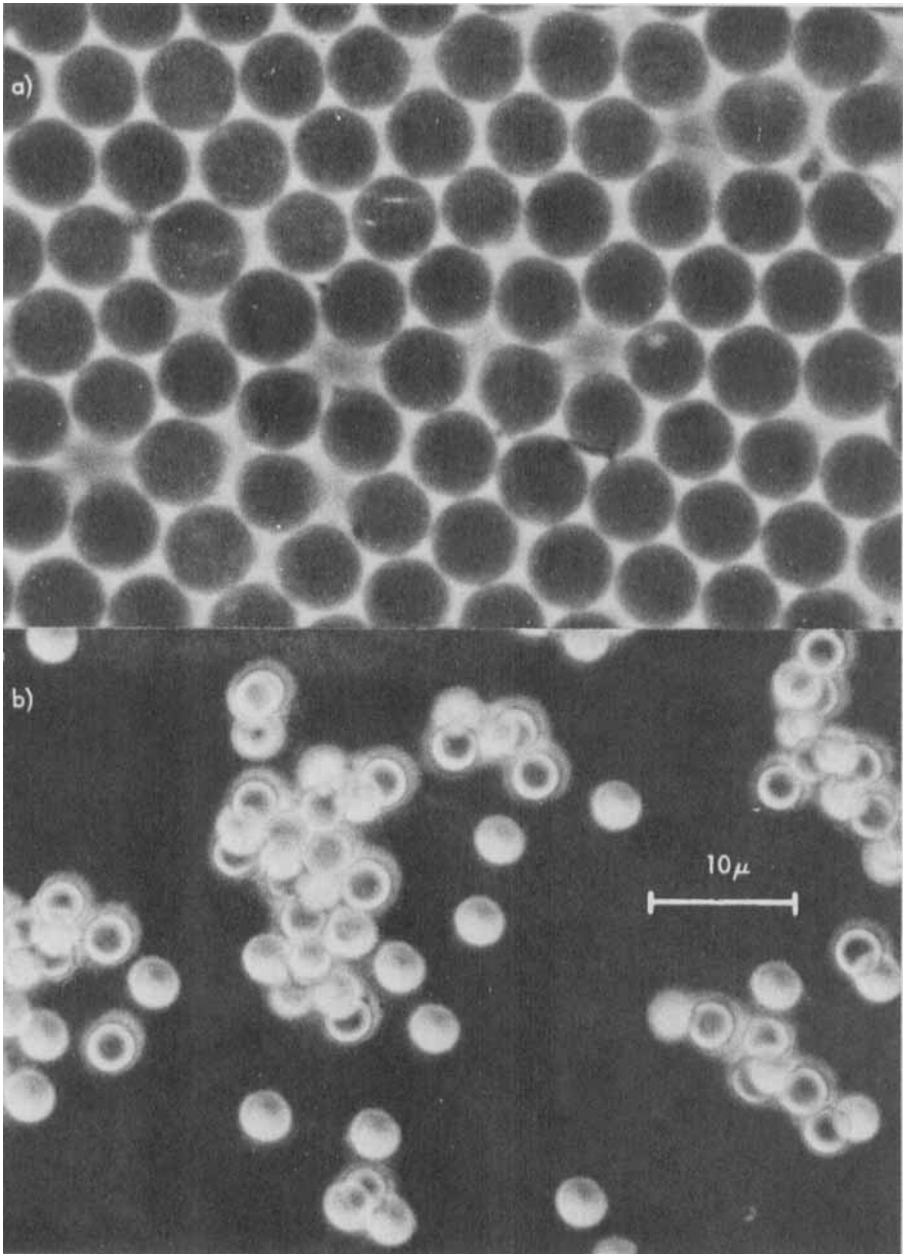

FIG. 17. Phase microscope photomicrographs of PVP microspheres: (a) at pH 3 in absence of salts; (b) at pH 6-9.

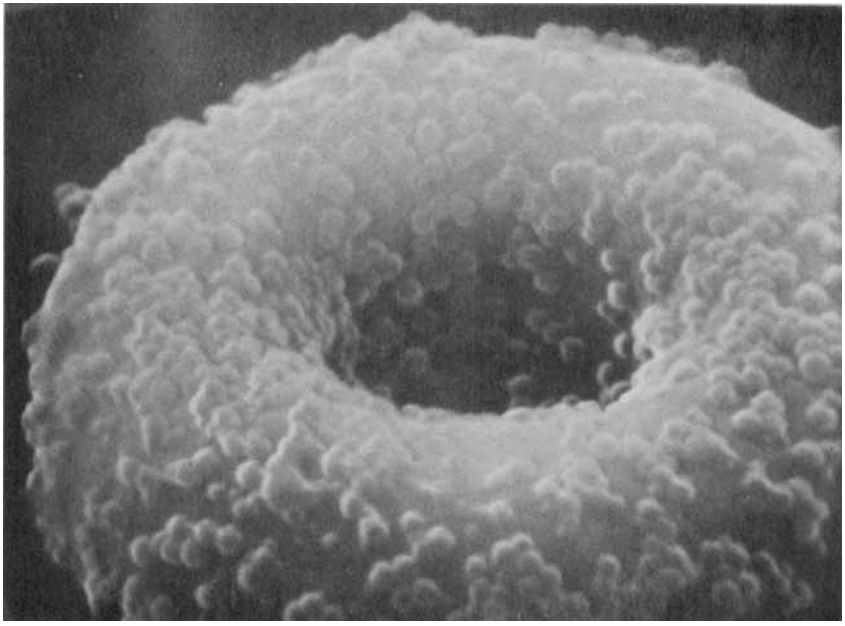

FIG. 18. SEM photomicrograph of human rbc sensitized with rabbit x human erythrocytes and labeled with PGL goat x rabbit microspheres.

(0.2 Mrad at 0°C for 60 min). The Fe microsphere suspension was purified on a mixed-bed ion-exchange column and centrifuged at 30,000g for 45 min at 4°C on a discontinuous gradient consisting of 20% (w/w) sucrose in the upper layer and 60% (w/w) in the lower layer. Three fractions (brown in color) were recovered: an upper band in the 20% sucrose fraction (Fe content 6.2%); a lower band in the 20% sucrose layer (Fe content 28.4%); and a pellet (Fe content 42.4%). The upper and lower fractions were used in cell labeling studies. The average size of these particles was 40 nm as measured by SEM.

Redox Polymerization. Synthesis of polymeric magnetic microspheres by redox polymerization was first disclosed in 1976 [45]. Some of the typical examples are shown in Table 4. The functional polymeric magnetic microspheres derived from hydrophilic copolymers either by radiation or redox polymerization were successfully tested as immunoreagents.

Subsequent investigations showed that T and B cells labeled with

TABLE 4.

Monomer mixture	Monomer	Composition (g/100 g H_2O)						
		SDS	Fe_3O_4[a]	$NaHSO_3$	$FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$	$K_2S_2O_8$	$(NH_4)_2S_2O_8$	Polymerization time (hr)
I[b]	3.2	0.1	0.1	0.04	0.0024	0.08	-	2.5
I	3.2	0.1	0.1	0.04	-	0.08	-	2.5
II[c]	3.36	0.1	0.1	0.04	-	0.08	-	2.5
I	3.5	0.1	0.1	0.04	-	0.08	-	2.5
III[d]	3.4	0.1	0.1[f]	-	-	-	0.01	3
IV[e]	3.4	0.1	0.15[f]	-	-	-	0.01	24

[a] Fe_3O_4 particles from Ferrofluidics, Burlington, Mass., unless otherwise noted.
[b] Composition of mixture I: MAA, 20%; HEMA, 20; EGDMA, 7%; MMA, 53%.
[c] Composition of mixture II; MAA, 19%; HEMA, 19%; EGDMA, 7%; MMA, 53%.
[d] Composition of mixture III: MAA, 10%; HEMA, 27.5%; EGDMA, 7%; MMA, 28%; AM, 27%.
[e] Composition of mixture IV: MAA, 16.0%; HEMA, 25.8%; EGDM, 6.6%; MMA, 25.8%.
[f] Fe_3O_4 particles prepared at JPL.

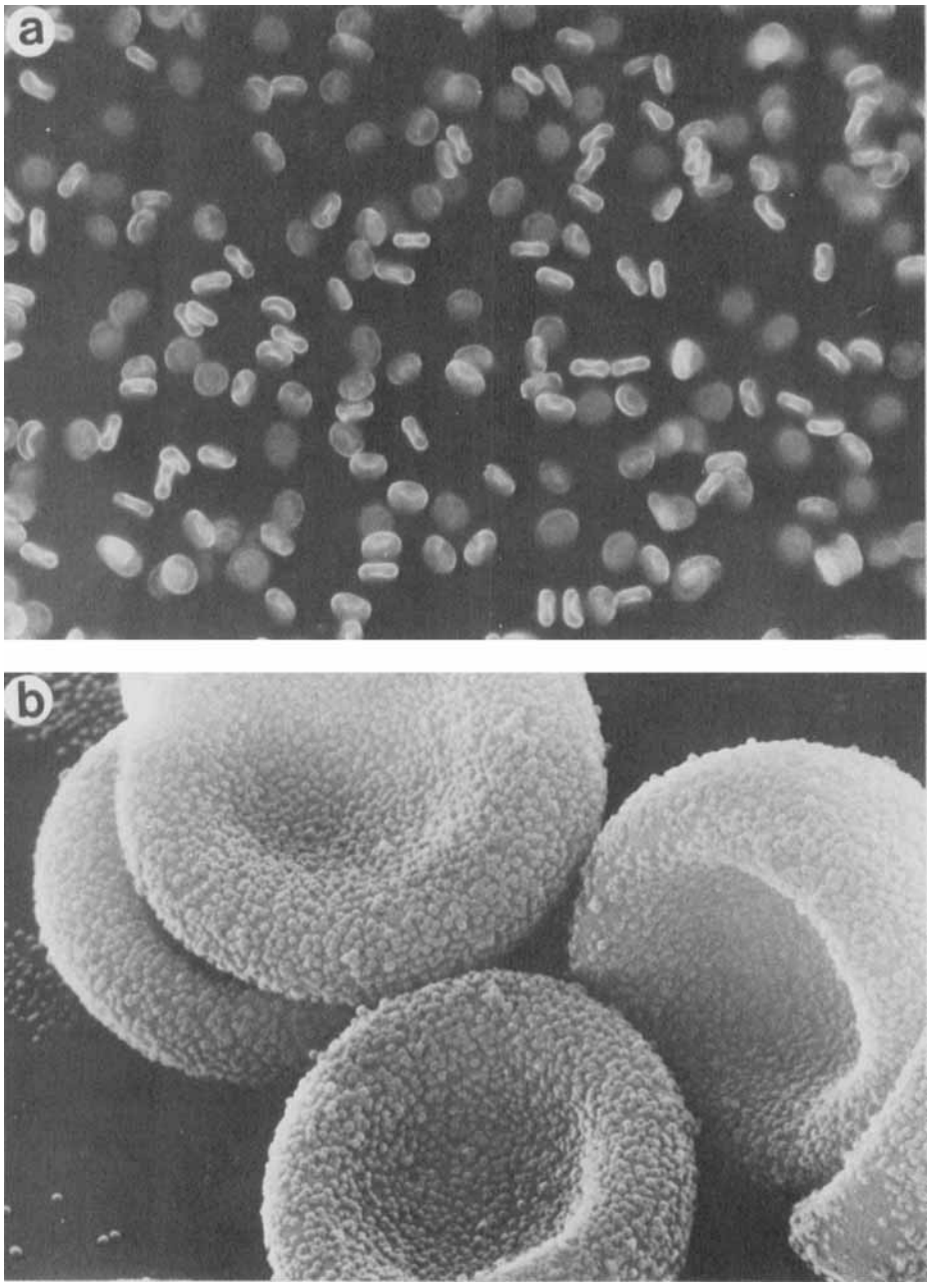

FIG. 19. Human red blood cells labeled first with rabbit anti-red blood cell antiserum and subsequently labeled with goat antirabbit antibodies covalently bonded to fluorescent polymeric microspheres (1000 Å diameter): (a) fluorescent micrograph; (b) scanning electron micrograph.

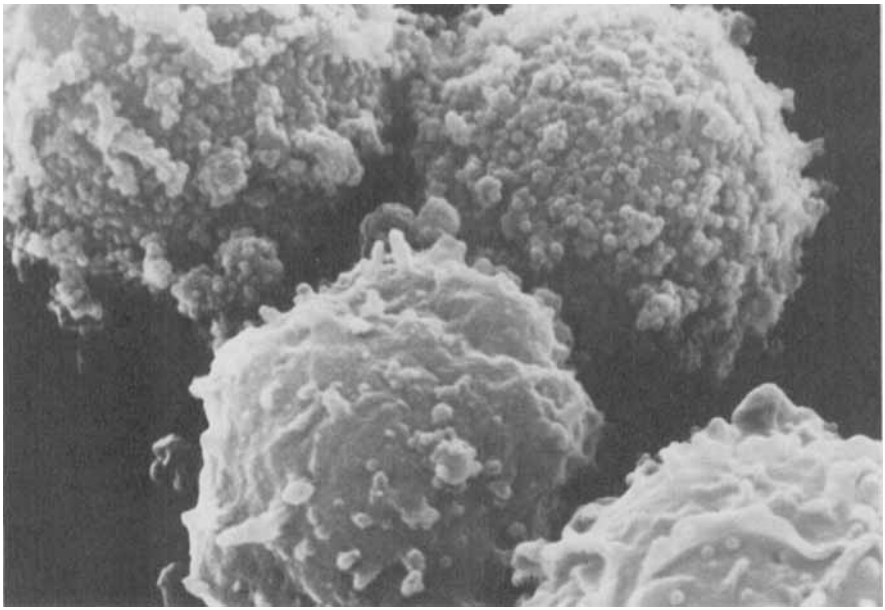

FIG. 20. Murine spleen lymphocytes fixed with glutaraldehyde and subsequently labeled for cell surface immunoglobulins (Ig) with immunomicrospheres. Only the cells with surface Ig are labeled.

magnetic immunomicrospheres can be efficiently separated by means of a permanent magnet; fluorescent microspheres coupled to an antibody (goat anti mouse Ig) were used to label mouse lymphocytes, and fluorescent microscope examination revealed that 37% of nonaggregated cells were labeled. The mixture of labeled and unlabeled cells was then subjected to a magnetic field by means of which fluorescent cells could be separated out leaving behind a T cell subpopulation. An independent confirmation of the feasibility of this technique was recently reported [46].

Application of Polymeric Microspheres as Visual Markers for Light and Scanning Electron Microscopy

Immunomicrospheres have been used to identify specific populations of cells by fluorescent light and SEM and to study the distribution of antigens on cell surfaces. In general the indirect immunological labeling technique has been used most frequently. This method

involves labeling the cell with antibodies (immunoglobulins) directed against specific cell surface antigens. These cells are then relabeled with immunomicrospheres consisting of microspheres coupled to anti immunoglobulin antibodies. Figures 19 and 20 illustrate the use of immunomicrosphere reagents in labeling cells. Microspheres coupled to lectins have also been used to study the distribution of membrane glycoproteins on cell surfaces [4, 34].

CONCLUSIONS

Polymeric microspheres bound to antibodies serve as versatile immunological reagents for cell surface labeling studies. These immunomicrospheres can be used to identify specific populations of cells and study the distribution of surface antigens by light and scanning electron microscopy. Preliminary studies further indicate that microspheres containing iron oxide have potential applications in the separation of cells, membranes and receptors.

An important requirement is that such microspheres must not bind nonspecifically to cell surfaces. The first class of microspheres (EM) we had prepared were shown not to bind to red blood cells, thymocytes, Dictyostelium discoideum cells and glutaraldehyde fixed lymphocytes. These reagents are indeed an improvement over commercially available polystyrene latex microspheres. More recent studies in our laboratory, however, indicate that this class of microsphere show some degree of nonspecific binding to unfixed B-lymphocytes and several other cell types. We have therefore prepared several classes of microspheres with different functional groups and a more hydrophilic surface as described in this review. These microspheres appear to show a significantly lower degree of nonspecific binding to cell surfaces. Such microspheres should prove valuable in labeling specific cells for biochemical and immunological studies.

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