Table VI
Test of Empirical Equations[a]

| System | Φ (polymer) | Deviation, % | | | |
|---|---|---|---|---|---|
| | | Eq A | Eq B[b] | Eq C[b] | Eq D[b] |
| PIB-B | 0.1989 | 0.04 | 0.11 | 0.13 | 2.88 |
| | 0.4355 | 0.09 | 0.03 | 0.03 | 0.64 |
| | 0.5923 | 0.07 | 0.02 | 0.06 | 2.61 |
| PIB-C | 0.1971 | 0.81 | 0.79 | 0.06 | 2.30 |
| | 0.3731 | 1.56 | 1.23 | 0.20 | 3.95 |
| | 0.5445 | 1.03 | 0.71 | 0.19 | 3.41 |
| PDMS-C | 0.3530 | 3.35 | 3.53 | 0.16 | 0.93 |
| | 0.5017 | 6.15 | 6.17 | 0.01 | 1.40 |
| PMDS-HMDS | 0.4370 | 2.74 | 2.55 | 0.00 | 0.90 |
| | 0.6497 | 3.57 | 3.45 | 0.06 | 3.43 |

[a] Deviations between calculated and measured quantities at 25 °C. Deviation = 100(calcd − meas)/(meas). [b] At 800 bars.

## Conclusion

The volumetric measurements obtained in this work show that at high pressures, Flory's equation of state cannot correctly represent the PVT behavior of pure liquid polymers, pure liquid solvents, and their mixtures, when equation-of-state parameters are evaluated from volumetric data at 1 atm. This deficiency may be significant for polymer–solution thermodynamics since equation-of-state contributions are important in calculating excess functions of polymer mixtures. It may, therefore, be desirable to consider possible improvements in Flory's equation of state.

**Acknowledgment.** For financial support the authors are grateful to the National Science Foundation, the donors of the Petroleum Research Fund, administered by the American Chemical Society, and to the Commission of Educational Exchange between the USA and Spain.

**Supplementary Material Available:** tables for experimental relative volumes (18 pages). Ordering information is given on any current masthead page.

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# Functional Polymeric Microspheres Based on 2-Hydroxyethyl Methacrylate for Immunochemical Studies[1]

**A. Rembaum,*[2a] S. P. S. Yen,[2a] E. Cheong,[2a] S. Wallace,[2a] R. S. Molday,[2b] I. L. Gordon,[2b] and W. J. Dreyer[2b]**

*Jet Propulsion Laboratory and Biology Division, California Institute of Technology, Pasadena, California 91125.* *Received September 30, 1975*

ABSTRACT: Co γ irradiation of 2-hydroxyethyl methacrylate in the presence or in the absence of other acrylic monomers was found to constitute an effective technique for the synthesis of hydrophilic functional microspheres in the size range of approximately 0.3 to 3 μ in diameter. The effect of monomer concentration, steric stabilization, and electrostatic interaction on the particle size was investigated. Experimental conditions were determined to obtain desired particle sizes of relatively narrow distribution. It was shown that particles may be formed without intermediate micelles, i.e., by homogeneous nucleation, and the rate of particle formation is affected primarily by the rate of particle coalescence in the initial stages of the reaction. When covalently bound to antibodies these microspheres were successfully used to label murine and human lymphocytes.

We have recently reported[3] a new, generally applicable technique which has been used to label antigens on the surface of red blood cells and lymphocytes. This technique consisted of (a) the synthesis by emulsion copolymerization of spherical particles containing hydroxyl and carboxyl groups on their surface in the sizes of 30–340 nm in diameter; (b) the covalent binding of amino acids, fluorescent molecules, and antibodies to the latex spheres by means of the cyanogen bromide, carbodiimide, and glutaraldehyde methods; (c) the interaction of the latex antibody conjugates with living cells and the examination of the labeled cells by means of scanning electron microscopy. In using transmission electron microscopy antigens on cell surfaces were previously visualized by means of ferritin,[4a] haemocyanin,[4b] enzymes,[5] or viruses[6] bound to antibodies. Haemocyanin and viruses have also served as markers for scanning electron microscopy[7,8] (SEM). Commercial polystyrene latex particles have been employed as immunological

markers for SEM work.[9,10] But applications of polystyrene microspheres are limited because the hydrophobic surface of the particles makes them stick nonspecifically to many surfaces of living cells and frequently induce a physiological response. Thus the interaction of polystyrene with blood platelets, for example, results in their activation, release of enzymes, and eventual clotting. In contrast the highly hydrophilic polyacrylamide under identical conditions does not produce any observable platelet changes.[11] In spite of the nonspecific "stickiness" of polystyrene latices, apparently due to its aromatic and/or hydrophobic character, they were used successfully in a number of agglutination and radioimmuno assays. These are based on the principle that physically adsorbed antibodies on polystyrene particles interact with specific antigens in blood sera causing particle aggregation. A number of diagnostic tests including the pregnancy test, are based on this phenomenon. However, because of the weak adsorption forces between the polystyrene particles and antibody molecules and also because of the nonspecific interaction of polystyrene with living cells the agglutination tests are not always satisfactory.[12]

Polymeric spheres to which molecules of biochemical interest are bound have also been widely used as immunoadsorbents for antigen and antibody purification[13] for specific affinity chromatography and for immobilization of enzymes. Agarose polymers most widely used as matrices appear particularly ideal for enzyme immobilization[14] and polyacrylamides are successful alternatives.[15] A promising technique making use of polyamides and nylon was recently described.[16] Most of these polymeric solid supports in chromatographic columns contain relatively large particles (10 to 60 $\mu$) and are therefore not suitable for cell labeling by means of SEM or light microscopy. In order to achieve cell labeling under the light microscope there is a need for biocompatible polymeric microspheres in the size range of about 0.4 to 2 $\mu$ in diameter with functional groups capable of covalent bonding with proteins or fluorescent molecules. A gel like nature and high porosity of particles seems to meet the biocompatibility requirement.[17] From a preparative point of view, the problem facing a polymer chemist working in this field is to select the optimum technique to attach the appropriate chemical function to a macromolecular backbone. Basically this can be achieved by two principal methods. The first is to polymerize or copolymerize suitable monomers already containing the desired functions. The second method involves performing one or more modification reactions on a suitable macromolecule thus introducing the desired functions on a presynthesized polymer.

In this paper we describe the design, synthesis and properties of gel like latex spheres containing a variety of functional groups by polymerization and copolymerization of water-soluble monomers. Co $\gamma$ radiation was found to constitute a convenient technique for the synthesis of hydrophilic and cross-linked therefore insoluble functional microspheres in the size range of approximately 0.3 to 3 $\mu$ in diameter. With the choice of suitable comonomers it is possible to incorporate into the particles hydroxyl, carboxyl, amido, amino, dimethylamino, and hydrazido functional groups. Suitable experimental conditions were determined to obtain desired particle size of relatively narrow distribution. For a number of applications it was desirable to tag the particles with fluorescent dyes. In order to ensure permanent attachment of fluorescent molecules to the latex spheres we have designed fluorescent monomers and copolymerized with nonfluorescent acrylic monomers. The fluorescent tagging and introduction of other functional groups could also be achieved by reacting the presynthesized polymeric microspheres with diamino alkanes through the cyanogen bromide procedure and subsequently with fluorescent compounds. When covalently bound to antibodies or lectins, these reagents were successfully used to label murine and human lymphocytes.

## Experimental Section

**Materials.** The purification of 2-hydroxethyl methacrylate (HEMA), methacrylic acid (MA), methyl methacrylate (MMA), ethylene glycol dimethacrylate (EGDM), and *N,N'*-methylenebisacrylamide (BAM) was previously described.[3b]

Additional monomers fractionally distilled and then used in copolymerization experiments were: 2-dimethylaminoethyl methacrylate (DMA, bp 68.5 °C (10 mm) from Rohm and Haas). Acrylamide was recrystallized from chloroform (mp 85 °C). Methacryloylhydrazide (MAH) was prepared according to the method of Harada et al.[18] Allylamine was distilled at atmospheric pressure (bp 56.5 °C). Dansyl chloride (Sigma Chem. Co.) was recrystallized from methylene chloride (mp 69 °C). Fluorescein isothiocyanate, rhodamine B isothiocyanate (Nutritional Biochem Co., Cleveland, Ohio), and Lissamine rhodamine sulfonylchloride (99% pure, Eastman Chem. Co.) were used as received. Polyethylene oxide (PEO) was acquired from Union Carbide (sample WSR 301). Commercial HEMA is quoted by the manufacturer to be 94% pure and contains higher boiling homologues of 2-hydroxyethyl methacrylate as well as MA (3.5%) and EGDM (1.5%). For comparison purposes highly purified HEMA (courtesy Dr. M. V. Rostoker of Hydron Lab Inc., New Brunswick, N.J.) was used. This material contained MA (0.01%) and EGDM (0.37%) and was vacuum distilled to ensure absence of inhibitor.

**Methods. (a) Polymerization.** Most polymerizations and copolymerizations were carried out by means of ionizing radiation using a Co $\gamma$ source and a dose of 0.8 mrad at 20–30 °C in homogeneous aqueous solutions purged with nitrogen or argon. The pH of the reaction medium was 3 to 5 and 7 to 9 for mixtures of HEMA with MA or acrylamide and HEMA with basic comonomers, respectively. Post irradiation effects were minimized by opening the containers to the air and diluting with water immediately after irradiation. The percentage yield of polymer particles and size distribution was determined before centrifugation. The average diameter and the yields were estimated after centrifugation, which served to remove unreacted monomers and impurities. The yield was obtained from a known volume of a well dispersed latex or suspension evaporated to dryness at atmospheric pressure and then dried in a vacuum oven at 100 °C.

A fluorescent monomer was synthesized by reacting allylamine (5 mmol) with dansyl chloride (5 mmol) in acetone (50 $cm^3$) in the presence of triethylamine (5 mmol) for 6 h at 0 °C, evaporating to dryness and dissolving the residue in dichloromethane. The solution was washed with sodium bicarbonate solution (3%) and a dilute solution of acetic acid (3%). After drying it was recrystallized from petroleum ether (mp 81 °C). Its ir spectrum is shown in Figure 1. Fluorescein containing particles were obtained by admixture of allylamine (1%) and fluorescein isothiocyanate (0.05%) to the monomers before the initiation of polymerization.

The average size of particles was established by measuring the diameter of about ten particles in several SEM photographs or from enlarged photographs (25×) taken under an oil immersion lens (100×) of a light microscope. In the latter case, the actual diameter was calibrated by comparison with SEM photographs. The average diameter $\bar{D}_v$ was calculated from

$$\bar{D}_v = (\sum n\bar{D}_v^3/\sum n)^{1/3}$$

where $n$ is the number of measured particles. The size distribution was determined by means of an instrument recently developed for cell sorting and other biological applications.[19] In this instrument cells or particles are observed individually in suspension in the central stream of a very small coaxial liquid jet as they pass through two laser beams. The low-angle scattered light produced by a helium–neon unit operating at 632.8 nm yields a signal related to particle volume. The second laser (454–514 nm) offers information of particle size distribution based on their fluorescence. The coefficient of variation ($C_v$) of a Gaussian or near Gaussian distribution is given by $C_v = \frac{1}{2}(w/x)$: where $w$ is the width of the distribution curve at 0.6 of its height and $x$ is the distance from the origin to the peak.

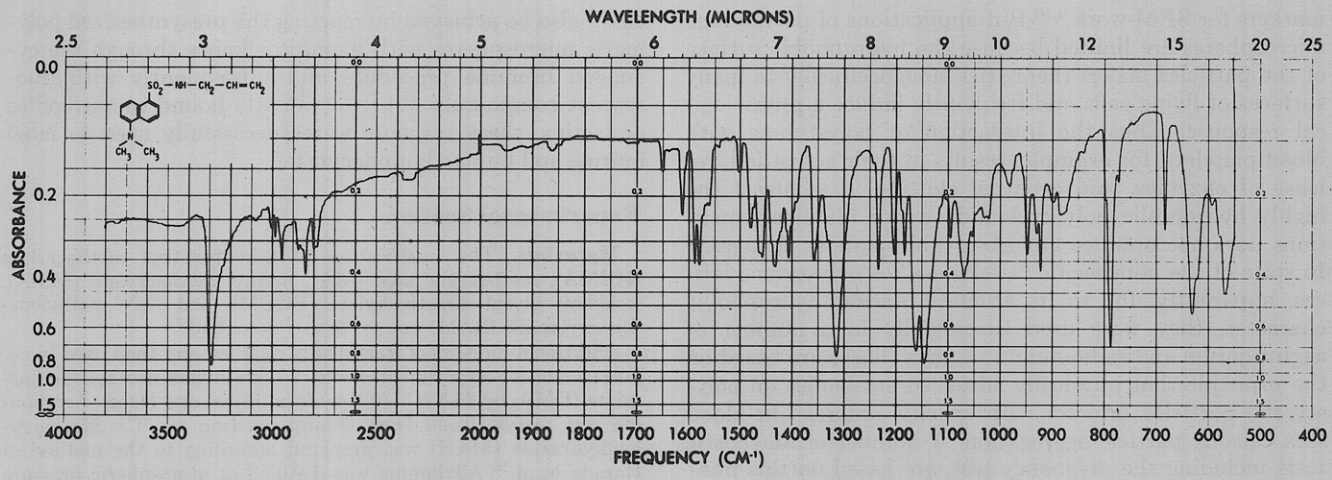



**Figure 1.** Ir spectrum of dansylallylamine (NH stretch 3280 $cm^{-1}$; olefinic CH 3000 $cm^{-1}$; C=C 1650 $cm^{-1}$)

**(b) Preparation of Microsphere–Antibody Conjugates.** Microspheres derivatized with diaminoheptane were coupled to antibody molecules by a two-step glutaraldehyde reaction as previously described.[3b] Typically, 1.5 ml of an aqueous solution of diaminoheptane (0.5 M) was added to 13.5 ml of a fluorescent microsphere suspension (50 mg/ml) and the pH of the solution was adjusted to 7.0 by the addition of 1 N HCl. After equilibration at 4 °C, 100 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was added and the reaction mixture was stirred for 4 h. Excess diaminoheptane was removed by dialysis first against 1 l. of 0.1 M NaCl and then against 2–3 changes (1 l. each) of 0.01 M sodium phosphate buffer pH 7.0. Presence of free amino groups was confirmed using trinitrobenzene sulfonate color test.[20]

Amino groups on the spheres were activated by adding to the latex suspension a 25% (w/v) aqueous glutaraldehyde solution until a final concentration of 1.25% w/v glutaraldehyde was reached. The reaction mixture was stirred for 1 h at 25 °C after which the excess of glutaraldehyde was removed by dialysis against three changes of 1 l. each 0.01 M sodium phosphate buffer pH 7.0 at 4 °C over a 16-h period. Goat antirabbit immunoglobulin or goat antimouse immunoglobulin antibody purified by affinity chromatography[21] was coupled to the spheres by adding 2.6 ml of antibody (3.1 mg/ml) to 7.4 ml of glutaraldehyde activated latex in 0.01 M sodium phosphate buffer pH 7.0. Sodium azide (1 mM) was added to inhibit microbial growth. After 12–24 h the reaction was stopped by the addition of 100 mg of glycine.

Microsphere–antibody conjugates (5 ml) were separated from unbound antibody by centrifugation on a discontinuous gradient consisting of 4 ml of 60% sucrose overlayed with approximately 25 ml of 20% sucrose. Centrifugation was carried out at 5 °C in a Beckman Sw-27 swinging bucket rotor for 30 min at 15 000 rpm. The conjugated microspheres were collected at the interface between the two sucrose layers. This washing procedure was repeated, to ensure complete removal of unbound antibody. Sucrose was removed by dialysis against PBS containing 1 mM EDTA at 4 °C. The final antibody latex conjugate was adjusted to a volume of 6 ml and stored in the dark at 4 °C until used.

**(c) Labeling of Lymphocytes with Microspheres.** Mouse lymphocytes were isolated from a suspension of spleen cells by centrifugation on a Ficoll-isopaque gradient[22] and washed in bicarbonate buffered Hank's balanced salt solution (Gibco) supplemented with 5% fetal calf serum. In a typical indirect labeling experiment $4 \times 10^6$ lymphocytes were incubated in 0.3 ml of rabbit antimouse lymphocyte serum or rabbit antimouse immunoglobulin serum in small conical centrifuge tubes with occasional agitation at 4 °C. After 30 min, the cells were washed by repeated centrifugation at 4 °C. The sensitized lymphocytes in 0.1 ml buffer were then labeled with 0.1 ml of goat antirabbit immunoglobulin–microsphere conjugate for 15 min at 4 °C. Finally the cells were separated from unbound conjugate by centrifugation on a dense solution consisting of 7.5 parts 34.8% w/v sodium metrizamide (Nyegaard Co.) and 2.5 parts 0.01 M phosphate–0.15 M sodium chloride buffer. Centrifugation was carried out for 15 min at 1000 g in a clinical centrifuge. Free microspheres pelleted to the bottom of the tube, while labeled and unlabeled cells banded at the interface with the dense solution. The lymphocyte band was removed with a capillary and resuspended in PBS or in 1% glutaraldehyde PBS and placed on a microscope slide for examination of fluorescence. For examination of labeled cells in an ordinary light microscope the lymphocyte band was suspended in fetal calf serum centrifuged onto a cover slip and stained with Wright stain.

In *direct* labeling experiments antibody–microsphere conjugates were mixed with cells which were not pretreated with an antiserum.

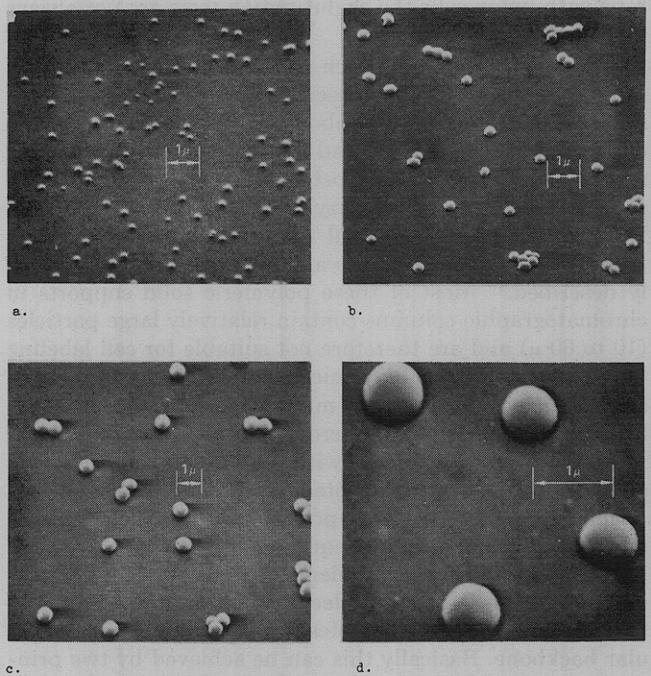
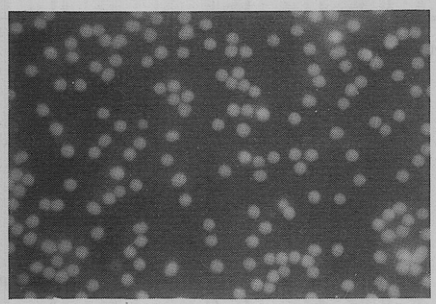


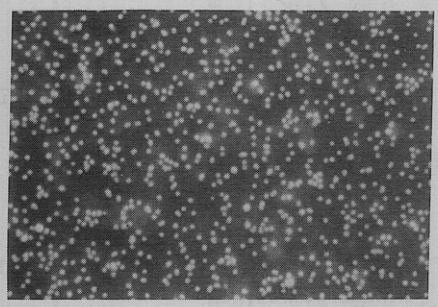

**Figure 2.** SEM photographs of polyHEMA particles: (a) copolymerized with DMA, dry $\bar{D}_v$ = 0.25 μ, 2143× (total monomer concentration, 3% w/v, monomer composition, HEMA 80% w/w, DMA 20% w/w in $H_2O$); (b) copolymerized with MA, dry $\bar{D}_v$ = 0.68 μ, 2143× (total monomer concentration 7.5% w/v, monomer composition, HEMA 80% w/w, MA 20% in 0.4% w/v PEO); (c) copolymerized with MA and BAM, dry $\bar{D}_v$ = 0.7 μ, 2952× (total monomer concentration 3% w/v, monomer composition, HEMA 70% w/w, MA 20% w/w, BAM 10% w/w in 0.4% w/v PEO); (d) copolymerized with MA and BAM, $\bar{D}_v$ = 0.68 μ, 11905× (total monomer concentration, 3% w/v, monomer composition, HEMA 75% w/w, MA 20% w/w, BAM 5% w/w in $H_2O$).

## Results

**(a) Parameters Affecting Particle Size.** In Figure 2 are shown SEM photographs of functional polymeric mi-



a.



b.

**Figure 3.** Light microscope photographs of fluorescent polyHEMA particles (100×, oil immersion objective). (a) PolyHEMA copolymerized with BAM and dansylallylamine $\bar{D}_v$ (hydrated) = 1.7 $\mu$ (total monomer concentration, 5% w/v, monomer composition, HEMA 90% w/w, BAM 10% w/w, dansylallylamine 0.1% w/w). (b) PolyHEMA copolymerized with BAM, MA, and fluorescein isothiocyanate adduct of allylamine. $\bar{D}_v$ (hydrated) = 0.7 $\mu$ (total monomer concentration, 5% w/w, monomer composition, HEMA 70% w/w, MA 20% w/w, BAM 10% w/w, fluorescein–allylamine 0.1% w/w PEO).

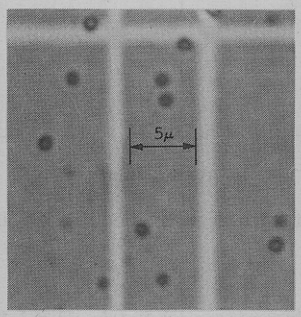
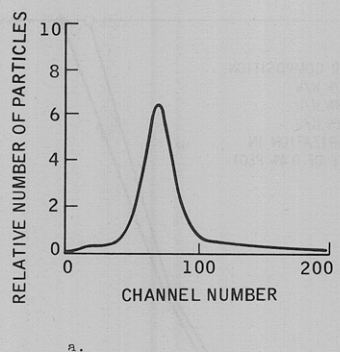


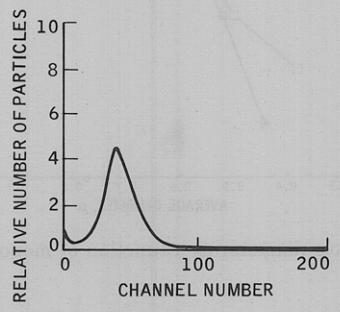

**Figure 4.** Light microscope photograph of nonfluorescent polyHEMA particles. $\bar{D}_v$ (hydrated) = 1.0 $\mu$ (total monomer concentration 4% w/v, monomer composition, HEMA 75% w/w, BAM 15% w/w, MA 10% w/w in 0.4 w/w % PEO).

crospheres of various compositions, sizes, and magnifications. Figures 3 and 4 are light microscope photographs of fluorescent and nonfluorescent polyHEMA copolymers, respectively. Figure 5 represents the size distribution of polyHEMA based particles obtained by means of the low angle scattered light mode of the cell sorter.[19] The size distribution was determined on diluted crude preparations prior to purification by centrifugation.

Freshly distilled commercial HEMA in the presence of




a.




b.

**Figure 5.** Size distribution of unpurified polyHEMA particles based on the light-scattering mode of the cell sorter.[19] (a) Dry $\bar{D}_v$ = 0.8 $\mu$, total monomer concentration 5% w/w, monomer composition, HEMA 70% w/w, MA 20% w/w, BAM 10% w/w, fluorescein–allylamine 0.1%, in 0.4% w/w PEO, $C_v$ = 12%. (b) Dry $\bar{D}_v$ = 1.16 $\mu$, total monomer concentration 10% w/w, monomer composition, HEMA 65% w/w, DMA 30% w/w, BAM 5% w/w, in 0.4% w/v PEO, $C_v$ = 28%.

cross-linking agents (2 to 4% of EGDM or 2 to 20% of BAM) yielded microspheres containing mainly hydroxyl groups. Although this is a convenient system for investigations of the mechanism of latex formation without stabilizer or emulsifier, it is limited to a low total monomer concentration (up to ~5%). Most of our results were therefore obtained with copolymer systems prepared in the presence of PEO which permitted the use of higher concentrations of monomers. In Figure 6 are compared particle diameters obtained by the use of distilled commercial HEMA (Rohm and Haas) with purified HEMA (Hydron Co.). In both cases the monomer composition on a weight percent basis was HEMA 78%, MA 20%, and BAM 2%, in distilled water containing 0.4% w/v of PEO. Figure 7 shows the effect of cross-linking agent concentration on size (the amount of BAM was increased at the expense of HEMA) and the total monomer concentration was kept constant (3%). The presence of PEO resulted in decrease of average diameter (Figure 7). This effect was confirmed by the study of particle size at constant BAM concentration (Figure 8). Figure 9 shows clearly the reduction of particle size due to copolymerization with methacrylic acid. The functional group on the acrylic comonomer has pronounced effects on the particle size; in Figure 10 are recorded the sizes of microspheres obtained under an identical set of experimental conditions but changing the structure of the comonomer. In these experiments the amounts of HEMA and BAM were kept constant. Copolymerization with acrylamide results in larger particles than those from copolymerization with 2-dimethylaminoethyl methacrylate.

In Table I are recorded the sizes of resulting particles as a function of total monomer concentration in the absence and in the presence of MA (compare with Figure 10). The

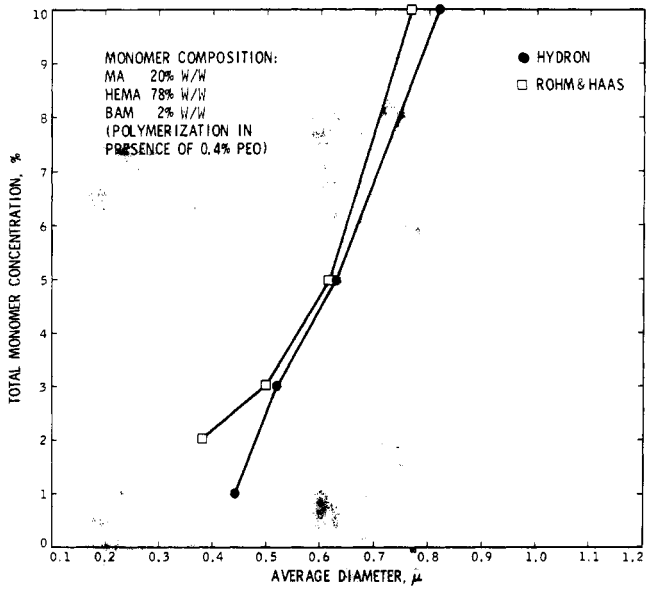



**Figure 6.** Particle diameter as a function of monomer concentration.

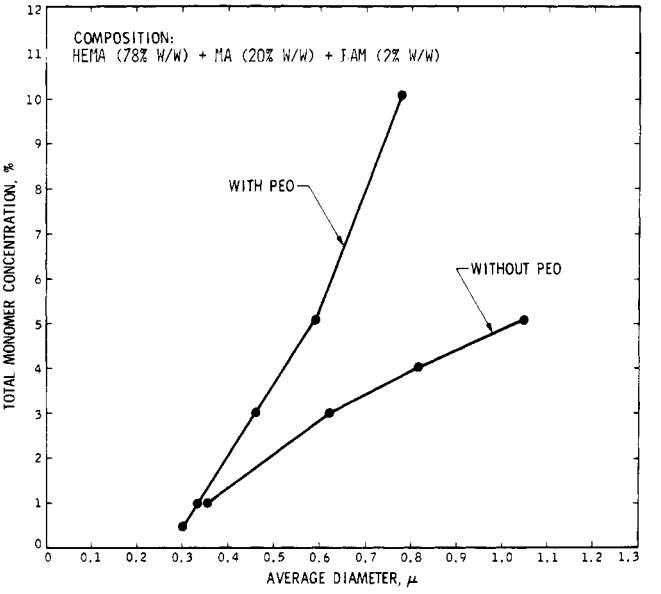



**Figure 8.** Effect of PEO on particle size.

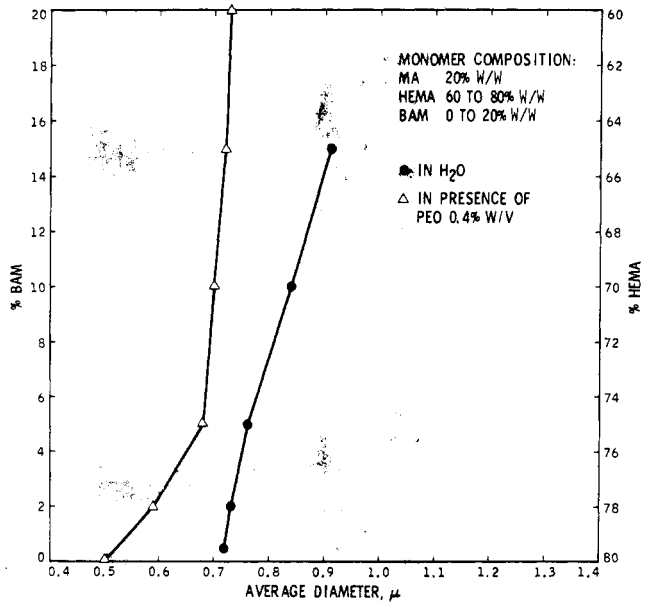



**Figure 7.** Particle diameter as a function of concentration of cross-linking agent.

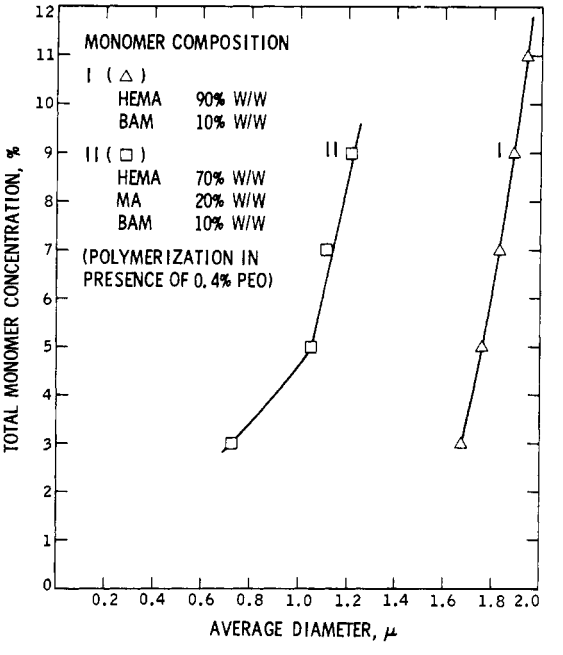



**Figure 9.** Effect of methacrylic acid on particle size.

decrease of particle size on addition of MA (Table I) is consistent with the finding that carboxyl groups lead to size reduction (Figure 9). The increase in standard deviation values (Table I) is probably due to a decreased stabilizer (PEO) monomer ratio.

With the exception of the polymerization experiments where fluorescent monomers were used, all the results described so far involved monomers, miscible in water in all proportions. However, the Co $\gamma$ polymerization technique is not limited to monomers completely soluble in water. An emulsion system was formed by using mixtures of HEMA and MMA in the presence of PEO giving latices after irradiation. The addition of increasing amounts of MMA resulted in considerable decrease in particle size and yield (Figure 11).

**(b) Aggregation and Surface Properties.** Since most biological studies are carried out in physiological saline or buffered solutions containing salts, the effect of salt concentration on aggregation was of direct interest. Addition of definite amounts of sodium chloride to latices produced particle aggregation. The salt concentration causing aggregation showed a marked dependence on the amount of cross-linking agent used in the polymerization. Figure 12 shows that increasing amounts of sodium chloride can be used before the onset of aggregation occurs, as the amount of cross-linking agent in the polymer increases. The aggregation tendency is also significantly less if the polymerization is carried out in the presence of PEO in which case the amount of sodium chloride causing aggregation depends on the number of rinsings which progressively remove PEO adsorbed to the particles.

The absence of initiator or emulsifier in the polymeriza-

Table I
Copolymerization of HEMA with Acrylamide in the Absence or the Presence of MA with BAM as Cross-Linking Agent[a]

| Wt % monomer composition | | | | Total monomer concn, w/v, % | pH | Yield, % | Av diameter,[b] μ |
|---|---|---|---|---|---|---|---|
| HEMA | Acrylamide | BAM | MA | | | | |
| 78 | 20 | 2 | | 1 | 5.0 | 98.0 | 0.33 ± 0.033 |
| 78 | 20 | 2 | | 3 | 3.5 | 96.0 | 0.83 ± 0.09 |
| 78 | 20 | 2 | | 5 | 3.3 | 95.5 | 1.2 ± 0.2 |
| 78 | 20 | 2 | | 8 | 3.2 | 93.7 | 1.3 ± 0.3 |
| 68 | 20 | 2 | 10 | 1 | 3.3 | 97.5 | 0.30 ± 0.03 |
| 68 | 20 | 2 | 10 | 3 | 3.2 | 96.7 | 0.55 ± 0.06 |
| 68 | 20 | 2 | 10 | 5 | 3.2 | 99.1 | 0.82 ± 0.09 |
| 68 | 20 | 2 | 10 | 8 | 3.2 | 99.3 | 0.98 ± 0.10 |

[a] In water containing 0.4 wt % PEO. Radiation dose 0.8 mrad. [b] Determined by means of SEM.

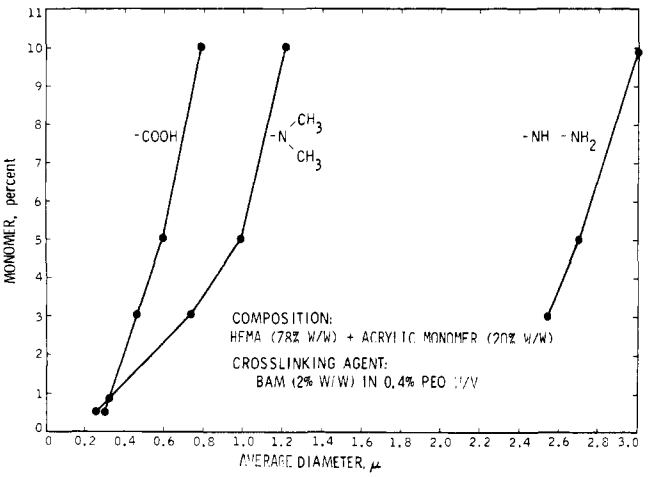



**Figure 10.** Effect of monomer functional group on particle size.

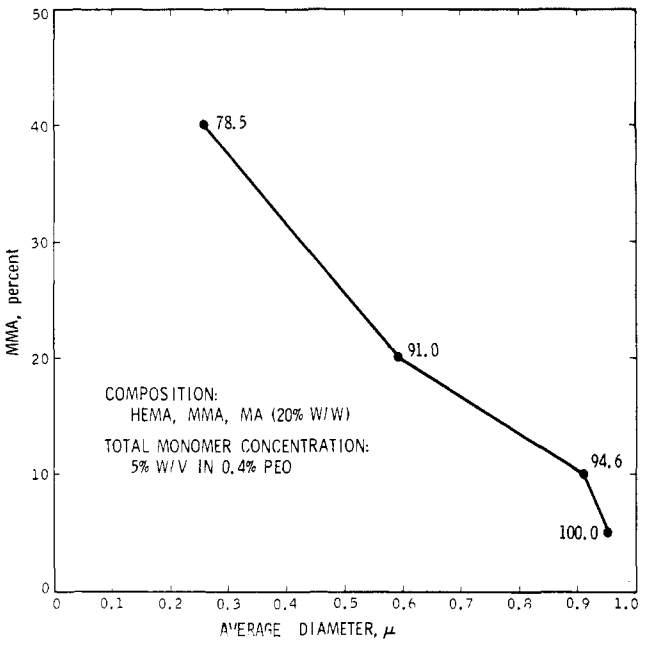



**Figure 11.** Effect of varying concentration of MMA and HEMA on particle size and yield (numbers near data points are percent yield).

tion mixture permits the determination of a number of carboxyl groups accessible for further reaction by simple titration procedures. In Table II are recorded the neutralization equivalents and the number of carboxyl groups per unit

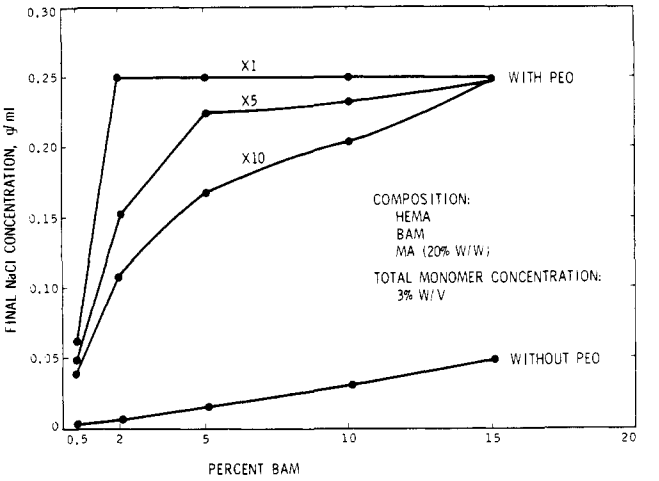



**Figure 12.** Aggregation of particles (0.6 μ, 4 × $10^8$ microspheres/ml) synthesized in the presence and the absence of PEO. (×1, one rinsing, ×5, five rinsings, ×10, ten rinsings.)

area of particles of different sizes containing the same amount of carboxylic acid. The neutralization equivalent decreases with increase of particle diameter indicating that some carboxyl groups are buried inside the particles and are not accessible for titration with alkali. No substantial change in neutralization equivalent values was observed by extending the time of titration to 30 min.

In order to incorporate amino groups into the microspheres the copolymerization of allylamine with HEMA was investigated in the presence of MA but in the absence of cross-linking agent. To ascertain that allylamine copolymerized with the acrylic monomers the percent nitrogen in the copolymer was determined as a function of allylamine concentration. The results are shown in Table III.

Table III indicates that it is possible to copolymerize small amounts of allylamine with HEMA and MA. The copolymerization of allylamine–fluorescein isothiocyanate adduct in low concentrations, with HEMA, MA, and BAM, yielded particles which when suspended in water remained intensely fluorescent for a period of at least 1 year.

**(c) Immunological Studies.** In order to visualize cell labeling in the light microscope, we have selected particles of 0.5 to 1 μ in diameter (swollen) by polymerization of the following mixture: HEMA 69%, MA 20%, BAM 10%, allylamine 0.95%, fluorescein isocyanate 0.05%. (Total monomer concentration 2 to 4% w/v.) The labeling of cells was achieved by means of the procedures described in the experimental section. Figures 13 and 14 show that cells can be labeled under the microscope by the use of particles of 0.5 to 0.8 μ in diameter.

Table II
Correlation of Particle Size with Carboxyl Content (Radiation Dose, 0.8 mrad)

| Total monomers,[a] % w/w | $\overline{D}_v$ (hydrated), $\mu$ | Yield, wt % | Neturalization equiv, mequiv/g | Area, $cm^2 \times 10^8$ | Area/g, $cm^2/g \times 10^{-4}$ | No. of carboxyl groups, Å$^2$ | Remarks |
|---|---|---|---|---|---|---|---|
| 0.5 | 0.5 | 16.6 | | | | | In presence |
| 1.0 | 0.55 | 59.6 | | | | | of 0.4% PEO |
| 3.0 | 0.76 | 98.0 | 1.52 | 1.84 | 6.32 | 1.45 | |
| 5.0 | 0.98 | 99.1 | 1.12 | 3.03 | 4.93 | 1.37 | |
| 10.0 | 1.30 | 98.7 | 1.09 | 5.31 | 3.29 | 1.76 | |
| 1.0 | 0.58 | 45.9 | 1.85 | 1.05 | 8.5 | 1.31 | In absence |
| 3.0 | 1.04 | 95.1 | 1.64 | 3.39 | 4.64 | 2.12 | of PEO |
| 5.0 | 1.66 | 95.7 | 1.27 | 8.16 | 2.61 | 2.53 | |

[a] Monomer composition: methacrylic acid 20%; HEMA 78%; BAM 2%.

Table III
Copolymerization of Allylamine with HEMA and MA in 0.4% w/v Aqueous PEO Solutions[a]

| HEMA wt, g | MA wt, g | Allylamine wt, g | Yield, % | N found, % |
|---|---|---|---|---|
| 3.95 | 1.00 | 0.5 | 95.0 | 0.09 |
| 3.90 | 1.00 | 1.0 | 97.0 | 0.12 |
| 3.85 | 1.00 | 1.5 | 94.0 | 0.15 |
| 3.80 | 1.00 | 2.0 | 98.0 | 0.20 |
| 3.75 | 1.00 | 2.5 | 95.0 | 0.40 |

[a] Radiation dose 0.8 mrad. Total monomer concentration 5% w/w.

In direct labeling experiments, human lymphocytes were mixed with microspheres bonded to goat antihuman IgG. Microscopic examination showed that approximately 20% of cells were labeled (Figure 14). Details of this work will be described in a future publication.

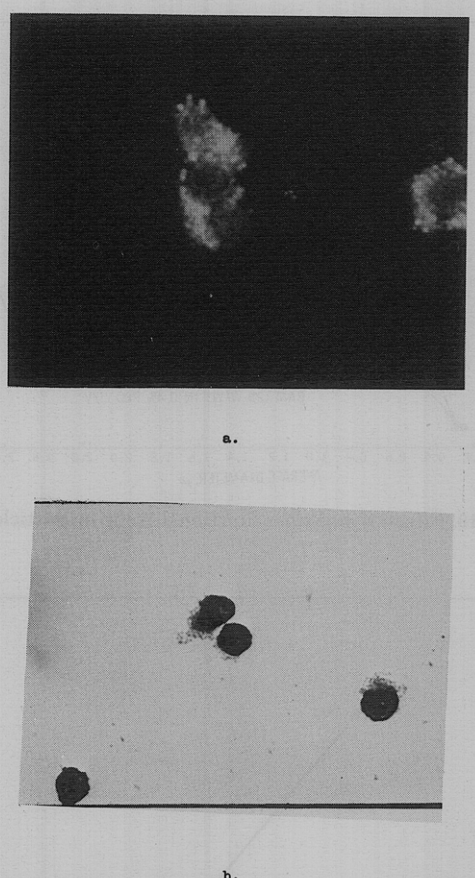



**Figure 13.** Microscope photograph of mouse lymphocytes sensitized with rabbit antimouse immunoglobulin and labeled with goat antirabbit immunoglobulin microsphere conjugates. (a) Fluorescent light, not stained (particle diameter 0.7 $\mu$). (b) Ordinary light, Wright stain (particle diameter 0.5 $\mu$).

## Discussion

**Hydrophilic Character and Particle Size.** It appears that one of the dominant requirements for covalent binding as well as for the retention of biological activity of biomolecules to reactive polymers is the hydrophilic character or gelphase formation in water of the adsorbent.[17] This requirement is met by polyHEMA as well as the copolymers of polyHEMA with MA, DMA, acrylamide, acryloylhydrazide, and even MMA. PolyHEMA swells in water to the extent of 30 to 40% forming porous structures known as xyrogels.[17] In addition, when copolymerized with MA it provides hydroxyl and carboxyl groups which can be utilized for covalent bonding of amino groups containing biomolecules. The hydrophilicity of polyHEMA can be modified by the use of less water soluble monomers, e.g., methyl methacrylate (Figure 11) in which case the hydrophilic functional groups of HEMA and MA remain on the surface of the microspheres at the completion of the polymerization process. An effort was made to determine the factors that determine particle size, since the latter is one of the essential criteria for immunological applications. The average diameter of polyHEMA microspheres can be gradually changed by varying the total monomer concentration (Figures 6 to 10). Variation of the water-soluble cross-linking agent (BAM) concentration does not alter the size of particles significantly (Figure 7). This is, however, not the case of PEO in the presence of which the particle size is reduced in particular at relatively high total monomer concentrations (Figures 7 and 8). Increasing amounts of MA in the monomer composition reduces the average diameter of particles as compared with "pure" HEMA; however, copolymerization with DMA and methacroylhydrazide has the opposite effect (Figure 10). The electrostatic effect of polar carboxyl groups and the high hydrophilicity imparted by dimethylamino and hydrazido groups may be responsible for these results. In contrast to available latices prepared in the presence of organic emulsifiers and inorganic initiators the determination of carboxyl groups is facilitated in the present case and can be carried out by a simple potentiometric titration because no acidic groups (e.g., sulfates) originating from initiator or emulsifier are incorporated into the surface of particles.

In Table II are recorded the numbers of carboxyl groups per Å$^2$ as a function of particle diameter. It is noted that the number of carboxyl groups per particle decreases with increased size of the particle and that values of Table II in-

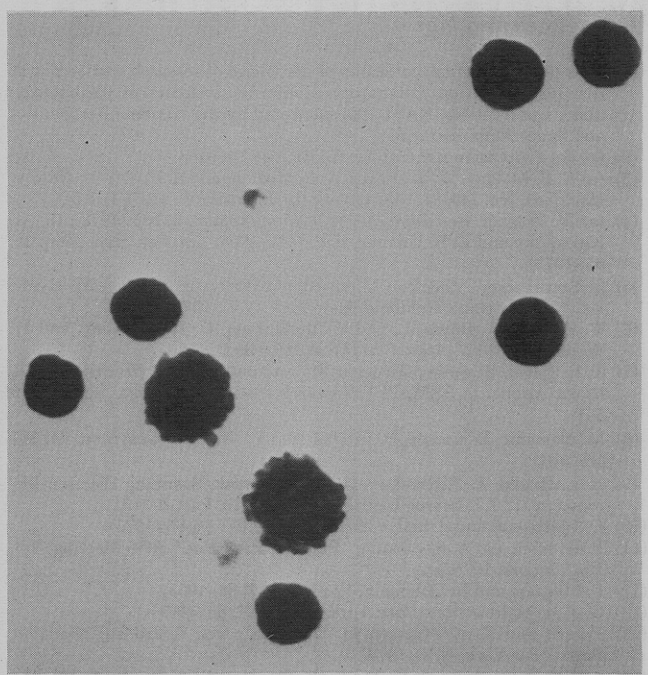


**Figure 14.** Microscope photograph of human lymphocytes labeled by means of the direct method with goat antihuman IgG–microsphere conjugates. (Particle diameter 0.8 $\mu$, Wright stain.)

dicate up to 2.5 carboxyl/Å$^2$. Since a carboxyl group would require considerably more than 1 Å$^2$ of surface area it is concluded that a certain number of carboxyl groups are located inside the microspheres and some, but not all, are still accessible to aqueous reagents due to the high porosity and hydrophilic character of polyHEMA. These deductions assume that HEMA and MA yield copolymers of the same composition when the total initial monomer concentration varies from 3 to 10%. The high yield of products indicates that this assumption is likely to be correct. It should also be noted that the neutralization equivalents of polymeric microspheres prepared in the absence of PEO are higher than in its presence. This is most probably caused by the known interaction of PEO which is adsorbed on the surface of the particles. Figure 12 provides a definite proof of PEO adsorption which is responsible for the absence of particle aggregation on addition of relatively high concentrations of salt solutions. Each point (Figure 12) represents the onset of aggregation on addition of a dilute sodium chloride solution under the same conditions (stirring rate and particle concentration). With increased amount of cross-linking agent larger amounts of NaCl are needed to produce aggregation. In addition, microspheres prepared in the presence of PEO are considerably more resistant to aggregation at all levels of cross-linking agent. In spite of repeated extraction with water (see curve labeled ×10) there is much less tendency to aggregate for microspheres prepared in the presence of PEO than in the absence of PEO. It is obvious therefore that the water washings do not remove all the adsorbed PEO.

**Mechanism.** In spite of the fact that emulsion polymerization was known at least since 1926, a detailed quantitative theoretical interpretation capable of predicting the behavior of most emulsion systems is still not available.[24,25] In the majority of previously investigated systems the monomers were either practically insoluble or only partially soluble in the aqueous medium and involved a free-radical initiator as well as an emulsifier. Present theories are based on Harkins[26] and Smith and Ewart[27] models in which micelles growing to form polymer particles play a dominant role. The state of the art is summarized in a number of excellent reviews[25,28–31] and it is now generally assumed that most emulsion polymerization systems proceed in three stages or intervals. Interval I begins with the onset of free-radical generation and proceeds until all micelles are consumed. In interval II polymer particles originating from the micelles are formed. These particles which are swollen with monomer are considered to be the sole loci of polymerization. It is assumed that no flocculation occurs at this stage and the number of particles remains constant. The diffusion of monomer from the remaining emulsion droplets into the particles is believed to be sufficiently rapid to maintain equilibrium saturation swelling. Interval III begins immediately after the emulsion droplets vanish and ends when the conversion of monomer is complete.

In most emulsion polymerization systems the initiators consist of either organic and inorganic peroxides such as benzoyl peroxide or a persulfate; the rate of polymerization is therefore dependent on time and temperature. High-energy radiation offers a source of free-radical initiation which varies only slightly with time and temperature.[32] Furthermore we have shown that the Co $\gamma$ irradiation yields colloidal particles from homogeneous aqueous solutions of monomers in the absence of surface active agents. Thus the investigation of the polymerization mechanism should present less complexity than the conventional systems.

The results obtained so far may be summarized as follows. (1) In all examined systems and irrespective of whether one or more monomers were used for polymerization, the size of particles could be considerably increased by increasing the total monomer concentration.[33] By exceeding this concentration over a certain limiting value which varied with such conditions as pH presence or absence of surfactant, structure of comonomer, etc., a coagulum was obtained. (2) Addition of PEO decreased particle size in homo as well as copolymerization in the presence and in the absence of a water-soluble cross-linking agent. PEO was found to stabilize the latices and to adsorb strongly to particles containing carboxyl groups. (3) Increased concentration of BAM while keeping all other conditions constant altered the particle size to a small extent only. (4) Addition of methacrylic acid decreased the particle size as compared with sizes obtained in homopolymerization of HEMA from two different commercial sources. However, copolymerization with DMA, acrylamide,[34] and methacryloyl hydrazide had the opposite effect. The effectiveness of the functional group in increasing particle size of polyHEMA obeys the following order $NH\text{-}NH_2 > CONH_2 > N(CH_3)_2$, provided the pH of the initial mixture of reactants does not cause significant ionization of the functional groups in which case gelation effects were observed. (5) Copolymerization with a relatively water-insoluble monomer, e.g., MMA, resulted in a decreased particle size.

In the absence of detailed kinetic measurements only a tentative interpretation of these results can be made; the formation of polyHEMA latices in the present work was achieved without micelle formation and in the absence of monomer droplets, since HEMA is miscible with water in all proportions. For the same reason the diffusion of monomers postulated to take place from insoluble monomer droplets to polymer particles in interval II probably occurs in our system directly from aqueous medium. The mechanism could therefore be visualized as involving only two intervals; interval I in which homogeneous nucleation occurs yielding particles of low molecular weight and consequently

prone to coalescence and interval II in which monomer-swollen polymer particles grow in the absence of coalescence or with a low coalescence rate. These general conclusions are in agreement with a theory outlined by Fitch[35] who proposed that the rate of particle formation is

$$dN/dt = R_i - R_c - R_f \quad (1)$$

where $N$ is the number of particles, $R_i$ the rate of initiation, $R_c$ the rate of radical capture, and $R_f$ the rate of coalescence of particles. In our present system radicals are generated in the aqueous phase and polymerization can therefore be achieved by homogeneous nucleation without micelle formation as suggested by Fitch.[35] The monomers are soluble in water initially and insoluble polymeric particles are formed as soon as the cross-linked chain length reaches its solubility limit. A high level of the cross-linking agent concentration should decrease the solubility and therefore decrease $R_f$. If we assume that $R_i \gg R_c$ and the main polymerization loci are the particles swollen with monomer, then the number of particles will be mainly dependent on $R_f$.

Thus the effect of PEO (Figures 7 and 8) is explained by a decreased $R_f$ value due to steric stabilization as described by Alexander.[31] The $R_f$ value can also be decreased through electrostatic repulsion,[26] e.g., in case of copolymerization with monomers containing polar functional groups such as carboxyl groups (Figure 9). The dramatic increase in size during copolymerization with DMA or methacryloylhydrazide is probably due to the high swellability of particles in these monomers. The attractive force between particles is inversely proportional to the distance between them[36] and the more hydrophilic the polymer the larger the particles formed, the smaller the interparticle distance (interval I), leading to an increase in the $R_f$ value, hence in an increase of the final size. This conclusion is supported by the fact that MMA, a more hydrophobic monomer than HEMA, DMA, etc., leads to a drastic decrease of particle size because MMA does not swell polyHEMA particles to any significant extent. However, it should be emphasized that the copolymerization of a hydrophobic monomer with a monomer highly soluble in water becomes considerably more complicated[37,38] than the copolymerization in a homogeneous system and the observed effect of MMA cannot be precisely interpreted without further extensive investigations.

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