Polychloromethylstyrene Microspheres: Synthesis and Characterization

SHLOMO MARGEL,* ELIAHU NOV, and INNA FISHER

The Department of Chemistry, Bar-Ilan University Rmat-Gan 52100, Israel

SYNOPSIS

The synthesis and characterization of polychloromethylstyrene microspheres are described. The effect of various factors, e.g., monomer concentration, surfactant concentration, solvents, crosslinker, etc., on the yield, diameter, and size distribution of these microspheres was elucidated. Conditions to covalently bind various primary amino ligands to these microspheres in aqueous solution were also established. The possible routes to modify the surface of these microspheres to obtain functional groups other than chloromethyl are discussed.

INTRODUCTION

The synthesis and biological uses of polystyrene microspheres have been summarized in a report by Vanderhoff in 1964.[1] Since then, many publications described the preparation, characterization and applications of polystyrene microspheres.[2-5] In most cases these microspheres were produced by mechanical disruption of the monomer styrene in water into submicron or micron size stabilized droplets, followed by radical polymerization. However, the obtained microspheres had a broad range of size distribution. Recently, in order to improve the quality and usefulness of polymeric microspheres, significant progress has been made in the synthesis of microspheres with relatively narrow size distribution.[6] The main methods developed to obtain highly uniform microspheres are based on step swelling polymerization,[7] polymerization in outer space where zero gravity forces exists,[5] and polymerization in the presence of appropriate solvents and surfactants.[9-12]

Polystyrene microspheres have some desired properties, e.g., high rigidity and the know-how of how to control the diameter and size distribution of these microspheres. However, polystyrene does not contain functional groups through which covalent binding of desired ligands, e.g., amino ligands such as proteins, is possible. Significant efforts have been directed in the design and synthesis of functional type polystyrene derivative microspheres.[12-18] Here, we describe the synthesis and characterization of polychloromethylstyrene (PCMS) microspheres of various diameters. The influence of different factors, e.g., monomer concentration, surfactant concentration, solvents, and crosslinker, on the diameter, size distribution, and yield of the obtained microspheres was studied. Conditions to covalently bind amino ligands to these microspheres have been established. The possible ways to modify the surface of the PCMS microspheres to obtain functional groups other than chloromethyl, e.g., amine, hydroxyl, and carboxylate groups, are also discussed.

EXPERIMENTAL

Chemicals

The following analytical-grade chemicals were obtained from commercial sources: chloromethylstyrene (CMS) from Polysciences, 1,3-diaminopropane (DAP), 1-amino-3-propionic acid (APA), 1-amino-

* To whom correspondence should be addressed.

Journal of Polymer Science: Part A: Polymer Chemistry, Vol. 29, 347-355 (1991)
 CCC 0887-624X/91/030347-09$04.00

3-propanol (APOH), polyvinylpyrrolidon (PVP), divinylbenzene (DVB, 50% in ethylvinylbenzene), (2-chloroethyl)-benzene, acetic acid, sodium acetate, sodium carbonate, sodium bicarbonate, dimethylsulfoxide (DMSO), methanol, ethanol, propanol, butanol, isopropanol, isobutanol and azo(bisiso)butyronitrile (AIBN) from Aldrich, affinity purified sheep immunoglobulin (sheep IgG) and protein A from Sigma. The alcoholic solvents were dried over activated 4 Å molecular sieves. DMSO was distilled from CaH_2 and then kept over 4 Å molecular sieves. AIBN was recrystalized from methanol. The monomer CMS was used without purification.

Synthesis of the PCMS Microspheres and Powder

The polymeric microspheres were prepared in a 25 mL, three-neck round-bottom flask, equipped with a condenser. The flask was immersed in a constant temperature silicone oil bath at a preset temperature. Appropriate amounts of solvent (ethanol), co-solvent (DMSO), surfactant (PVP), initiator (AIBN), and monomer (CMS) were placed into the reaction flask and magnetically stirred. Nitrogen was bubbled through the solution for 15 min to exclude the air, then a blanket of nitrogen was maintained over the solution during the polymerization period. The obtained microspheres were washed with ethanol by four centrifugations at 3000 $\times$ g for 20 min. The microspheres were then freeze-dried. The dried microspheres were placed in aqueous solutions which were then sonicated (Heat Systems Ultrasonic Processor, W-380, Farmingdale, NY) to break the agglomerated microspheres to separated single microspheres.

PCMS powder was prepared by a similar procedure, deleting the presence of the surfactant PVP.

Kinetics of the Microspheres Formation

Samples of 0.1 mL were taken from the reaction solution at different intervals during the polymerization period. The samples were then diluted with 0.1 mL of 2% ethanol solution of the internal standard, ((2-chloroethyl)-benzene. The residual monomer was then determined by injecting the mixtures into a gas chromatograph equipped with a hydrogen flame detector and an XE-60 column heated to 100°C.

Molecular Weight Determination

The molecular weight distributions were measured by GPC on an Aerograph 8500 (Varian) HPLC with μ-Styragel columns (Waters) 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3}. THF was used as a carrier solvent. The peaks were detected spectrophotometrically at 254 nm.

Recovery

The reaction mixture was centrifuged for 10 min at 5000 $\times$ g. The supernatant was then decanted and the remaining precipitate was repeatedly washed by four centrifugations, and dried then under vacuum.

Diameter and Size Distribution of the Microspheres

The diameter of the microspheres was determined by scanning electron microscopy (SEM), JSM-840, Jeol, as previously described.[11] The size distribution patterns of the microspheres were measured by the Computerized Inspection System (Galai CIS1, Migdal Haemek, Israel).

Nephelometric Studies

The stability of the PCMS microspheres at various pH conditions and salt concentrations was studied with a spectrophotometer (Varian, DMS 100S). A significant decrease in the turbidity of the microsphere suspension, indicated by the decrease in the absorption at 750 nm, is an indication of the instability of the microspheres under these conditions.

Reaction of the PCMS Microspheres with Amino Ligands

Aqueous solutions of the ligands were prepared by dissolving a known amount of the desired ligand in water and adjusting the solution to the desired pH with NaOH or HCl aqueous solutions. Then, aqueous buffered solutions were added to obtain the desired volume. pH 10.0 and 11.5 were prepared from 0.1 M carbonate buffer, pH 7.0 from 0.1 M phosphate buffer, and pH 4.5 from 0.1 M acetate buffer. The reaction was accomplished by adding the desired

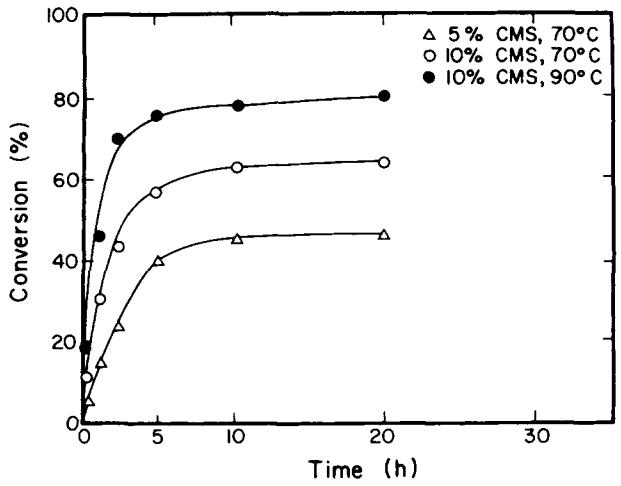

Figure 1. Rates of polymerization of CMS, with (△) 5% (v/v) CMS at 70°C, (○) 10% (v/v) CMS at 70°C, and (●) 10% (v/v) CMS at 90°C. Air-free ethanol solutions (total volume 10 mL) containing 115 mg PVP and 0.5 mL or 1 mL CMS were polymerized with 20 mg AIBN.

amount of dried microspheres to the buffered aqueous solution containing the appropriate amount of the studied amino ligand. The mixture was then shaken at 25 or 60°C for 24 h. The reaction mixture was washed with water by 4 centrifugations and the obtained product was then dried under vacuum and analyzed for nitrogen. The amount of ligand bonded to the microspheres was determined from the percent nitrogen in the product.

The amount of proteins (sheep IgG and protein A) bonded to the microspheres was measured according to the method of Lowry et al.[19]

RESULTS AND DISCUSSION

Rate of Polymerization and M.W. Distribution

The percent conversion as a function of time was calculated by the following equation: % Conversion $= [(M_o - M)/M_o] \times 100$, where M_o is the initial concentration of the monomer and M is the concentration of the monomer at time T. The rate of polymerization of CMS, as presented by the percent conversion, is increased as the polymerization temperature or the monomer concentration increases (Fig. 1). The MW of the resulted PCMS microspheres was determined by GPC. The number average MW is 12,000, the weight average MW is 57,000; the molecular weight distribution (MWD) is thereby 4.75.

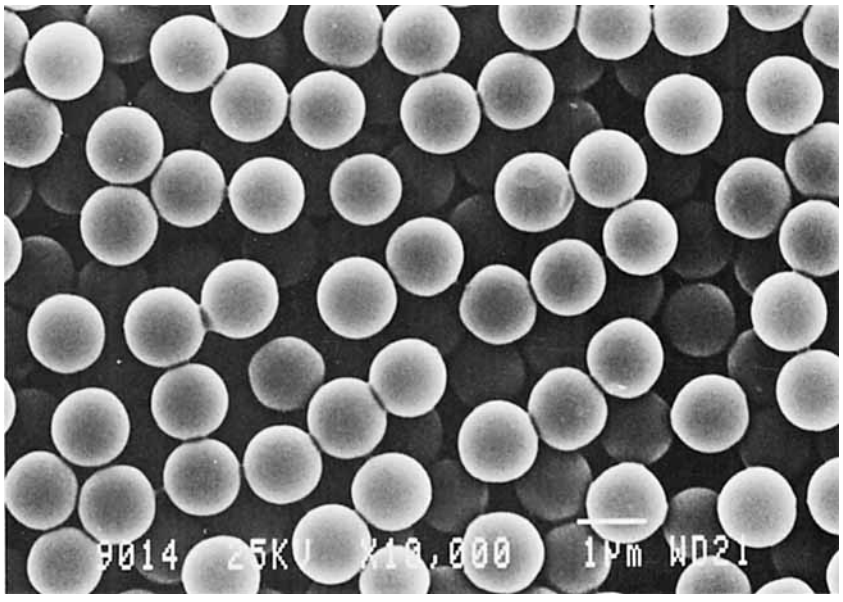

Figure 2. SEM photomicrograph of PCMS microspheres prepared by polymerizing CMS (0.5 mL) at 70°C in air-free ethanol solution (total volume 10 mL) containing 1 mL DMSO, 115 mg PVP, and 10 mg AIBN.

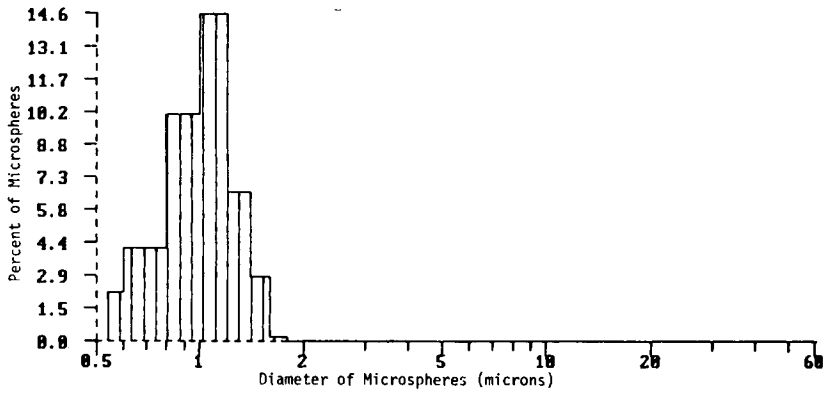

Figure 3. Particle size distribution pattern of PCMS microspheres prepared by polymerizing 0.5 mL CMS at 70°C in air-free ethanol solution (total volume 10 mL) containing 1 mL DMSO, 115 mg PVP, and 10 mg AIBN.

Stability, Diameter, and Size Distribution

A scanning electron microscopy (SEM) photomicrograph of PCMS microspheres is given in Figure 2. The size distribution pattern of these microspheres, as presented in Figure 3, shows a unimodal size distribution pattern. The average diameter is 1.01 μm with a standard deviation of 0.24 μm. The stability of the aqueous microsphere suspension towards different pH conditions and salt (NaCl) concentrations was studied by observing the turbidity of the solution under these conditions. A significant decrease in the absorbance of the microsphere suspension is an indication of the instability of these microspheres, due to agglomeration process. Figure 4 does not show any significant decrease in the absorbance at 750 nm of the microsphere suspension at pH range between 1 and 13 and at a salt concentration up to 10%, indicating the stability of these microsphere suspension under these conditions. The stability of the microspheres in other pH conditions or higher NaCl concentrations was not studied, since these conditions are usually not practical.

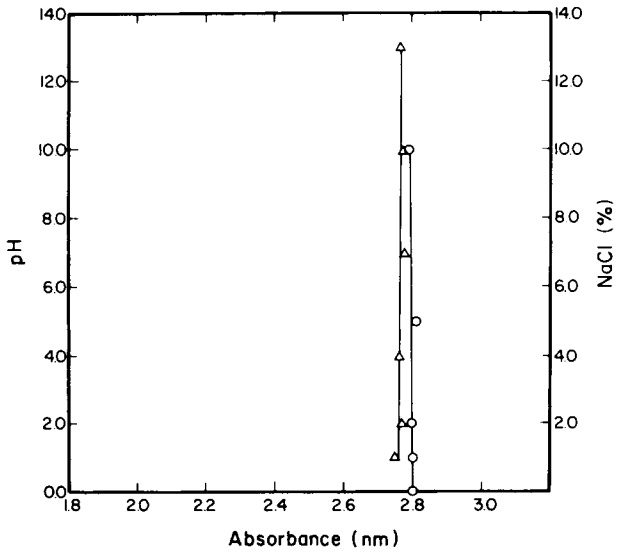

Figure 4. Aggregation properties of PCMS microspheres of 0.5 μm average diameter as a function of sodium chloride concentration (○) and as a function of pH (△). The absorbance of aqueous microsphere suspension (1 mg/mL) containing different NaCl concentrations or at different pH was measured at 750 nm. The desired pH was obtained with NaOH or HCl aqueous solution.

Table I. Effect of DMSO on the Recovery and Diameter of the PCMS Microspheres[a]

DMSO (%)	Recovery (%)	Average Diameter (μm)	Remarks
0	52	0.5	
5	49	0.7	
10	48	1.0	
25	19.6	0.1–0.5	
40	0	—	Soluble polymer
60	0	—	Soluble polymer

[a] CMS (0.5 mL) was polymerized at 70°C for 24 h in air-free ethanol solution (total volume 10 mL) containing PVP (115 mg), AIBN (10 mg), and variable amounts of DMSO.

Table II. Effect of Solvent on the Recovery and Diameter of the PCMS Microspheres[a]

Solvent (%)	Recovery (%)	Average Diameter (μm)
Methanol	55.7	1.1
Ethanol	51.1	1.0
Propanol	41.4	1.1
Butanol	38.2	1.2
Isopropanol	62.1	0.5–1.1
Isobutanol	45.1	0.5–1.1

[a] CMS (0.5 mL) was polymerized at 70°C for 24 h in air-free atmosphere in different alcoholic solutions (total volume 10 mL) containing DMSO (1 mL). PVP (115 mg), and AIBN (10 mg).

Effect of Different Variables on the Recovery and Diameter of PCMS Microspheres

Table I presents the effect of co-solvent, DMSO, on the recovery and the diameter of the PCMS microspheres. Addition of DMSO, 0, 5, 10, 25, 40, or more, gradually decreased the yield of the microspheres, 52, 49, 48, 19.6, and 0%, respectively. The decrease in the yield of the PCMS microspheres by the addition of DMSO may be explained by the increased solubility of the microspheres. The gradual addition of DMSO (0, 5, and 10%) increased the average diameter of the microspheres (0.5 μm, 0.7 μm, and 1.0 μ, respectively). The obtained microspheres were monodispersed with deviation in their diameter of ± 12%. At DMSO concentration of 25%, polydispersed microspheres were obtained with diameters ranging between 0.1 and 0.5 μm. At DMSO concentration of 40% or higher, a soluble polymer was formed.

Table II presents the effect of solvent on the recovery and diameter of the PCMS microspheres. By changing the polymerization solvent (methanol, ethanol, propanol and butanol), the yield of the microspheres decreased (55.7, 55.1, 41.4, and 38.2%, respectively). On the other hand, the diameter of the formed microspheres was similar in all solvents, approximately 1.0 ± 0.12 μm. Figure 5(a) presents the monodispersed microspheres obtained in butanol. The change in the polymerization solvent from isopropanol to isobutanol decreased the yield of the microspheres from 62.1 to 45.1%, respectively. Also, in both solvents polydispersed microspheres were obtained with diameters ranging between 0.5 and 1.1 μm.

The effect of monomer concentration on the recovery and diameter of the microspheres is given in Table III. The increase in the CMS concentration from 1.2 to 9.9% increased the recovery of the microspheres from 27.6 to 63.9%, respectively. A further increase in the monomer concentration did not significantly change the percent recovery of the formed microspheres. Below 1.2% CMS, the obtained particles did not have a distinct round shape. At monomer concentration ranging between 2.5 and 19.8%, the diameter of the formed microspheres were similar; 1.0 ± 0.12 μm diam. At monomer concentration of 50%, polydispersed microspheres were formed with diameters ranging from approximately 0.2 μm up to approximately 10 μm. Figure 5(c) is a SEM photomicrograph of these polydispersed microspheres. Figure 6 indicates the particle size distribution pattern of these microspheres.

The effect of surfactant concentration on the re-

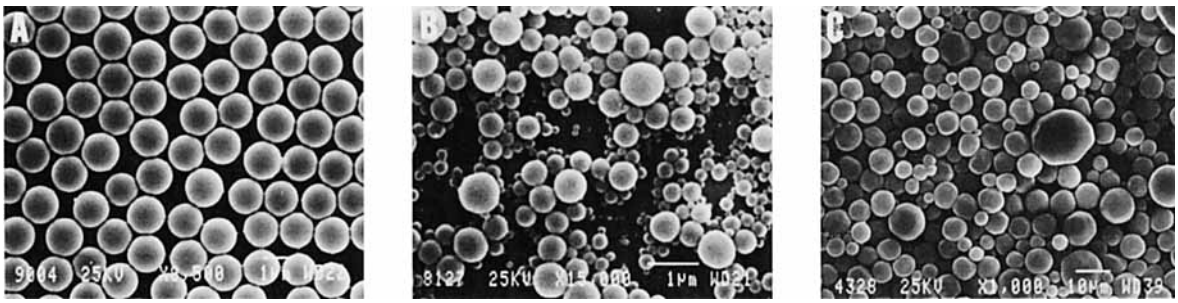

Figure 5. SEM photomicrographs of PCMS microspheres prepared by polymerizing CMS at 70°C in air-free organic solution (total volume 10 mL) containing 1 mL DMSO, 115 mg PVP, and 10 mg AIBN: (A) solvent-butanol, 0.5 mL CMS, (B) solvent-ethanol, 0.45 mL CMS and 0.05 mL DVB, (C) solvent-ethanol, 5 mL CMS.

Table III. Effect of CMS Conccentration on the Recovery and Diameter of the PCMS Microspheres

CMS (%)	Recovery (%)	Average Diameter (μm)	Remarks
1.2	27.6	—	Particles without a spherical shape distinct
2.5	35.0	1.2	
5.0	47.6	1.0	
9.9	63.9	1.0	
19.8	61.7	1.1	
50.0	62.9	0.2–10	

[a] Different amounts of CMS were polymerized at 70°C for 24 h in air-free ethanol solution (total volume 10 mL) containing DMSO (1 mL), PVP (115 mg), and AIBN (10 mg).

covery and diameter of the PCMS microspheres is given in Table IV. The percent recovery of the microspheres significantly increased through the increased amount of the surfactant. Below surfactant concentration of 0.04%, agglomerated polymer was obtained. This may indicate that this concentration is below the critical micelle concentration (CMC) of PVP in the working ethanol solution. Between surfactant concentrations of 0.13–3.5%, microspheres of approximately 1.0 ± 0.12 μm diam were obtained. At surfactant concentration of 10.5%, polydispersed microspheres with diameters ranging from 0.02 μm up to 0.7 μm were formed.

Table V demonstrates the effect of the crosslinker DVB on the recovery and diameter of the PCMS microspheres. The percent recovery of the microspheres is significantly increased through the increased amount of the crosslinker. On the other hand, by the addition of the crosslinker the diameter of the microspheres decreased and polydispersed microspheres were formed. Figure 5(b) is a SEM photomicrograph of the polydispersed microspheres obtained due to the crosslinking process of the PCMS microspheres.

Reaction of Amino Ligands with PCMS Microspheres

Tables VI and VII illustrate the interaction of the PCMS microspheres with various amino ligands. The extent of reaction between these microspheres and the ligands is increased (Table VI) under higher temperatures or increasing pH. The binding capacity of these microspheres towards proteins is illustrated in Table VII, for sheep IgG the binding capacity is approximately 40 mg/g microspheres and for protein A the binding capacity is approximately 60 mg/g microspheres. The rate of reaction of PCMS micro-

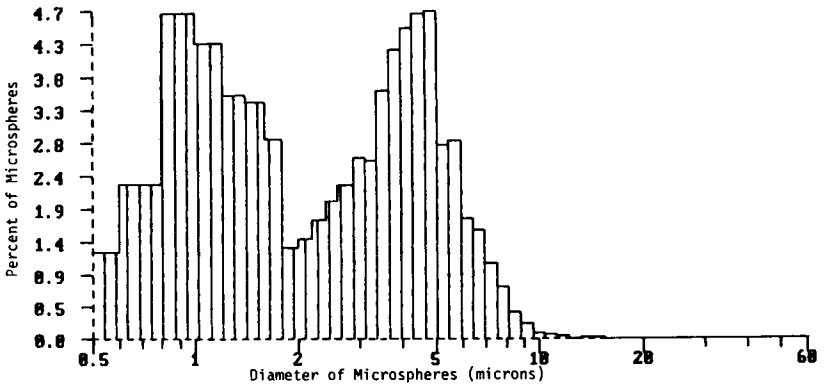

Figure 6. Particle size distribution pattern of PCMS microspheres prepared by polymerizing 5 mL CMS at 70°C in air-free ethanol solution (total volume 10 mL) containing 1 mL DMSO, 115 mg PVP, and 10 mg AIBN.

Table IV. Effect of Surfactant Concentration on the Recovery and Diameter of the PCMS Microspheres[a]

Surfactant (%)	Recovery (%)	Average Diameter (μm)	Remarks
0.004	30.9	—	Agglomerated polymer
0.01	36.8	—	Agglomerated polymer
0.04	40.5	—	Agglomerated polymer
0.13	46.4	1.0	
0.38	48.4	1.0	
1.15	50.2	1.1	
3.5	55.5	1.0	
10.5	75.3	0.02–0.7	

[a] CMS (0.5 mL) was polymerized at 70°C for 24 h in air-free ethanol solution (total volume 10 mL) containing DMSO (1 mL), AIBN (10 mg), and variable amounts of PVP.

spheres with amino ligands, such as 1,3-diaminopropane, is much higher than that of the powder, as demonstrated in Figure 7. In most cases (diffusion control), the reaction rate is directly proportional to the surface area of the polymer. For a polymer with a spherical shape, the rate is proportional to the ratio $1/r$:

$$v \alpha n\pi r^2 = (3V/4\pi r^3) \cdot \pi r^2 = 0.75V(1/r) = k(1/r)$$

where v is the rate of reaction, V is the spherical volume, and n is the number of spheres having radius r.

Conclusions

In both academia and industry, polystyrene microspheres are the most investigated microspheres. Polystyrene microspheres have some significant advantages, e.g., high rigidity and the know-how of to prepare microspheres with different diameters and extremely low size distribution.[1-10] However, polystyrene particles have also some major disadvantages: (1) nonspecific adsorption of proteins and cells onto these particles, because of its hydrophobic character, (2) lack of functional groups through which covalent bonding of ligands is possible, and reliance on just physical adsorption is not always satisfactory. Here, we described the synthesis of PCMS microspheres which may obviate, at least partially, these disadvantages. A recent publication described the preparation of micron-size monodisperse polymeric microspheres having chloromethyl groups.[17] This was accomplished by seed copolymerization of styrene and chloromethylstyrene in the presence of monodispersed microspheres of polystyrene with 1.9 μm diam. In a previous publication by the authors,[13] the synthesis of PCMS microspheres has been described. However, these microspheres were limited in their size (up to 2 μm) and could not be used efficiently in aqueous solutions because of their tendency to agglomerate in water. The PCMS microspheres described here can be considered as a more advanced and investigated PCMS microspheres. They are stable in both organic solvents, e.g., ethanol, and in water. Nephelometric studies demonstrated their stability against agglomeration in aqueous solution in pH ranging between 1 and 13 and in high salt concentrations (e.g., 10% NaCl). The effects of various factors, e.g., monomer concentration, cosolvent, crosslinker, etc., on their diameter, size distribution, and yield have been studied. Furthermore, the interaction of these microspheres with amino ligands, such as proteins and antibodies, have also demonstrated. For biological applications,[20,21] in order to decrease nonspecific

Table V. Effect of Crosslinker (DVB) on the Recovery and Diameter of the PCMS Microspheres[a]

CMS (μL)	DVB (μL)	Recovery (%)	Average Diameter (μm)
500	—	51.8	1.0
490	10	55.0	0.6–1.0
475	25	64.5	0.1–1.0
425	75	70.2	0.07–0.7
400	100	71.5	0.07–0.7
350	150	76.9	0.07–0.7

[a] Different amounts of CMS and DVB (total amount 0.5 mL) were copolymerized at 70°C for 24 h in air-free ethanol solution (total volume 10 mL) containing DMSO (1 mL), PVP (115 mg), and AIBN (10 mg).

Table VI. Effect of pH and Temperature on the Reaction between PCMS Microspheres and Variety of Amino Ligands[a]

pH	Temperature (°C)	Bound Ligand (mmol/g microspheres)		
		OAP	APOH	APA
4.5	25	4.0	0.0	3.5
4.5	60	5.0	0.0	4.5
7.0	25	6.0	0.0	3.5
7.0	60	7.0	0.0	5.0
10.0	25	24.5	8.0	6.0
10.0	60	35.0	15.5	9.0
11.5	25	30.5	13.5	9.0
11.5	60	40.0	22.5	15.5

[a] 50 mg dried PCMS microspheres in 20 mL aqueous solutions were shaken with 250 mg of the amino ligands for 48 h at various pH and temperatures. Abbreviations: DAP, 1,3-diaminopropane; APOH, 3-amino-1-propanol; APA, 3-amino-1-propionic acid.

binding of the microspheres to proteins and cells, it may be necessary, after coupling the microspheres to the desired protein, to block the residue chloromethyl groups with hydrophilic amino ligands (e.g., ethanolamine or albumine), to obtain more hydrophilic specific surfaces.

The functionalization scheme of PCMS microspheres to obtain functional groups other than chloromethyl groups, e.g., amine, hydroxyl, and carboxylate groups, is presented in Figure 8. The synthesis of microsphere surfaces containing these functional groups through a linked spacer arm was accomplished by the covalent binding to the PCMS microspheres of ligands such as $NH_2(CH_2)_2X$, wherein $X = CH_2NH_2$, CH_2OH, and CO_2H, respectively, as demonstrated in Table VI. The possible synthesis of microspheres containing the same functional groups without a linked spacer arm is presented in the left-side of Figure 8. Primary amine groups may be obtained through Delepine reaction.[18,22] Formylpolystyrene microspheres may be prepared through Sommelet reaction.[13,23] The reduction or oxidation of these microspheres will result in the formation of microspheres containing hydroxyl groups or car-

Table VII. Binding Capacity of Proteins (Sheep IgG and Protein A) to PCMS Microspheres[a]

Ligand		
Type	Amount (mg)	Binding Capacity (mg/g microspheres)
Sheep IgG	15	30
Sheep IgG	40	40
Protein A	15	30
Protein A	40	50
Protein A	60	50

[a] 200 mg PCMS microspheres in 28 mL aqueous solution at pH 10 (0.1 M carbonate buffer) were shaken at room temperature for 24 h with different amounts of the proteins.

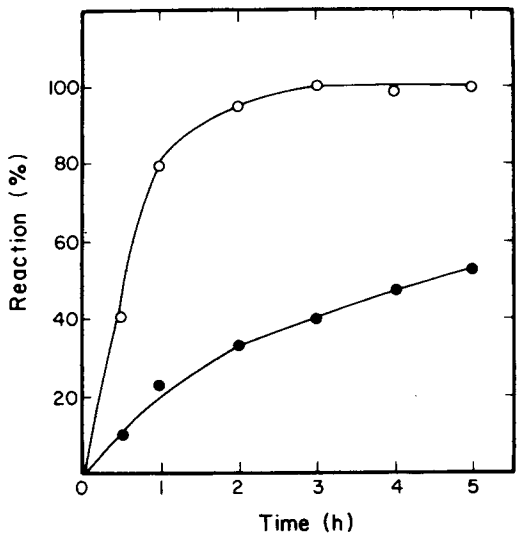

Figure 7. Comparison of the rate of reaction of 1,3-diaminopropane (DAP) with PCMS microspheres of 0.5 μm average diameter (○) and PCMS powder (●). 200 mg dried PCMS were shaken at room temperature with 100 mL aqueous solution at pH 11.5 containing 1 g DAP.

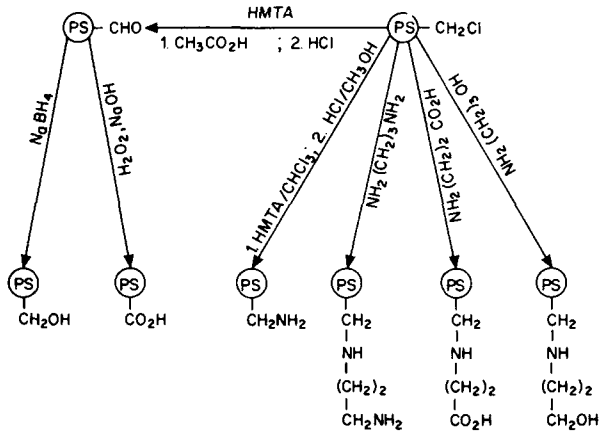

Figure 8. Functionalization of PCMS microspheres (PS $-CH_2Cl$) through a linked spacer arm (right) and without a linked spacer arm (left). HMTA-hexamethylenetetramine.

boxyl groups, respectively.[24] Further studies, concerning the synthesis, characterization, and applications of PCMS microspheres are ongoing in our laboratories.

Thanks to Dr. E. Eyal and Mrs. O. Bernstein from The Israel Institute for Biological Research, The Department of Environmental Physics, Ness-Ziona, Israel, for their help in carrying out the size distribution patterns of the microspheres.

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Received January 17, 1990
Accepted July 16, 1990