Microspheres of acrolein homopolymers and copolymer with hydrophilic comonomers such as methacrylic acid and/or hydroxyethylmethacrylate are prepared by cobalt gamma irradiation of dilute aqueous solutions of the monomers in presence of suspending agents, especially alkyl sulfates such as sodium dodecyl sulfate. Amine or hydroxyl modification is achieved by forming adducts with diamines or alkanol amines. Carboxyl modification is effected by oxidation with peroxides. Pharmaceuticals or other aldehyde reactive materials can be coupled to the microspheres. The microspheres directly form antibody adducts without agglomeration.

21 Claims, 4 Drawing Figures
**Fig. 1.**

- **ALDEHYDE MICROSPHERE** + NH₂OH
- **HYDROXYL-AMINE**
- **H₂N-(CH₂)ₙ-OH**
- **HYDROXYALKYLAMINE**
- **NH₂-R-NR₂**
- **DIAMINE**
- **CH=N-R-NR₂**
- **POLYAMINE**
- **CH=N-(CH₂)ₙ-OH**
- **POLYOXIMES**
- **+ NH₂-FLUOROCHROME**
- **+ NH₂-ANTIBODY**
- **+ NH₂-PHARMACEUTICAL**
- **+ OXIDIZING AGENT**
- **POLYCARBOXYL**
- **PHARMACEUTICAL-N=HC**
- **MICROSPHERE PHARMACEUTICAL ADDUCT**
- **ANTIBODY-N=HC**
- **IMMUNO REAGENT**
- **FLUOROCHROME-N=HC**

**Fig. 2.**
Fig. 3.

Fig. 4.
POLYACROLEIN MICROSPHERES

ORIGIN OF THE INVENTION

The invention described herein was made in the performance of work under a NASA contract and is subject to the provisions of Section 305 of the National Aeronautics and Space Act of 1958, Public Law 83–568 (72 Stat. 435; 42 USC 2457).

This is a division of application Ser. No. 520,313, filed Aug. 4, 1983 U.S. Pat. No. 4,622,362 which in turn is a division of prior application Ser. No. 248,899, filed Mar. 30, 1981 U.S. Pat. No. 4,413,070.

TECHNICAL FIELD

The present invention relates to the synthesis of polyacrolein microspheres, functional derivatives thereof, fluorescent and magnetic variations thereof, protein conjugates thereof and to the use of the conjugates in biological and chemical research and testing.

BACKGROUND OF THE PRIOR ART

The isolation and characterization of cell membranes and their components is essential for an understanding of the role in which surface membranes play in regulating a wide variety of biological and immunological activities. The present techniques used for this purpose are not quite satisfactory.

Knowledge of the nature, number and distribution of specific receptors on cell surfaces is of central importance for an understanding of the molecular basis underlying such biological phenomena as cell-cell recognition in development, cell communication and regulation by hormones and chemical transmitters, and differences in normal and tumor cell surfaces. In previous studies, the localization of antigens and carbohydrate residues on the surface of cells, notably red blood cells and lymphocytes, has been determined by bonding antibodies or lectins to such molecules as ferritin, hemocyanin or peroxidase which have served as markers for transmission electron microscopy. With advances in high resolution scanning electron microscopy (SEM), however, the topographical distribution of molecular receptors on the surfaces of cell and tissue specimens can be readily determined by similar histochemical techniques using newly developed markers resolvable by SEM.

Recently, commercially available polystyrene latex particles have been utilized as immunologic markers for use in the SEM technique. The surface of such polystrene particles is hydrophobic and hence certain types of macromolecules such as antibodies are absorbed on the surface under carefully controlled conditions. However, such particles stick non-specifically to many surfaces and molecules and this seriously limits their broad application.

The preparation of small, stable spherical Poly-Hema particles which are biocompatible, i.e., do not interact non-specifically with cells or other biological components and which contain functional groups to which specific proteins and other biochemical molecules can be covalently bonded is disclosed in U.S. Pat. No. 3,957,741.

Smaller, more evenly shaped acrylic microspheres are disclosed in U.S. Pat. No. 4,138,383. Microspheres having a density differing from that of cell membranes are disclosed in U.S. Pat. No. 4,035,316 and fluorescent-
The monomer mixture can contain other agents such as stabilizing, suspending as emulsifying agents. Radiation accelerators such as halides or metal salts may be added to the reaction mixture. Though the polyacroleins prepared by Bell et al have a high degree of available aldehyde function, there was no recognition of the use of such material as a biological reagent. Furthermore, the presence of extraneous ingredients interferes with the purity of the polymer and it would not be suitable as a biochemical protein bonding agent. Furthermore, specific modification of the material by copolymerization with certain comonomers designed to impart further properties such as non-specific binding and modification to add other functional groups for introduction of dyes, proteins or other materials would improve the flexibility of use of the material.

DESCRIPTION OF THE INVENTION

Novel acrolein interpolymer microspheres and functional, modified reaction products and protein adducts thereof, are produced in accordance with the invention. The size and properties of the microspheres can be controlled by selection of polymerization conditions and especially by selection of comonomers. The microspheres of the invention exhibit exceptional stability and can be derivatized by reaction with amines or with proteins without aggregation. The non-aggregating microspheres are produced in accordance with this invention by the high-energy initiated interpolymerization of an unsaturated aldehyde such as acrolein and at least 20% by weight of at least one addition copolymerizable comonomer having a hydrophilic functional substituent selected from hydroxyl, amino or carboxyl.

Another manner of introducing functionality other than aldehyde onto the microspheres is by adduct reaction of the microspheres with compounds of the formula:

\[ R^1 \text{N} - R - Z \]

where \( R^1 \) is hydrogen or a hydrocarbon group which may be aliphatic or aromatic preferably aryl such as phenyl or alkyl of 1 to 10 carbon atoms, \( R \) is a divalent hydrocarbon group such as alkylene of 1 to 20 carbon atoms and \( Z \) is a functional group such as amine or hydroxyl or \( RZ \) can be hydroxyl. Representative compounds are hydroxylamine or ethylene diamine. The microspheres can be modified to introduce carboxyl groups by oxidation with an agent such as hydrogen peroxide.

The microspheres of the invention exhibit little or no aggregation during or after derivatization reaction to introduce large amounts of antibodies or other proteins, fluorochromes, etc. The microspheres are insoluble, have functional groups directly reactive with protein, are easily dispersed and bind specifically to receptors and can be readily prepared in sizes from 100 Angstroms to 2,000 Angstroms, or up to 10 microns or larger if desired.

The derivatization procedure is simplified. The hydroxyl modified microspheres can be used to chelate metals as a purification media or as a support for a catalyst. The microspheres can be formed into a strong transparent film by drying on a surface or can be formulated to contain metals which can be utilized to form election dense magnetic non-aggregating particles or magnetic coatings or films. The microspheres of the invention provide a reliable, simple method to label cells for research or analysis.

The microspheres of the invention can also be utilized as a substrate to bind pharmaceuticals containing functional groups reactive with aldehyde, the hydrophilic hydroxyl, carboxyl or amine substituent or the functional group \( Z \) of the adduct. The microsphere-pharmaceutical adduct is less likely to migrate and should reduce side effects. Furthermore, antibodies can be attached to the microsphere so that it migrates to specific cells having corresponding antigen receptor sites. Magnetic microspheres can be accumulated at a specific location in a subject by application of a magnetic field to that location.

These and many other features and attendant advantages of the invention will become apparent as the invention becomes better understood by reference to the following detailed description when considered in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a series of graphs showing the effect of addition of comonomers on the size of acrolein copolymer microspheres.

FIG. 2 is a series of schematic reactions of polyacrolein microspheres and various modifying and aducting reagents;

FIG. 3 is a pair of curves illustrating the aldehyde and carboxyl content of oxidized Acrolein-Methacrylic Acid copolymer microspheres; and

FIG. 4 is a pair of curves demonstrating the kinetics of reaction of polyacrolein microspheres with an antibody.

DETAILED DESCRIPTION OF THE INVENTION

Initiation of copolymerization by high energy radiation in absence of chemical initiators or acid materials provides a purer and more evenly sized and shaped microsphere. The microspheres are produced by addition polymerization of a liquid polymerization system optionally including a dispersion of the metal particles in a monomer mixture containing a covalently bondable unsaturated monomer. More uniformly sized and shaped beads are formed in very dilute aqueous monomer mixtures of no more than 5% by weight, preferably 1 to 4% by weight of dissolved monomers. Surfactants may be present to aid in the dispersion of the metal particles and in suspending the microspheres.

The polymerization proceeds with or without stirring with application of high energy radiation capable of generating free radicals in the aqueous system. The radiation source is suitably a cobalt 60 gamma source or cesium source and doses of 0.05 to 2.0 megarads are sufficient for polymerization. It is believed that polymer chains grow from the surface of metallic particles. The reaction is preferably conducted under oxygen excluding condition, generally by applying vacuum to the reaction vessel or by displacing oxygen gas from the system with an inert gas such as nitrogen. After polymerization has proceeded to completion, the reaction mixture is made neutral by adding acid or base, passed through mixed ion exchange resins to remove emulsifi-
null
Synthesis

Acrolein or monomer mixtures consisting of HEMA and acrolein or HEMA, BAM, MA and acrolein formed homogeneous solutions in distilled water containing 0.4% PEO or 64 mM of SDS. After deaeration with nitrogen the mixtures were irradiated in CO gas mixture source at room temperature (dose rate 0.12 Mr/hour) for 4 hours. The reaction product was purified by three centrifugations and kept in distilled water.

Methods

The aldehyde content was determined from the percent nitrogen of the oxime prepared by the reaction of an aqueous suspension with hydroxylamine hydrochloride [P.J. Bochert Kunststoffe 51 (3) 137 (1961)]. IR spectra were obtained with a Fourier transform IR (fts-15C, Houston Instruments) spectrophotometer.

EXAMPLE 1

Pure acrolein (5% v/v) in water containing PEO produced colloidal particles (approximately 1,000 Angstroms in diameter) after cobalt gamma irradiation. Repeat of the procedure substituting 64 mM SDS for PEO resulted in 170 Angstrom microspheres in higher yield.

EXAMPLE 2

Acrolein - HEMA copolymer microspheres of eight different HEMA contents were prepared by cobalt gamma irradiation of a 5% (v/v) monomer solution in water containing 0.4% PEO. The diameter of the resulting microspheres decreased with increasing acrolein content as shown in FIG. 1. Over the middle of the concentration range studied, monomer ratios had little effect on size; permitting the preparation of microspheres of similar size but different degrees of hydrophobicity.

EXAMPLE 3

Seven of the copolymers were reacted with hydroxylamine chloride to form hydroxyl functional microspheres. The aldehyde content was analyzed by this procedure as shown in Table I.

### TABLE 1

**HEMA ACROLEIN MICROSPHERES**

<table>
<thead>
<tr>
<th>% (v/v) Acrolein</th>
<th>Mole % Acrolein</th>
<th>Yield</th>
<th>Microspheres/mg</th>
<th>% Nitrogen per mg</th>
<th>% Nitrogen per microsphere</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>44</td>
<td>61.1</td>
<td>0.84</td>
<td>3.06</td>
<td>1.4</td>
</tr>
<tr>
<td>40</td>
<td>55</td>
<td>50.0</td>
<td>3.1</td>
<td>4.02</td>
<td>1.8</td>
</tr>
<tr>
<td>50</td>
<td>65</td>
<td>37.9</td>
<td>4.84</td>
<td>2.2</td>
<td>5.9</td>
</tr>
<tr>
<td>60</td>
<td>74</td>
<td>37.8</td>
<td>4.6</td>
<td>2.5</td>
<td>5.4</td>
</tr>
<tr>
<td>70</td>
<td>82</td>
<td>36.3</td>
<td>6.5</td>
<td>3.0</td>
<td>7.6</td>
</tr>
<tr>
<td>80</td>
<td>88</td>
<td>21.6</td>
<td>5.2</td>
<td>4.0</td>
<td>7.8</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>17.6</td>
<td>6.4</td>
<td>6.4</td>
<td>10.0</td>
</tr>
</tbody>
</table>

4 Mole % of acrolein in monomer mixture.
5 Calculated using spherical microspheres of diameter shown in FIG. 1 and a density of 1.2 g/ml.
6 Theoretical nitrogen for homopolymer is 19.72%.

The acrolein homopolymer (100% acrolein) was found to contain approximately 65% of the expected aldehyde groups. The presence of aldehyde groups was further confirmed by IR spectra analysis which showed a high intensity peak at 1725 cm⁻¹. Adducts and reaction products are depicted in FIG. 2.

EXAMPLE 4

The hydroxylamine modified copolymer microspheres containing 35% mol HEMA were impregnated with an aqueous solution of copper salt. The copper ions reacted with the microspheres to form metal chelate adducts.

EXAMPLE 5

Cross-linked microspheres containing acid functions were produced by adding MA and BAM to the HEMA - Acrolein monomer mixture. The porosity of the microsphere was significantly increased as evidenced by swelling (uptake of liquid). However, the size of the cross-linked microspheres closely approximated that of the HEMA - ACROLEIN microspheres of Example 2 as shown in FIG. 1. By addition of increasing amounts of BAM to acrolein the hydrophilicity of acrolein microspheres could be progressively increased.

EXAMPLE 6

One of the BAM-MA-HEMA-Acrolein copolymers was reacted with various diamine to form amine-modified adducts. The results are shown in Table 2.

### TABLE 2

**REACTION OF POLY ACROLEIN MICROSPHERES WITH DIAMINES**

<table>
<thead>
<tr>
<th>Reactant</th>
<th>pH</th>
<th>% Nitrogen</th>
<th>Number of Free Amino Groups</th>
<th>pH</th>
<th>% Nitrogen</th>
<th>No. of Free Amino Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>L,2 diaminooctane</td>
<td>3.0</td>
<td>1.43</td>
<td>3.2 × 10⁻¹⁷</td>
<td>2.4 × 10⁻¹⁰</td>
<td>11.3</td>
<td>4.88</td>
</tr>
<tr>
<td>L,2 diaminohexane</td>
<td>3.0</td>
<td>0.78</td>
<td>8.6 × 10⁻¹⁰</td>
<td>6.6 × 10⁻⁸</td>
<td>11.7</td>
<td>3.11</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>3.2</td>
<td>0.40</td>
<td>8.8 × 10⁻¹⁰</td>
<td>6.8 × 10⁶</td>
<td>9.0</td>
<td>2.41</td>
</tr>
</tbody>
</table>

4 Reaction mixture for microsphere synthesis: 35.9 mole % acrolein, 35.9 mole % HEMA, 5.9 mole % methacrylic acid 1.3 mole % bisacrylamide.

When the acrolein homopolymer microsphere suspension was evaporated to dryness, a brittle film was formed. However, evaporation of the HEMA - copolymer (35 mol percent HEMA) microsphere suspension to dryness results in a strong, flexible film.

It was found that at high pH the number of free amino groups was comparable to the number of aldehyde groups found by hydroxylamine analysis. This reaction allows the efficient conversion of aldehyde functions to...
amino functions, removed from the surface of the spheres by a two to six carbon spacer arm.

The monomer mixture utilized in the experiment in Table 2 was modified by maintaining the ratio of HEMA, MA and BAM constant while adding increasing amounts of acrolein. As shown in Table 3 which follows, the aldehyde content increased with increasing acrolein content proving that acrolein was being incorporated into the copolymer.

TABLE 3

<table>
<thead>
<tr>
<th>MOLE % ACROLEIN</th>
<th>% N</th>
<th>No. ALDEHYDE GROUPS/mg x 10^-18</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.9</td>
<td>3.26</td>
<td>1.35</td>
</tr>
<tr>
<td>58.5</td>
<td>4.32</td>
<td>1.93</td>
</tr>
<tr>
<td>75.8</td>
<td>6.57</td>
<td>2.90</td>
</tr>
<tr>
<td>95.3</td>
<td>12.16</td>
<td>5.83</td>
</tr>
</tbody>
</table>

EXAMPLE 7

The copolymer of Example 6 was reacted with an adduct of fluorescein isothiocyanate (FITC) and 1,6-diamino-n-hexane which resulted in microspheres of high fluorescent intensity.

EXAMPLE 8

An allyl amine adduct of FITC was prepared. Addition of 0.1% by weight of the adduct to the polymerization system of Example 6 resulted in an addition interpolymerized fluorescent copolymer microsphere.

EXAMPLE 9

An adduct of 1,6-diaminohexane (DAH) and FITC was prepared. Addition of 0.1% of the adduct to the polymerization system of Example 6 resulted in introduction of fluorescent chromophore by condensation with aldehyde groups of the addition polymerized copolymer.

EXAMPLE 10

Dispersible iron oxide was prepared by dissolving 10 g of ferrous chloride and 13.5 g of ferric chloride in 210 cc of 1% w/v polyethylene imine (M.W. 1800) aqueous solution. 50% NaOH was added to pH 7. The reaction mixture was mixed for 3 hours, dialyzed extensively against water and separated magnetically three times from non-magnetic particles. The magnetic polyethylene imine-iron oxide particles were dispersed in water and then sonicated with a clinical sonicator for 10 minutes. Magnetic particles having a diameter of 300 Angstroms with amine groups on the surface were formed. When 1% of the polyethylene imine-iron oxide is added to the solution of monomers of Example 6 and subjected to gamma irradiation microspheres containing a dispersion of magnetic iron particles is produced.

EXAMPLE 11

The Acrolein methacrylic acid copolymer microspheres were oxidized to convert the aldehyde groups to carboxyl adding 30.5 ml H2O and 30 ml of 1M H2SO4 sohtion and irradiated for 4 hours with a cobalt gamma source at 0.12 Mr/h and centrifuged 3 times. The particles were not visible. Their size as determined under an electron microscope was about 300-500 Angstroms.

When the SDS content was increased to 50 mg, the particles were too small to be centrifuged. After dialyzing for several days their size as determined by SEM was about 170 to 340 Angstroms.

The marking of cell surface receptors by means of fluorescent, non-fluorescent or magnetic fluorescent PGL microspheres was found to be simple and efficient as evidenced by numerous tests using fixed human or animal antibody labeled cells.

The reactivity is similar to polyglutaraldehyde microspheres. However, no significant aggregation was observed during reactions with amines, diamines or proteins under a variety of experimental conditions. The microspheres are preferably very small in size from 100 Angstroms to 100 microns, generally from 500 Angstroms to 10 microns so that specific receptor sites on a cell surface can be tagged.

EXAMPLE 13

To 2.5 ml of a water suspension of acrolein microspheres (total 15 mg) was added 0.5 of a 2 mg 1 ml solution of 125 Iodine labeled goat immunoglobulin G (spec. activity 1 x 10^5 cpm/mg) in PBS. The mixture was rotated for 3 hrs. and 400 microliters aliquots were taken at 0,30,60,120 and 180 minutes. Aliquot were immediately added to 400 ml of a 1% (w/v) solution of egg albumin in PBS and centrifuged at 15,000 x g for 4 min., resuspended and washed once in PBS as above.

The acrolein microspheres exhibited direct binding of about 7-9% by weight of antibody whereas a control HEMA-BAM microsphere was able to bind less than 1% by weight of the microsphere. Results are illustrated in FIG. 4.

EXAMPLE 14

Binding of Methotrexate to Polyacrolein Microspheres

I. Preparation of Microspheres

[10%] - Total monomer concentration

90% Acrolein 10% Methacrylic Acid

in 25 ml 0.4% PEO 100,000 MW

pH 2.8

Degas with Nitrogen

Co Gamma Radiation 5h Dose - 0.12 Mic/hr.

Wash 3X

Resuspend 36 ml H2O

Conc: 27.5 mg/ml

Yield: 46.13%

II. Reaction of Microspheres with 1-6 Diaminohexane

50 mg of microspheres

0.6 ml DAH (80% aqueous solution)

Repeat 4 hr. with shaking at room temperature

Wash 3X

Resuspend in 10 ml H2O

III. Reaction of Microspheres with Carbodiimide

Add 20 mg of carbodiimide to 50 mg of DAH microspheres

Sonicate 10 minutes

Adjust pH to 6 w/Na2HPO4

Add 10 mg methotrexate in 2 ml H2O

Check to be sure pH is still 6.0

Sonicate 2 minutes

Shake overnight at room temperature
Spin down 3×
Take spectrum of first supernate. Spectrum indicates
that more than 90% of methotrexate adducted with
the microspheres.

A new convenient immunoreagent in form of acro-
lein copolymer microspheres was synthesized in a vari-
ety of sizes and with a relatively narrow size distribu-
tion. High intensity of fluorescence can be imparted to
the microspheres during or after polymerization. The
aldehyde functional groups permit covalent bonding
with antibodies, enzymes and other proteins in a single
step. Therefore this immunoreagent eliminates the pre-
viously used intermediate steps in which the cyanogen
bromide and carbodiimide reaction was used. The high
specificity of the microspheres, at least as far as human
rbc is concerned is also a desirable property. A minor
synthetic modification yields fluorescent, magnetic mi-
crospheres for a large number of potential applications.
The polyacrolein copolymer microspheres of this in-
vention contain approximately twice as many aldehyde
groups as the comparable glutaraldehyde copolymer
microspheres.

The use of magnetic particles has created a great deal
of interest in biochemical research and clinical medicine
when used as supports for immobilized enzymes. Their
easy retrieval from liquids containing colloids and un-
dissolved solids should be of practical value. The separa-
tion of proteins and chemical compounds by affinity
chromatography can be simplified by elimination of
tedious centrifugation procedures and column chroma-
tography steps. Magnetic particles have also recently
been tested in radioimmunoassay techniques in hyper-
thermia treatment of cancer, in guidance of magnetic
particles to a vascular malformation such as cerebral
eaneurism with the intent to seal the defect by inducing
thrombosis.

Other proposed applications have been as tracers of
blood flow or vehicles for drug delivery. The first suc-
sessful application of magnetic immunomicrospheres to
the separation of B and T cells has been demonstrated.
There is little doubt that physical sorting of cell subpop-
ulations has become a necessity. Many separation meth-
ods, while useful are limited by the restricted set of
parameters upon which separation can be based and by
the fact that they are batch techniques. New flow cy-
rometers and sorters permit quantitative multiparameter
measurements and sorting based on these measurements,
but are limited as far as the number of cells that can be
separated in a given time. Magnetic cell sorters have the
potential of cell separation in a continuous process. Evidence obtained using model cell
systems indicates that magnetic immunomicrospheres
of desirable sizes can be conjugated with proteins in a
simple and convenient manner, therefore offer a poten-
tial for large scale immunological cell sorting as well as
other applications.

It is to be understood that only preferred embodi-
ments of the invention have been described and that
numerous substitutions, modifications and alterations
are permissible without departing from the spirit and
scope of the invention as defined in the following
claims.

1 claim:
1. A method of preparing small polymeric micro-
spheres comprising the steps of:
forming a solution of less than 5% by weight of a
monomer mixture containing at least 10% by
weight of an unsaturated aldehyde selected from

the group consisting of acrolein and C2 to C4 aryl,
aldehydes or cycloaldehyde derivatives thereof and at least
20% by weight of at least one addition copolymer-
izable monomer having a hydrophilic substituent
selected from hydroxyl amino or carboxyl and 0.1
to 20% of a polyunsaturated crosslinking agent;
irradiating the solution with radiation capable of initi-
ating polymerization; and
recovering said microspheres.
2. A method according to claim 1 in which the alde-
hyde is present in the mixture in an amount from 20% to
90% by weight.
3. A method according to claim 1 in which the alde-
hyde is acrolein.
4. A method according to claim 2 in which the com-
onomer is present in an amount from 10 to 50% of
the mixture and comprises a mono-unsaturated, freely
water-soluble acrylic monomer substituted with amino,
carboxyl or hydroxy.
5. A method according to claim 4 in which the com-
onomer is selected from acrylamide, methacrylamide,
acrylic acid, methacrylic acid, dimethylaminometha-
crylate or compounds of the formula:

CH2=O

R1

\[ \text{II} \quad \text{II} \]

R1-C=C-R2-Z

where R1 is hydrogen or lower alkyl of 1-8 carbon
atoms R2 is alkylene of 1-12 carbon atoms and Z is
—OH or R3-N-R4 where R3 or R4 are individually se-
lected from H, lower alkyl or lower alkoxy of 1-8 car-
on atoms.
6. A method according to claim 5 in which the com-
onomer comprises hydroxyethyl methacrylate.
7. A method according to claim 4 in which the com-
onomer imparts a negative charge to the microsphere.
8. A method according to claim 7 in which said co-
nonomer is methacrylic acid.
9. A method according to claim 1 in which the cross-
linking agent further includes functional groups.
10. A method according to claim 9 in which the cross-
linking agent is bis-acrylamide.
11. A method according to claim 2 further including
0.05 to 5% by weight of a copolymerizable fluorescent
chromophore monomer
12. A method according to claim 11 in which the fluo-
scent monomer contains functional groups reactive
with aldehyde.
13. A method according to claim 12 in which the fluo-
scent monomer contains addition polymerizable
unsaturated groups.
14. A method according to claim 2 in which said
monomer solution contain a dispersion of metal particles.
15. A method according to claim 14 in which the
metals are magnetizable.
16. A method according to claim 1 in which the solu-
tion contains a suspending agent.
17. A method according to claim 16 in which the
suspending agent is selected from polyalkylene oxide
liquid polymers and an alkali metal alkyl sulfate contain-
ing 8 to 20 carbon atoms.
18. A method according to claim 17 in which the
agent is selected from sodium lauryl sulfate and sodium
dodecyl sulfate.
19. A method according to claim 1 further including
the step of oxidizing the recovered microspheres to
convert the aldehyde groups to carboxyl groups.
20. A method according to claim 1 further including the step of reacting the recovered microspheres with a compound of the formula:

\[ R^1 \backslash \bigg\{ N-R-Z \bigg\} \]

where \( R^1 \) is hydrogen or a hydrocarbon group, \( R \) is hydroxyl or \( R \) is selected from aliphatic or aromatic and \( Z \) is amine, hydroxyl or carboxyl to form an adduct.

21. A method according to claim 1 further including the step of further reacting the adduct with a material reactive with \( Z \) to form a further adduct.