Amine containing polymeric microspheres such as polyvinyl pyridine are complexed with metal salts or acids containing metals such as gold, platinum or iron. After reduction with sodium borohydride, the salt is reduced to finely divided free metal or metal oxides, useful as catalysts. Microspheres containing covalent bonding sites can be used for labeling or separating proteins.
Fig. 2.

![Graph showing the relationship between hydrogenation rate (ml H₂/min) and amount of catalyst (mg).](image)
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IMPREGNATED METAL-POLYMERIC FUNCTIONAL BEADS

This is a division of application Ser. No. 718,103, filed Aug. 27, 1976, now issued on Oct. 31, 1978, as U.S. Pat. No. 4,123,396.

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).

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to metal-containing polymeric microspheres and to a method of preparing such materials and to the use thereof in catalysis and a labelling, separation and analysis of protein materials.

2. Description of the Prior Art

The isolation and characterization of cell membrane and their components is essential for an understanding of the role in which surface membranes play in regulating 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 binding antibodies or lectins to such macromolecules as ferritin, hemocyanin or peroxidase which have served 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 polystyrene 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 severely limits their broad application.

The preparation of small, stable spherical particles which are bio-compatible, i.e., do not interact non-specifically with cells or other biological components and which contain functional groups to which specific proteins and other bio-chemical molecules can be covalently bonded is disclosed in copending application Ser. No. 434,124, filed Jan. 17, 1974 now issued on May 18, 1976, as U.S. Pat. No. 3,957,741.

Smaller, more evenly shaped microspheres are disclosed in Ser. No. 634,935, filed Nov. 24, 1975, now issued on Feb. 6, 1979, as U.S. Pat. No. 4,138,383 and microspheres having a density differing from that of cell membranes are disclosed in Ser. No. 634,429, filed Nov.

24, 1975 now issued on July 12, 1977, as U.S. Pat. No. 4,035,316.

The hydroxyl groups can be activated by cyanogen bromide for covalent bonding of proteins and other chemicals containing amino groups to the polymeric matrix. Methacrylic acid residues which impart a negative charge onto the particles are likely to prevent non-specific binding to cell surfaces and to provide carboxyl groups to which a variety of bio-chemical molecules can be covalently bonded using the carbodiimide method. Cross-linking of the polymeric matrix is preferable in order to maintain the stability and size of the particles in both aqueous solution and in organic solvents commonly used in the fixation and dehydration of biological specimens for electron or light microscopy.

These polymeric microspheres ranging in diameter from 300 Å to 2000 Å have been successfully utilized as biocompatible immunochemical markers of red cells and lymphocytes in scanning electron and light microscopy. The variable density microspheres have been utilized in separation of labeled cells to which they are attached.

The metal containing microspheres can be readily detected by x-ray electron probe methods since the presence of metal causes the microspheres to be electron dense.

Optimum catalysis requires both selectivity and efficiency. A metal containing functional microsphere prepared by polymerization of the microsphere in presence of finely divided metal particles or compounds thereof has been disclosed in Ser. No. 694,151 filed June 9, 1976, now abandoned. However, the distribution of metal within the microspheres is on a random basis, since it is difficult to maintain the particles suspended during polymerization. Impregnation or porous polymeric microspheres on the surfaces of non-porous microspheres with aqueous solutions of metal compounds is not effective since the metal salt dissolves during use in aqueous media.

SUMMARY OF THE INVENTION

Polymeric microspheres containing controlled and uniform distribution of metal atoms are readily custom synthesized according to the invention. Porosity, hydrophilic properties and type and amount of covalent bonding sites are a result of selection of monomer and polymerization conditions of the microsphere substrate.

The metal containing microspheres will find use in a variety of industrial olefin addition processes such as hydrogenation. The catalysts have excellent selectivity due to the control of porosity, metal content and spacing and surface area of the microspheres. Functionally substituted metal-polymeric microspheres have a significant density differential from protein and will find use in separation of labelled cells or cell fragments. The metal containing microspheres are electron dense and can readily be detected by x-ray electron probe methods. Protein labelled with functional polymeric microspheres containing magnetically attractive particles such as iron or magnetite can be separated from non-labelled protein by applying a magnetic field to the cell mixture.

The metal-containing microspheres are hydrolytically stable, biocompatible and have good mechanical strength. The microspheres are of well characterized structure, of outstanding purity and the hydrophilic properties, size, mechanical properties and metal distri-
The polymeric support must be insoluble in the polymerization and in the catalytic olefin addition reaction media. The polymeric support may be in the form of particles, sheets, films, strands, hollow fibers or a coating on a surface. The polymeric substrate is preferably a high area substrate such as glass particles.

Vinyl pyridine polymers in which the monomer mixture contains at least 20% by weight of vinyl pyridine are preferred due to the ready availability of the pendant pyridyl group for ionic or coordinating reaction with the metal atom. 2-vinyl, 4-vinyl or 2-methyl-5-vinyl pyridine polymers are suitable. The polymers may be obtained commercially, or can be prepared according to procedures disclosed in application, Ser. No. 671,085, filed Mar. 29, 1976, the disclosure of which is expressly incorporated herein by reference.

The monomer mixture contains at least 20% by weight of vinyl pyridine, preferably at least 50% by weight. The remaining comonomers are soluble in the aqueous medium, compatible, unsaturated compounds capable of addition polymerization with vinyl pyridine.

The comonomers preferably contain a hydrophilic group such as carboxyl, amino or hydroxyl.

Mono-unsaturated comonomers are suitably selected from amino, carboxyl or hydroxyl substituted acrylic monomers. Exemplary comonomers are acrylamide (AM), methacrylamide (MAM), acryl acid, methacrylic acid (MA), dimethylaminomethylacrylate or hydroxyl-lower alkyl- or amino-lower-alkyl-acrylates such as those of the formula:

where R1 is hydrogen or lower alkyl of 1-8 carbon atoms, R2 is alkylen of 1-12 carbon atoms, and Z is 

These 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 curves showing the volume of H2 absorbed in ml as ordinate versus time in minutes as abscissa by the hydrogenation of 1-hexene with poly-4-vinyl pyridine containing 20% by weight of platinum; and

FIG. 2 is a curve showing hydrogenation rate in ml absorbed H2/min. versus amount of catalyst in mg for the hydrogenation of 1-hexene with the catalytic microspheres of this invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The polymeric microsphere is a solid particulate support containing an amine ligand capable of ionic reaction or coordination with a metal containing acid or salt. The amine ligand is preferably a tertiary amine such as CH2NMe2—PhNMe2 —pyridyl and the like. The ligand is preferably pendant from the polymer backbone. Representative polymers are cross-linked polystyrene-divinylbenzene substituted with —CH2NMe2 polyethylene imine, polyvinylbenzimidazole and polyvinylpyridine.

The polymeric support must be insoluble in the polymerization and in the catalytic olefin addition reaction media. The polymeric support may be in the form of particles, sheets, films, strands, hollow fibers or as a coating on a surface. The polymeric substrate is preferably a high area substrate such as glass particles.

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pletion, the polymerization suspension is centrifuged, washed and the trapped water is then removed by azeotropic distillation or freeze drying to yield white, round microspheres at least 80% of which and preferably 90% of which have an average diameter of less than 100 μm, preferably from 100 Å to 60 microns and a standard deviation in most cases of no more than 5–40%.

The microspheres can be coalesced into a film by application to a substrate such as a planar surface or particulate surface such as glass beads or polymerized in situ in the presence of the substrate to form a coated substrate. The film consists of a high concentration of microspheres.

The size of the microspheres is dependent on monomer concentration. The vinyl pyridine monomers are soluble in water up to about 3%. Copolymerization with water soluble monomers permits increased water solubility of the monomer mixture up to 6–10% or more. Microspheres produced at 3% vinyl pyridine monomer concentration or less will have a diameter below 3500 Å. Solubility of the vinyl pyridine is increased by the addition of a water-miscible solvent thereof such as by replacing up to 50% by weight of the water with a lower alkanol or di or trialkanol such as methanol. The polymerization rate is considerably decreased requiring irradiation of the solution for a longer period. Examples of practice follow.

**EXAMPLE 1**

4-vinyl pyridine (2.7 g) and bisacrylamide (0.3 g) were irradiated in an aqueous solution containing 0.1% polyethylene oxide (M.W. 600,000) and 3% total monomer with 1.2 megarads from a Co-60 gamma source. The diameter of the microspheres obtained was 2300 Å.

The metal is added to the beads as an aqueous solution of an acid or salt of the metal such as a transition metal compound of metals of Groups IV, VI, VII, or VIII of the Periodic Table having a valence lower than the maximum valence. Typical metals are iron, gold, nickel, rhodium, manganese, chromium, titanium, ruthenium, tantalum or iridium. Electron dense metals suitable for bioassay are heavy metals having an atomic number above 50, preferably above 75, such as cobalt, platinum, gold, or iron. The metal may be magnetically attractive such as Fe, Ni, Co, or alloys thereof or an inorganic magnetic compound such as a metal oxide. Representative water soluble compounds impregnating the microspheres are chloroplatinic acid (H₂PtCl₆.6H₂O), chloroauric acid (HAuCl₄.3H₂O), nickel chloride, ferrous sulfate (FeSO₄), ferrous chloride (FeCl₂), cobalt chloride (CoCl₂.6H₂O) or the like.

The amount of metal added to the microspheres can be varied from 0.01% by weight to stoichiometric amounts based on amine content of the microsphere and is usually from 5 to 50% by weight thereof. Metal-containing acids react easily in the cold with polyvinyl pyridine forming a strong quaternary bond. Metal salts do not appear to quaternize the pyridyl groups but form a strong ionic bond therewith. Addition of a water-miscible polar or non-polar solvent for the salt such as a lower alkanol containing 1-6 carbon atoms, suitably methanol or ethanol, and/or heating the impregnating solution appears to provide better interaction of the metal salt with the tertiary amino nitrogen.

The metal containing beads are reduced to the free metal or a lower valence metal compound by reaction with a water soluble reducing agent suitably a borohydride or aluminum hydride. Representative materials are cyanoborohydride, lithium aluminum hydride and sodium borohydride. The amount of reducing agent depends on the metal content of the microspheres, the initial valence of the metal and the degree of reduction desired. The reducing agent is usually added in considerable excess with respect to the microspheres, typically at least 1/1 by weight generally 2/1 to 20/1 by weight.

**EXAMPLE 2**

The microspheres of Example 1 were suspended in a 10 mole % CoCl₂.6H₂O ethanolic solution. An almost quantitative absorption of Co⁺² was observed.

**EXAMPLE 3**

Suspension of the microspheres in an ethanolic solution containing 1:1 molar ratio yielded a higher Co⁺² content.

**EXAMPLE 4**

The microspheres of Example 1 were suspended in an absolute ethanolic solution of anhydrous FeCl₃ at a 1:1 molar ratio. An almost quantitative absorption of Fe⁺³ was observed.

**EXAMPLE 5**

The PVP microspheres (100 mg) were suspended in 5 cc of aqueous methanol (15%) to which was added 400 mg of chloroauric acid. After reaction for one (1) hour at room temperature, the slightly yellow supernatant was separated by centrifugation and the dark yellow microspheres were washed several times with methanol and re-suspended in methanol.

0.2 g of NaBH₄ in 70 cc of H₂O was added and left overnight. The black microspheres were then passed through a mixed ion-exchange resin, bed, centrifuged several times and redispersed in water. Analysis showed the microspheres contained 30% gold.

**EXAMPLE 6**

100 mg of the PVP microspheres of Example 1 in 5 cc of water were treated with varying amounts of 5% solution of chloroplatinic acid, and then separated and reduced according to the procedure of Example 5.

The resulting microspheres containing 20% by weight of platinum (6.0, 4.0, 3.1 and 2.0 mg) were utilized to hydrogenate 1-hexene (0.2 ml/10 ml methanol) at 24.5° C. The olefin was hydrogenated in 100% yield at room temperature and pressure demonstrating that the finely divided platinum metal associated with the PVP microsphere is a very active, high surface area heterogeneous catalyst. This is surprising since pyridine nitrogen usually poisons platinum catalysts. FIGS. 1 and 2 show that the rate of hydrogenation is dependent on the amount of platinum relative to olefin.

**EXAMPLE 7**

Example 4 was repeated with a PVP copolymer prepared by irradiating an aqueous solution containing 2% total monomer including 60% 4-VP, 30% acrylamide and 10% bisacrylamide. The resulting beads (1000 angstroms in diameter) contained 30% gold are electron dense. These microspheres do not exhibit non-specific binding to living cells, e.g. murine lymphocytes and were found to be capable of binding to antibodies by the
cyanogen bromide or carbodiimide reaction, J. Cell Biol. 64 75.

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

What is claimed is:

1. A method of labelling protein comprising the step of covalently bonding the protein to a microsphere composition comprising polymeric microspheres having a diameter below 100 microns containing at least 20% by weight of a tertiary amine substituted monomer having the amine ligand pendant from the polymer backbone selected from the group consisting of cross-linked polystyrene-divinylbenzene substituted with —CH₂NMe₂, polyethylene imine, polyvinylbenzimidazole and polyvinylpyridine, said ligands being capable of ionic reaction or coordination with Group IV, VI, VII or VIII metal compounds selected from oxides, salts or acids to form complexes therewith and said microspheres containing; at least 0.1 weight percent of finely divided, free metal associated with the amine ligands formed by reduction of the compound in situ within the microsphere by reaction with a reducing agent selected from the group consisting of borohydrides and aluminum hydrides and at least 20% by weight of an acrylic monomer having a covalent bonding group selected from hydroxyl, carboxyl or amine.

2. A method according to claim 1 in which the metal is a heavy metal.

3. A method according to claim 2 further including the step of separating microsphere labelled protein from nonlabelled protein.

4. A method according to claim 1 in which the tertiary amine substituted monomer is vinyl pyridine.

5. A method according to claim 4 in which the microspheres contain from 5 to 50% by weight of metal.

6. A method according to claim 1 in which the metal is selected from iron, gold, platinum, cobalt, nickel, rhodium manganese, chromium, titanium, ruthenium, tantalum or iridium.

7. A method according to claim 6 in which the metal is a transition metal which forms a coordination complex with the tertiary amine group.

8. A protein specific reagent comprising polymeric microspheres covalently bonded to a conjugate of said protein, said microspheres comprising polymeric microspheres having a diameter below 100 microns containing at least 20% by weight of a tertiary amine substituted monomer having the amine ligand pendant from the polymer backbone selected from the group consisting of cross-linked polystyrene-divinylbenzene substituted with —CH₂NMe₂, polyethylene imine, polyvinylbenzimidazole and polyvinylpyridine, said ligands being capable of ionic reaction or coordination with Group IV, VI, VII, or VIII metal compounds selected from oxides, salts or acids to form complexes therewith and said microspheres containing; at least 0.1 weight percent of finely divided, free metal associated with the amine ligands formed by reduction of the compound in situ within the microsphere by reaction with a reducing agent selected from the group consisting of borohydrides and aluminum hydrides and at least 20% by weight of an acrylic monomer having a covalent bonding group selected from hydroxyl, carboxyl or amine.

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