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Surface-functionalized, probe-containing, polymeric nanospheres for biomedical imaging

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Abstract. Nanoparticles may be bound covalently to biological macromolecules for imaging, reporting, or delivery of therapeutic agents. The nanospheres of this report range from 15 nm to 30 nm in diameter, depending on the composition, although each type of nanosphere varies only ±5 nm from its mean. These nanospheres are smaller than an antibody, so may be used in the same way as other antibody labels such as enzymes in immunoassay. A double micelle forms the core of these nanospheres, in which a water pool contains approximately 1200 ionic probes such as fluorescent lanthanides, ferrite, borohydrides for BNCT, or hydrophilic therapeutic agents. Stability is imparted to the double micelle by a polymeric net that is woven into the hydrophobic zone, allowing control of diffusion. Imagable atoms may be incorporated as monomers into this polymeric net, providing as many as 200,000 fluorine atoms for ^{19}F MRI or 40,000 iodide atoms for CAT. The surfaces of the nanospheres may be tailored with hundreds of active groups such as acids or amines for later conjugation, and also polar groups such as esters, alcohols, and amino acids to suit the intended environment. The finished nanospheres may be conjugated to macromolecules using rapid, simple reactions.

1. Introduction

Surface-functionalized, probe-containing, polymeric nanospheres for use in immunoassay, imaging, and drug delivery were developed under a research contract with the USEPA for multi-analyte environmental immunoassay [1]. Different fluorescent probes were conjugated to different antibodies in order to quantify up to four compounds of environmental interest simultaneously in non-extractive immunoassay. The fluorescence responses were sufficiently different to allow for simultaneous quantification using a fluorescence spectrophotometer.

The nanospheres range in size from 15 to 30 nm, depending on the probe contained. Approximately 1200 ion pairs are contained in the water pool of each nanosphere. By manipulating the quantities of reagents, this value may be increased to more than 2000 probe ions, but the nanospheres increase to about 40 nm in diameter. A nanosphere may be conjugated to proteins such as an antibody or vector easily and with high efficiency through long-chain acid groups on its surface. Other types of linking functionalities may be selected at the time of synthesis to tailor the nanosphere to the desired application. The suspension and affinity characteristics of the nanospheres may be modified easily during synthesis to suit the application. The probes may be selected from a wide variety of options. To date, lanthanoids, borate, ferrite, several transition metals, and a chemotherapy drug have been encapsulated. Porosity may be selected to control the release rate of the probe over a wide range, including zero release. Ionic probes may be structured for zero release in isotonic solutions, but allow for extraction by aqueous organic

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ligand solutions in minutes. The polymer that provides stability to the structure may be selected during synthesis for optimum fate and adsorption characteristics.

In addition to including detectable ions in the core of the nanospheres, the polymeric shell may be modified to include 40,000 iodine atoms or 200,000 fluorine atoms. Up to 1,000 other ions may be attached to the surface of each nanosphere through ligands incorporated into the structure, either during or after synthesis. Combinations of added ions make the nanospheres suitable for many types of imaging, including radiolabeling, MRI, PET, CAT, and electron microscopy. Other potential applications include multi-analyte immunoassay, boron neutron capture therapy, targeted drug delivery, magnetic separations, nanoscale localized heating in an oscillating magnetic field, and quantum dots.

2. Theoretical model

Based on known constants for micelles of dioctylsulfosuccinate (AOT) in organic solvents [2], the number of molecules of each reagent in each nanosphere may be approximated. The radius of the water pool inside the AOT micelle has been calculated over a range of solvent : AOT ratios, all of which exceed the critical micelle concentration. Within certain limits, the volume of the water pool may be calculated from a linear regression performed on the experimental data. Dividing the total volume of added water by the volume of one water pool (derived from the mass of AOT and volume of heptane) yields the number of micelles in the solution. Since all the other reagents are also added in measured quantities, and because they may be assumed to distribute themselves homogeneously, the number of molecules of each reagent per nanosphere may be approximated. Table 1 shows the numbers of molecules of each reagent in one of the many types of nanospheres. Although the numbers are given as if precise, they are only the products of a theoretical construct, not empirical measurements. However, these estimates have been in agreement with the performance of the nanospheres in many experiments [3].

An Excel spreadsheet may be constructed based on the empirical values of AOT molecules per micelle to model the impact of varying the relative quantities of components. An example is shown in Table 2 for styrene/divinyl benzene nanospheres. Conditional formatting was used to flag parameters that exceeded known limits, such as saturation of terbium ions, critical micelle concentration, or maximum water capacity of a micelle.

Nanosphere synthesis proceeds from the formation of AOT reverse micelles (each with a water pool containing the probe ions) to the inclusion of those micelles in aqueous sodium dodecyl sulfate (SDS)

Table 1
Theoretical number of molecules of reagents in one nanosphere

Methyl methacrylate nanospheres	
Compound	Quantity
Terbium chloride hexahydrate	1166
Water	51665
Sodium dioctylsulfosuccinate	1224
Sodium dodecyl sulfate	3029
Methyl methacrylate	43595
2,3-Dimethyl-1,3-butadiene	4808
Methacrylic acid	1833
Citronellic acid	2949
2-Hydroxyethylmethacrylate	4486

Table 2

Typical Excel worksheet for modeling reagent ratios in nanospheres. Plain boxes are for user input. Double-line boxes show alarm ratios through conditional formatting

	FW	Density	µL added	mg added	moles	N/bead	
Heptane	100.2	0.6837	40000	27348	2.73E-01		
Sodium dioctylsulfosuccinate	444.55			350	7.87E-04	1224	**AOT**
Water	18.01528	0.998	600	599	3.32E-02	51665	
Terbium chloride hexahydrate	373.38			280	7.50E-04	1166	**Tb**

[Tb^{3+}]	w	AOT%	bead FW	Tb_2O_3 nm	% pool
1.250	42	1.3%	9.07E+06	3.6	3.1%

AOT r nm	AOT nm^3	micelles	moles	Tb_2O_3 ρ	Tb_2O_3 FW
7.2	1549	3.8742E+17	6.43E-07	7.42	365.8

	FW	Density	µL added	mg added	moles	N/bead	
Sodium dodecyl sulfate	288.38			562	1.95E-03	3029	**SDS**
Styrene	104.15	0.909	3000	2727	2.62E-02	40699	**Monomer**
Divinyl benzene	130.19	0.914	400	366	2.81E-03	4365	**Cross**
2,3-Dimethyl-1,3-butadiene	82.15	0.726	350	254	3.09E-03	4808	**Cross**
Citronellic acid	170.25	0.923	350	323	1.90E-03	2949	**Acid**
2-Hydroxyethylmethacrylate	130.14	1.073	350	376	2.89E-03	4486	**Ester**

low AOT	high AOT	max [Tb^{3+}]	w	r	m	b
1%	3%	1.5	11	2	0.167901	0.088889
			24	4	0.003733	0.119965
low w	**high w**	**Σmg**	35	6	0.999012	0.099381
16	50	5836	47	8	2023	2

micelles to form SDS/AOT double micelles in aqueous solution. The monomers are added to the aqueous solution of double micelles. They accumulate in the region of the hydrophobic tails of each double micelle. Different monomers are selected according to the surface character desired. The more polar monomers orient themselves with their polar portions pointing toward the aqueous regions. Since the exterior surface area is 30 times greater than the interior surface area (the surface area of the water pool in which the probe ions are sequestered), the vast majority of the polar monomers are oriented to the exterior surface of the nanosphere. Once polymerized, the active groups are permanently fixed in place. The cross-linking is intentionally low so that the polymeric net that holds each nanosphere together will have pores available for ligands to migrate in and out to extract the ionic probes. So that the looseness of the polymeric net does not result in leakage of the probe ions, methyl substituents must be included on the butadiene and the acrylate monomers. In the polystyrene/divinylbenzene version of the nanospheres, the phenyl rings perform the same function.

The methyl methacrylate nanospheres were synthesized with acid, alcohol, and ester groups to impart a larger negative charge to the finished nanosphere in order to improve its suspension characteristics and to reduce random adsorption in a soil sample. The methacrylic acid chain is very short, so the majority of the negative charge remains close to the surface, less accessible to the environment. Citronellic acid, having a long chain, providing a spacer for ease of conjugation through an amide linkage. The long-chain alcohol and ester groups are to provide polarity but without activity, improving the suspension characteristics and decreasing random adsorption.

3. Synthesis

The details for the synthesis of the multitudes of variations of nanospheres are readily available [4]. A synopsis follows containing comments and critical points not stressed in the patent description. The parameters given in Table 3 yield about 3.5 g of nanospheres. Batch size has not been investigated. Stirring in the polymerization step is critical, so scale-up will require significant research. Where indicated, solutions are sonicated at 50% full power using an ultrasonic processor, manufactured by Ultrasonics, 1938 New Highway, Farmingdale, NY 11735, and sold under model designation Heat Systems W-225. The ultrasonic processor is fitted with a water jacket receptor suitable for holding a 100 ml beaker. All reagents are from Aldrich.

Synthesis begins by dissolving the first surfactant, AOT, in 40 ml of an organic solvent such as heptane in a 100 ml beaker. Sonication may be used to disperse the solid surfactant. The solution should be clear. The mass of AOT and the volume of heptane may be varied to control the final size of the nanospheres. Higher concentrations of AOT yield smaller micelles. Lower quantities of heptane : AOT increases the amount of polymeric net in each nanosphere, but also causes an excess of the second surfactant in forming the double micelle. The final clean-up will remove the excess surfactant, but yields will be lower. Any of several nonpolar solvents may be used if the solvent has a density less than one, is insoluble in water, and has a boiling point less than 100°C. These requirements are to allow the easy removal of the solvent in a subsequent step. Other surfactants may be used in order to form different sizes of micelles. The concentration must be at least five times the critical micelle concentration or the micelle will not have sufficient structural integrity to withstand the following steps. The surfactant must have branching or the finished nanosphere will leak.

To the AOT micelles is added a concentrated solution of the probe ions, typically 600 μl at 1.25 M. Generally, a saturated solution is used in order to sequester the maximum number of ions per nanosphere. If several different ions are desired, they may be added in the same solution or sequentially, since the subsequent sonication will cause the micelles to collide and mix their water pools. This solution should be sonicated about two minutes to ensure homogeneity. The suspension should be clear.

To this reverse micelle suspension of probe ions is added solid sodium bicarbonate. This is not sonicated, or the solid will disappear into the water pools of the micelles. The purpose of the bicarbonate

Table 3
Typical masses of the components of nanospheres

Common components of nanospheres	mg
Sodium dioctylsulfosuccinate	350
Sodium dodecyl sulfate	562
Citronellic acid	323
2-Hydroxyethylmethacrylate	376
Components of PMMA nanospheres only	mg
Methyl methacrylate	2808
2,3-Dimethyl-1,3-butadiene	545
Components of polystyrene nanospheres only	mg
Styrene	2727
2,3-Dimethyl-1,3-butadiene	254
Divinyl benzene	366
Probes	mg
Saturated aqueous solution of probe ion(s)	100 to 400

is to buffer the water pools almost passively. The pH of a water pool is difficult to ascertain because of the large number of deprotonated organic acids and high ion strength. This small amount of buffering stabilizes the water pools. Also, the pH is near that at which most metal ions and lanthanoids precipitate as hydroxides. Theoretically, this causes the probe ions to associate more closely with the organic acids, making them less likely to diffuse out of the micelles during the subsequent steps.

If a solid probe is desired, the sodium bicarbonate can be replaced by other substances. For example, ammonium hydroxide was added after the water pools were filled with a combination of Fe(II) and Fe(III). This caused them to precipitate into 3 nm ferrite crystals inside of 15 nm polymeric spheres (Fig. 1). Alternately, semiconductor crystals (quantum dots) may be precipitated by adding the cations as the probes and sodium sulfide as the solid. However, solid probes often are too dense to be contained in the reverse micelles, becoming precipitates, leaving the empty micelles in solution. This process is being pursued in separate research.

In a second 100 ml beaker, the SDS is added to 40 ml of water, then sonicated three minutes. This suspension should be clear. The aqueous suspension is then poured into the organic suspension. The combined suspension is milky. This suspension is sonicated 10 minutes, causing the reverse micelles to be engulfed by the normal micelles. After sonication, the organic solvent separates quickly to form a clear top layer, which is evaporated at room temperature.

The aqueous solution is then transferred to a 250 ml three-necked, round-bottomed, glass reaction flask fitted with a gas inlet, a stopper, and a Friedrich condenser, having an output port that is vented by a rubber septum pierced with a needle. Before the aqueous solution is transferred to the flask, essentially all oxygen is purged from the flask and condenser, and is replaced by argon so that residual molecular oxygen does not interfere with the radical polymerization to follow. Argon flow is maintained throughout the polymerization. The reaction flask is immersed in a temperature-monitored, silicon oil bath, and the aqueous solution is stirred so as to create a vortex. Before the aqueous solution is heated, the main monomer, cross-linkers, surface-activating monomer, and surface-functionalizing monomers are added to the flask, and mixed until the solution is homogeneous. The initiator, 72 mg of potassium persulfate dissolved in about 3 ml distilled water, is then added to the flask. The reaction is heated to about 70°C, and maintained between 65°C and 75°C, for about 20 hours. When it reaches about 70°C, polymerization begins. Being an exothermic reaction, initiation will cause the temperature to rise rapidly, so special care

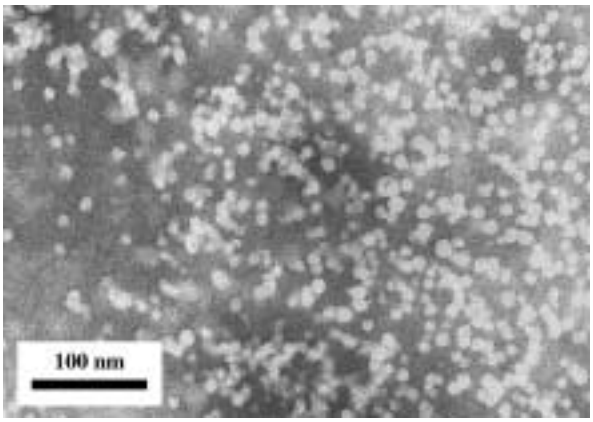

Fig. 1. Ferrite nanospheres (TEM).

must be taken that the heating bath not go higher than 75°C, or many of the micelles will rupture. After 20 hours, the reaction mixture is allowed to cool to room temperature while stirred under argon.

The nanospheres can be collected and cleaned by filtering, dialysis, ion exchange, or centrifugation. Filtration is generally less damaging to the nanospheres; less clumping occurs. Excess small molecules can be removed by dialysis. Excess ions and surfactants can be removed by ion exchange (e.g., AG501-X8 Resin, BioRad). The nanosphere suspension should not be exposed to the ion exchange resin more than 5 minutes, or the SDS molecules of the normal micelle will be extracted to a significant degree, changing the surface character and charge balance. Nanosphere suspensions may be lyophilized and reconstituted with less than 10% loss to agglomeration. Nanospheres with amine groups on the surface tend to oxidize in suspension after about 3 months. Nanospheres without amines have been kept in suspension for more than two years without introducing difficulty. Ionic probes such as lanthanoids have not diffused out to a measurable degree even after two years.

4. Applications

Five unique features suggest that polymeric nanospheres have the potential to act as bridges between proteins and thousands of probes:

1. Since these nanospheres may be stored in dry form, shelf life is very long, on the order of years.
2. Diffusional loss of the encapsulated probe ions is negligible in the physiological pH range.
3. The surface of a nanosphere can be chemically modified (functionalized) to promote attachment to various compounds of biomedical interest.
4. The surface of a nanosphere can be modified to present an infinite variety of characteristics to its environment. The polarity and functionality of the surface may be adjusted easily without affecting the probe-containing function so that the nanosphere may be tailored to avoid non-specific adsorption or uptake, and promote the desired affinity.
5. The toxicity issues of many probes may be avoided by sequestering them inside the nanospheres.

These nanospheres are fundamentally different than other types of antibody conjugates. Many micron-scale spheres are available [5]. Essentially, these are very small solid supports. The antibodies or other proteins that are adsorbed or bound to the surface are no longer able to remain in suspension for long periods. Because the microspheres are much larger than antibodies, the microspheres dictate the disposition of the antibodies. Thus, micron-scale spheres are used primarily for separations. A technique based on magnetic particles for separating antibodies from a slurry in a competitive immunoassay has been marketed [6]. Conversely, nanospheres are used much like enzyme probes. A nanosphere-linked antibody and an enzyme-linked antibody are each significantly larger than the original antibody, but both have good suspension characteristics and mobility.

Polymer-coated functionalized nanoparticles [7] have been reported, but they are on the order of 100 nm in diameter, and have a core of elemental metal. Semiconductor nanoparticles in reverse micelles [8] have been reported, but they are not spherical and are not functionalized.

The subject nanospheres are different from other nanoscale vesicles in that these polymeric nanospheres are hardy and may be prepared with an infinite variety of probes, functionalizations, other surface modifiers, and permeability. The nanospheres are held together structurally by a net of cross-linked polymer strands laced into a double micelle. Thus, the nanospheres can withstand significant physical stress, and the probe ions do not diffuse out. Yet, they are small enough to be enveloped by a

macrophage. The monomer for the polymeric net may be selected from a number of possibilities. Liposomes are not as flexible in the selection of their surface characteristics and have difficulty encapsulating water-soluble probes.

The nanospheres of this report can be conjugated to an antibody, virus, peptide, polyamine, or factor with relative ease. Successful conjugation already has been accomplished with *Rous Sarcoma* virus (*RSV-Praqual l*), *E. coli* 0157:H7, *E. coli* PBR, a host of antibodies, and avidin [9]. The bacterial couplings were accomplished via antibodies that were labeled with the nanospheres. The labeled virus was found to migrate inside chicken tumor cells *in vitro*, presumably via receptor mediated endocytosis. Recent work has demonstrated that nanosphere-labeled goat anti-rabbit IgG antibodies remain in solution and biologically active for extended periods, although at a somewhat reduced level of activity compared to the unlabelled antibody [10]. Since antibodies generally are targeted to the surfaces of tissues rather than the interior, targeting vectors based on dipeptides, polyamines, and viruses can be used as the biological transport mechanism [11].

4.1. Surface functionalization

When these nanospheres were originally synthesized, the first surface modifications were with fluorescene isothiocyanate [12] and Bordeaux Red dye [13]. This allowed a nanosphere suspension to be observed easily without expensive equipment. More importantly, these experiments demonstrated that the nanospheres could be substituted with various compounds after synthesis of the nanospheres. The simplest coupling reaction utilized a carbodiimide to couple an acid on the nanosphere with an amine on the functionalization. Biotin, avidin, protein-A, and a tobacco virus all were conjugated to nanospheres. These experiments showed that a wide variety of compounds can be added to the surface, including ligands for radiopharmaceuticals.

4.2. Multi-analyte immunoassay

Simultaneous assay using fluorescent nanospheres has been reported previously [3]. A numerical method for separating the signals is being developed under an SBIR research contract for real-time monitoring of metals in wastewater [14]. Although immunoassay and wastewater spectroscopies measure photons in very different energy regions, factor analysis and overdetermined matrices allow a complex spectrum to be resolved into its components. The columns of the body of the matrix are composed of the absorption or fluorescence constants (extinction coefficients, fluorescence yields, or mass absorption coefficients, depending on the energy range and type of spectroscopy) for the compounds or elements being analyzed. The rows are the energies or wavelengths of the spectrometer. The rightmost column is calculated from the spectroscopic measurement taken from the sample, along with other system constants. The non-responsive columns are removed via factor analysis, leaving far more rows than columns. This overdetermined matrix may be solved for a best fitting answer along with various statistical descriptors. The Solver function of Excel can solve most such matrices. Excel add-ins are available for a modest investment that will perform factor analysis and handle very large matrices. This form of mathematical treatment allows overlapping fluorescence responses to be resolved in an insignificant period of time. Four resolvable fluorescence responses from different nanospheres have been obtained using the same ligands. Therefore, a single immunoassay may be exposed to four antibodies, each tagged with a different nanosphere, simultaneously. The combined fluorescence may be separated into its component parts in a spreadsheet.

4.3. Boron carriers for BNCT

The success of Boron Neutron Capture Therapy (BNCT) relies on the presence of boron in a specific tissue that will capture thermal neutrons from an applied beam, resulting in radioactive decay and the emission of an alpha particle. The capture cross section of ^{10}B for neutrons is more than three orders of magnitude higher than for other nuclei common to living tissue, so the target region can be dosed with neutrons at a sizeable flux and still have only minimal effect on boron-free regions in the beam path. The potential range in which cell death will occur due to the alpha particle emission is approximately one cell diameter (10 μm) [15]. The accompanying prompt gamma emission is of low energy, so not particularly hazardous. The other by-product of the radioactive decay is lithium, again not presenting any biological or environmental hazard, although the recoil energy from the decay is sufficient to inflict damage within the one cell range. If boron can be transported to the target tissue with sufficient specificity and in sufficient quantity, quite specific cell death may be accomplished. The required boron concentration is generally estimated at 10^9 ^{10}B atoms (natural abundance 19.9%) per cell, which translates to approximately 35 μg ^{10}B per gram of tissue. To prevent damage to healthy tissue in the path of the neutron beam, the surrounding tissue should contain no more than 5 μg/g. Unfortunately, BNCT techniques have not been able to achieve the theoretical possibilities because the boron-transporting mechanisms have not been able to achieve the required threshold values consistently [16]. While several researchers have been able to transport the required number of atoms to the cells, either the tumor/blood ratio was disappointingly low or the boron was entirely on the outside of the tumor cells, requiring a significantly higher concentration in order to achieve the same statistical probability of cell death [17].

In preliminary work to test the hypothesis that nanospheres could be used as effective boron carriers, $B_{12}H_{12}^{2-}$ anions were encapsulated. The nanospheres were synthesized with that cargo, then exposed to both anion and cation exchange resins to remove salts (including residual, unencapsulated $B_{12}H_{12}^{2-}$). Finally, the nanospheres were ruptured and the borohydride oxidized with hydrogen peroxide. Subsequent analysis by ICP by a third party [18] showed that the encapsulation had been successful, with about 2000 boron atoms per nanosphere. The nanospheres have been shown by Transmission Electron Microscopy (TEM) to have a nominal diameter of 30 nm with a narrow ($\pm$10 nm) size distribution (Fig. 2). This would require that 500,000 nanospheres be transported into one cell, occupying a volume of slightly more than 7 μl.

In the same way, $B_{12}I_{12}^{2-}$ can be encapsulated to produce nanospheres that can be imaged by X-ray techniques and be a vehicle for BNCT. This combination strategy will permit confirmation of localization

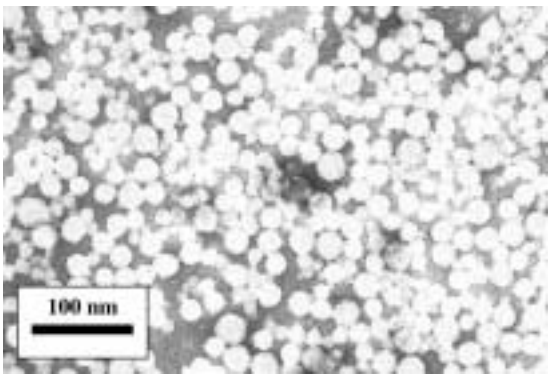

Fig. 2. Boron-containing nanospheres (TEM).

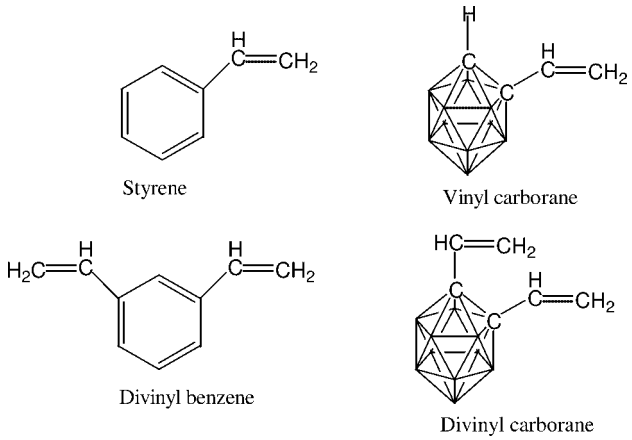

Fig. 3. Boronated monomers proposed for BNCT nanospheres.

of boron via X-ray imaging before application of a neutron beam. If localization fails, the treatment can be aborted and may be cleared by macrophages without release of toxicity or alpha particles.

The quantity of boron per nanosphere could be increased by a factor of 250 by devising new boronated monomers to be used in the nanosphere structure. By mole ratio, the components of a nanosphere may be described as 87% structural polymer, 7% surface functionalizing polymer, 5% surfactant, and 1% cargo. The next stage in this research will be to replace the 87% styrene or methyl methacrylate with the icosahedral $B_{10}C_2H_2$·moiety with one or two vinyl groups and requisite spacers (Fig. 3). The divinyl carborane would replace the present crosslinkers. Sterically, the carborane cage occupies about the same volume as a rotating phenyl group. This would decrease the number of nanospheres per cell required for a reasonable probability of cell death to about 2000.

4.4. Iodine carriers for CAT

Present X-ray imaging agents cause adverse effects in 8% to 10% of patients [19]. These can be avoided if the surfaces of the delivery vehicles are coated with hypoallergenic functionalities. Typical radiopaque solutions are about 30% iodide by mass. The side effects of such imaging agents stem mainly from the large change in osmolality caused by the use of a concentrated solution or emulsion of the imaging agent. Some toxic effects also stem from the nature of the molecules used. Iodinated nanospheres using allyl iodide as the monomer have a weight percent of iodine of 48%. The nanospheres may be suspended at concentrations that would deliver amounts of iodine similar to existing concentrations of X-ray imaging agents without the osmolality and toxicity effects. In addition, by covalently binding a nanosphere to a virus, antibody, or other vector, the iodine may be accumulated in a relatively short period at a target tissue in imagable quantities.

4.5. Fluorine carriers for ^{19}F MRI

Six types of perfluoroalkyl groups already have been included in the polymeric net of nanospheres under an air force STTR [20]. That modification was intended to mimic the action of the perfluorosurfactant used in Aqueous Film Forming Foam, the most popular fire suppressant for aircraft and shipboard fuel fires. However, that same substitution will allow the nanospheres to be tracked by ^{19}F fMRI *in vivo*.

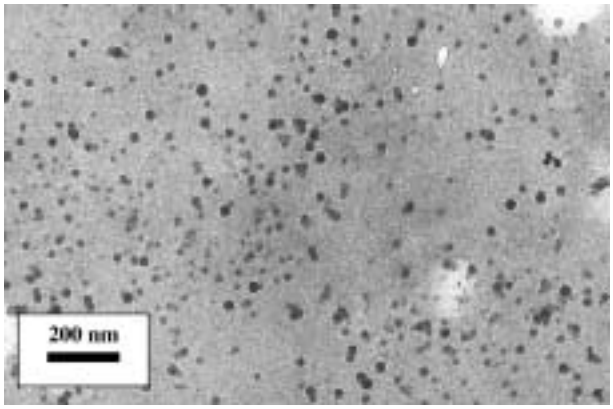

Fig. 4. Nanospheres with 28,000 fluorine atoms incorporated into the polymer net (TEM).

Approximately 28,000 fluorine atoms were incorporated into the polymer of each nanosphere. An example is shown in Fig. 4. The detection limit characteristically assigned to fMRI using a voxel size of 2 mm (8 μl) is between 2 μM and 10 μM [21]. This translates into 1.6×10^{-11} mol to 7.8×10^{-11} mol of fluorine in a voxel. Assuming that a cell diameter is about 10 microns, one voxel would contain about 8,000,000 cells. Therefore, each cell must contain an average of 1,200,000 to 5,870,000 fluorine atoms or 40 to 200 nanospheres. This estimate is improved dramatically by using pentafluorostyrene as the main monomer, incorporating 200,000 fluorine atoms in each nanosphere. Such nanospheres have been synthesized by simply replacing styrene with pentafluorostyrene in the normal synthetic method for nanospheres. An average concentration of nanospheres of between 6 and 28 nanospheres per cell would be imagable.

4.6. Targeted drug carriers

Delivering an encapsulated drug has several advantages over other methods. Some drugs are inherently caustic, inflicting damage to veins near infusion sites. Some drugs have reactive moieties, resulting in oxidation or other disabling reactions before arriving at the target. Some drugs have affinities for non-target regions, resulting in significant portions of the drugs being sequestered in undesired locations. This encapsulation technique avoids all of those disadvantages by separating the drug from the environment. The exterior of the nanosphere can be modified easily to avoid side reactions and undesired affinities.

Under an EPA SBIR grant for multi-analyte, non-extractive immunoassay [1], the nanospheres were modified several ways to control non-specific adsorption in natural matrices. In continuing work begun under that grant, to expand the scope of the resulting successful patent application, the porosity of the nanospheres was controlled such that a chemotherapy drug, 6-mercaptopurine, could be allowed to diffuse out of the nanospheres in a controlled way, including a leak rate of zero. The nanospheres were synthesized with that cargo, then exposed to both anion and cation exchange resins to remove salts. Finally, the nanosphere solution was analyzed by HPLC to determine the rate at which the 6-mercaptopurine was diffusing out of the nanospheres. By varying the combination of cross-linking monomers during synthesis, leakage half-lives from 1 to 3 days were determined. Finally, a leak rate of zero was achieved. The nanospheres have been shown by Transmission Electron Microscopy (TEM) to have a nominal diameter of 20 nm with a narrow ($\pm$5 nm) size distribution (Fig. 5).

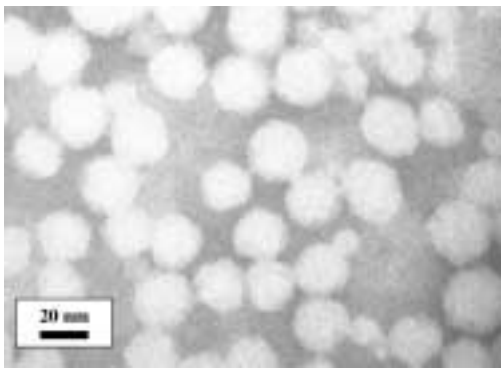

Fig. 5. Nanospheres containing 6-mercaptopurine (TEM).

5. Conclusion

Nanoparticles may be bound covalently to biological macromolecules for imaging, reporting, or delivery of therapeutic agents. The nanospheres of this report range from 15 nm to 30 nm in diameter, depending on the composition, although each type of nanosphere varies only ± 5 nm from its mean. A double micelle forms the core of these nanospheres, in which a water pool contains ionic probes such as fluorescent lanthanides, ferrite, borohydrides for BNCT, insoluble iodides for X-ray imaging, or hydrophilic therapeutic agents. To provide strength to the double micelles, a polymeric net is woven into the hydrophobic region. The surfaces of the nanospheres may be provided with active groups such as acids or amines for later conjugation, and also polar groups such as esters, alcohols, and amino acids to suit the intended environment. The finished nanospheres may be used in mild reaction, such as with a carbodiimide, without harm. The flexibility and simplicity of these nanospheres hold the potential for many biomedical imaging and therapeutic applications.

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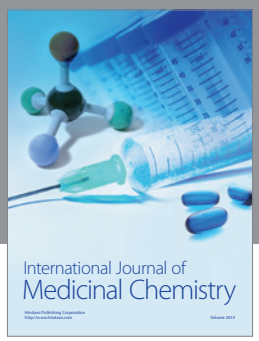

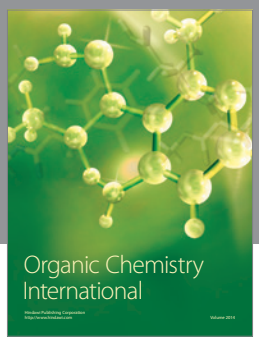

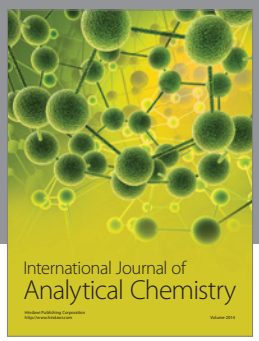

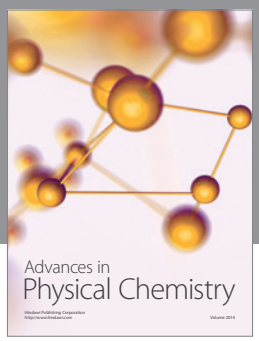

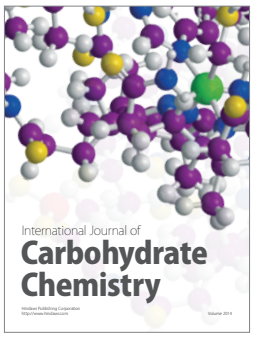

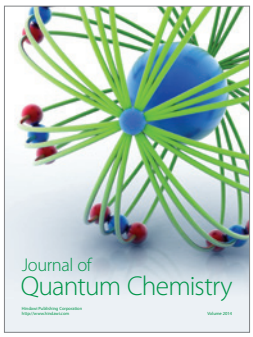

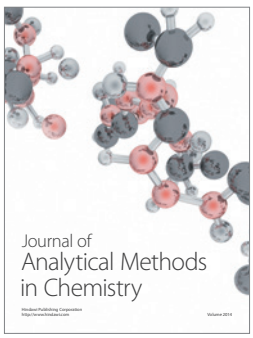

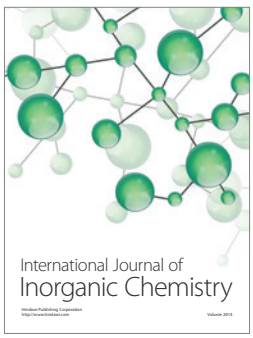

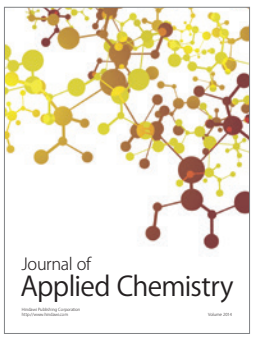

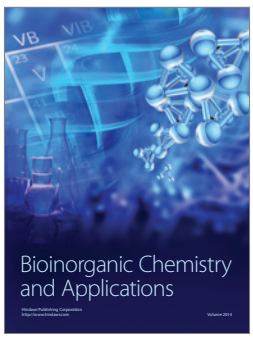

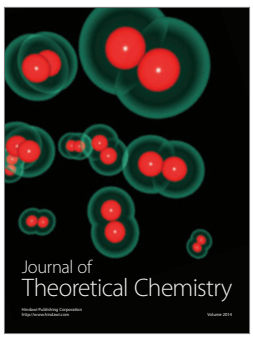

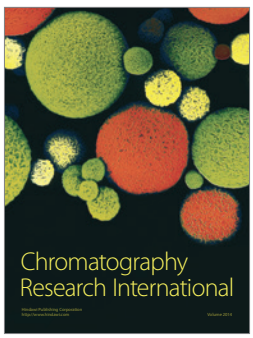

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