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(57) Abstract

The present invention relates to a highly efficient method of preparing microcapsules suitable for encapsulation or surface attachment of therapeutic and diagnostic agents. In one aspect of the invention, surface charge of the polymeric material is altered by conjugation of an amino acid ester to the polymer, providing improved targeting of encapsulated agents to specific tissue cells. Examples include encapsulation of hydrophilic radiodiagnostic agents in 1 μm capsules to provide improved opacification and encapsulation of cytotoxic agents in 100 μm capsules for chemo embolization procedures. The microcapsules are suitable for attachment of a wide range of targeting agents, including antibodies, steroids and drugs, which may be attached to the microcapsule polymer before or after formation of suitably sized microcapsules.

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EFFICIENT MICROCAPSULE PREPARATION AND METHOD OF USE

The invention relates generally to a reproducible, efficient method of preparing nonaggregated microcapsules. The microcapsules are suitable for encapsulation or conjugation with substances useful as diagnostic and therapeutic agents. The invention also relates to amino-acid surface modified microcapsules and microcapsules conjugated with agents having particular potential for drug targeting.

LIST OF ABBREVIATIONS

PLA	poly-(D,L)-lactic acid
PCL	polycaprolactone
PCLD	polycaprolactone diol
5-FU	5-fluorouracil
TX	Tamoxifen
EHEC	ethylhydroxyethyl cellulose
CDDP	cisdiamminedichloroplatinum, (cisplatin)
MAA	macroaggregated albumin
DTPA	diethyltriaminepenta-acetic acid
PBLG	poly-benzyl-L-glutamate

Microencapsulation is a well-studied art. It is basically the use of a matrix or encapsulating material to enclose gases, liquids or solids into particles of relatively small size (0.5-500 μm). The matrix is capsular material selected according to the intended use of the microcapsules.

Physical properties of encapsulated chemical entities may be modified because of the encapsulation. Other effects of encapsulation include dispersion of one

substance within another, stabilization of emulsions and alteration of solubility rate. One of the most useful properties of encapsulated therapeutic materials is controlled release (1,2).

Microcapsules have been prepared by many methods, including coacervation, interfacial polymerization, mechanical methods, polymer dispersion and matrix encapsulation. Sustained release microcapsules have been prepared from ethylcellulose (3) and poly-(D,L)-lactide (4). There is voluminous literature on the preparation and use of encapsulating polymers designed for sustained drug release (5,6).

Although many preparations of microencapsulated compounds have been reported, few describe microparticles in the size range below 10 μm. Particles of 1-250 μm are typically prepared by a solvent evaporation technique (7) while sizes from 1-10 μm have been made by emulsion deposition (8). One method using solvent evaporation claims to provide a range of sizes from 0.5 - 250 μm (9). Nevertheless, none of these methods appears to provide a homogeneous preparation of single-particle, nonaggregated microcapsules. Typical of these preparations is a claim to aggregate having an overall size of about 177 to 395 μm with 5-162 μm particles making up the aggregates (10). This technique requires sieving to remove larger agglomerates, leaving behind a wide range of particle sizes which, although composed of small spheres, are nevertheless in aggregated form.

Discrete microprills, polymeric particles in which a drug (for example, Mellarib™) is uniformly dispersed, have been disclosed (11). Although the microprills were reported to be nonaggregated, the average size range was 10-50 μm.

Lack of particle size homogeneity may cause severe problems in quality control and in clinical use. For example, in chemoembolization studies, the particle diameter is fairly critical in that only a limited range of sizes will lodge in a target area (12). If too large, damage to larger vessels may occur, while if too small, the particles pass through and drug is not released at the targeted site. Thus a homogeneous particle preparation is important.

Despite the proliferation of microencapsulation methods, there is a particular need for simple and efficient methods of producing homogeneous preparations of microencapsulated agents for clinical treatment and diagnosis, most particularly in small, nonaggregated particles ranging from 0.5 to 500 μm. A method of preparing encapsulated therapeutic agents in 1 μm and 100 μm particles would provide more effective agents, particularly for diagnostic imaging and chemoembolization.

Bioimaging agents microencapsulated in 1 μm particles would provide an ideal size particle for bioimaging studies, particularly if combined with capsular material selected to concentrate in the organ of interest. Additionally, the use of microencapsulation materials capable of targeting particular areas *in vivo* would enable improvements in biodistribution imaging studies as well as in drug delivery to specific organs.

The present invention is a highly efficient, reproducible method of obtaining homogeneous nonaggregated preparations of polymeric microcapsules in which therapeutic or diagnostic agents may be encapsulated or conjugated. The invention also includes microcapsules prepared from polymers conjugated to an

amino acid, enabling improved targeting of drug-laden microcapsules to a particular target organ or cell. The invention illustrates two important size ranges, 1 and 100 μm, of polymeric particles useful in clinical studies and in which imaging or therapeutic agents may be efficiently encapsulated.

An important aspect of the invention is the preparation of homogenous nonaggregated microcapsules having a diameter of approximately 1 μm. These microcapsules are prepared by combining a solution which may contain a drug or therapeutic agent, a nontoxic emulsifier and polymer dissolved in convenient solvent, and then vigorously agitating the mixture. Agitation is performed for a period of time sufficient for the development of microcapsules having a mean diameter below 5 μm. The formation of the microcapsules is monitored periodically, after which the organic solvent is removed and the microcapsules collected.

The nontoxic emulsifier may be selected from several commonly used emulsifiers, for example Tween-80, polyvinyl alcohol, sodium laurylsulfate, Span 20, Lubrol, Triton™ X-100, cetylpyridinium chloride and the like. Thus a wide variety of emulsifiers may be suitable, including anionic, cationic, and non-ionic types.

Likewise, a wide variety of materials may be used for the preparation of the capsules, including nonpolymers such as cholesterol, diglycerol, ethyl cellulose as well as numerous types of polymers. Microcapsules particularly useful for clinical or therapeutic purposes release their contents by erosion, degradation or diffusion. This is not to say that microcapsule polymers used for medical treatment must be biodegradable. For example, relatively permanent

implantable drug-containing polymers (*e.g.*, hydrogels) might be used for long-term sustained release in certain applications. Polymers particularly suitable for microencapsulation include poly-(D,L)-lactic acid, ethylhydroxyethyl cellulose, polycaprolactone, polycaprolactone diol, polylysine, polyglycolic acid, poly-benzyl-L-glutamic acid, polymaleic acid and the like.

Generally speaking, the emulsifier is soluble in water, while the polymer, typically water insoluble, is dissolved in an appropriate organic solvent. Water immiscible or miscible organic solvents may be used, depending on the nature of the polymer. Examples of solvents include, but are not limited to, acetone, water, ethyl acetate, chloroform, carbon tetrachloride and methylene chloride.

An important step in the preparation of nonaggregated microcapsules less than 5 μm in diameter is the vigorous agitation of the mixture containing polymer, emulsifier and, when desired, a diagnostic or therapeutic agent. Agitation may be carried out by stirring, sonication, or a combination of agitation methods. If stirring alone is used, a speed of approximately 1500 rpms is preferred; however, where 1 μm preparations are desired, it is preferable to use sonication alone or in combination with stirring. If both stirring and sonication are used, sonication at approximately 20 Khz and stirring at 500 rpms are preferred settings. Sonication and stirring are most preferably used simultaneously. Agitation is continued for a period of time sufficient to form individual microcapsules with an average size less than 5 μm, typically at least 5 min and more preferably 10 minutes. Under the general conditions described, somewhat longer periods of time may be

required depending on the polymer, the organic solvent used, the volume and concentration of starting material as well as pH and temperature. Microcapsule formation is typically monitored by periodically examining size and shape of the microcapsules as they form in solution. This step is particularly useful when optimizing time and agitating conditions to assure homogeneous preparations in the desired size range. Any method that detects size and shape of the capsules may be used, for example, removal of a drop of the solution and inspection under a light microscope at a magnification of approximately 600 fold.

After the microcapsules have formed, the organic solvent is removed from the mixture. A convenient method, particularly for lower boiling organic solvents, is to stir the reaction mixture at relatively slow rpms, for example about 350 rpm, for a period of several hours until the solvent is completely evaporated. The length of time depends on the type and volume of solvent in addition to other factors related to physical properties. For example, the solvent acetone require about six hours for complete evaporation. Other solvents with lower vapor pressure/higher boiling points may require longer periods of time. Evaporation, in this process, occurs at room temperature, but higher temperatures may be applied when different solvents are used. Monitoring of capsule size and shape continues to be important throughout the evaporation phase to assure that aggregation does not occur.

The microcapsules are collected, after complete evaporation of the organic solvent, preferably by filtration, for example, by filtration through a nylon mesh or other suitable filter that allows smaller particles to pass through while retaining the larger

particles. The resulting suspension containing 1 μm microcapsules may then be further processed to isolate and store or use the particles. This is conveniently accomplished by centrifuging the suspension after which any residual organic solvent or emulsifier can be removed by washing either with water or sterile saline. The aqueous layer may then be decanted and the microcapsules resuspended in a liquid for storage or for therapeutic use. When used therapeutically, phosphate buffered saline, pH 7.4 is a most preferred resuspension medium. This method has provided a high yield (99%) of nonaggregated 1 μm particles. The amount of material collected in the nylon sieve is rarely over 1%, and the microparticles prepared by this method are remarkably uniform with a narrow size distribution ranging from 0.5 to 5.0 μm with the highest percentage being approximately 1.0 μm.

These microcapsules may be used to enhance or modify properties of diagnostic or therapeutic agents by virtue of the encapsulation. For example, in order to alter biodistribution properties, an ionic radiographic contrast agent may be encapsulated in a nonionic coat using this microencapsulation process. In the first step of preparing an encapsulated drug, the diagnostic or therapeutic agent is added to a mixture containing an aqueous solution, an emulsifier, and a polymer dissolved in solvent. During microparticle formation, the drug is encapsulated. The yield depends on the material being encapsulated. For example, 1 μm and 100 μm capsules of meglumine diatrizoate have relatively high efficiencies of encapsulation of 66% and 46% by weight respectively. Therapeutic agents (cisplatin, 5-fluorouracil and Tamoxifen) and diagnostic agents (Ethiodol, Iohexol, diatrizoate and Hexabrix) have also been incorporated into 100 μm capsules. Encapsulation is not intended to

be limited to these particular drugs and it is envisioned that most therapeutic and diagnostic agents, whether water soluble or insoluble, could be encapsulated by this simple method.

Those skilled in the art will appreciate that this method of encapsulation of therapeutic or diagnostic agents will result in an entrapment of the material, which will be released from the microcapsule at different rates depending on the relative amount of polymer to amount of drug encapsulated. Other factors affecting the rate of release are the chemistry of the compound being encapsulated, the environment into which the microcapsule is being placed, temperature of the environment and the nature or chemical composition of the capsular material. The rate of release of drug will also be determined by the relative ratios of drug to polymer, the type of polymer, and the biodegradability of the polymer.

One μm microencapsulated imaging agents are ideal for diagnostic imaging procedures and are readily prepared by the method of the invention. First, a homogeneous nonaggregated preparation of a 1 μm microencapsulated imaging agent is prepared as previously described. The material may be any standard imaging agent, for example, an iodinated compound such as meglumine diatrizoate. The microencapsulated imaging agent can then be administered to an animal or human, preferably by intra-arterial or intravenous injection. The imaging agent is then detected by appropriate means such as computed tomography or intravenous urography.

The general method used for the preparation of 1 μm microcapsules can also be used to make microcapsules of somewhat larger sizes, for example, 100 μm. Non-aggregated microcapsules having a mean diameter of 100 μm

can be prepared by combining a polymer in a solvent with a solution of a nontoxic emulsifier. The mixture is emulsified by stirring at low speed, approximately 350 rpm, while monitoring microcapsule formation. The solvent is then evaporated and the microcapsules collected.

One difference between the procedure for preparing 100 μm microcapsules and preparing 1 μm microcapsules is stirring the mixture of the polymer and the emulsifier at a relatively lower speed when the larger particles are desired. The stirring speed is approximately 350-400 rpm. During the stirring process, the size and shape of the particles in the mixture are monitored, for example, by using a light microscope at approximately 125x magnification. After the desired size range of microcapsules has formed, the organic solvent is removed, preferably by evaporation and simultaneous stirring at room temperature. After collection and drying, the microcapsules are preferably sized. This may be accomplished by passing the particles through various sized filters, for example, first 600 μm mesh, then 600-500 μm mesh, then 500-355 μm mesh, then 355-212 μm mesh, and finally 106 μm mesh. The sieved particles yield a mixture containing size ranges of approximately 106-212 μm. Use of these mesh sizes is for illustration purposes only and, of course, any series of that same general size mesh could be used. In the final step the 106-212 μm particle mixture is sieved through a 106 μm mesh sieve and the particles that pass through the sieve are discarded. This provides a relatively uniform preparation. Using this method, consistently reproducible yields of approximately 70% of particle sizes in the size range of 100-200 μm have been obtained.

In preparing 100 μm diameter particles, any of a number of polymers may be used, including biodegradable polymers such as poly-(D,L)-lactic acid, ethylhydroxyethyl cellulose, polycaprolactone, polycaprolactone diol, polylysine, polyglycolic acid or polymaleic acid.

In the initial step of preparing 100 μm microcapsules, a selected polymer is first dissolved in an organic solvent then mixed with emulsifier. The solvents may include methylene chloride, chloroform, carbon tetrachloride, or other solvent in which the polymer is soluble. The emulsifier may be selected from any of a group of nonionic, cationic, or anionic emulsifiers. A nontoxic emulsifier is preferably chosen when the disclosed methods of microcapsule preparation are used to encapsulate therapeutic or diagnostic agents for *in vivo* use. The selected emulsifiers are preferably solubilized in saline, although water or buffers may be used. Hydrophobic or hydrophilic therapeutic or diagnostic agents may be microencapsulated in the 100-200 μm particles. These compounds generally release slowly from the microcapsules and the rate of release will depend on the nature of the compound encapsulated, as well as the type of polymeric material used for the microcapsule.

It has been found that the 100-200 μm microcapsules are ideal for chemoembolization. When chemoembolization is desired, a drug encapsulated in a biodegradable or nondegradable polymer is prepared. The microcapsule generally has a diameter of about 100 μm which is somewhat larger than the diameter of the tumor vessels in the targeted organ. Encapsulated material is administered intra-arterially causing occlusion of the arteries. By occluding the arterial supply to neoplasms

with 100 μm capsules, the ischemia results in death of the tumor cells. The rate of release will depend on the nature of the material used to prepare the microcapsules. Slow release over hours or weeks allows greater contact time between the cytotoxic agents and tumor cell in an anoxic environment which also increases capillary permeability.

Examples of microencapsulated drugs useful for chemoembolization are cisplatin, 5-fluorouracil and Tamoxifen. In one particular example, cisplatin was microencapsulated and administered into canine renal arteries. Poly-(D,L)-lactide capsules and ethylhydroxyethyl cellulose capsules, both loaded with cisplatin, exhibited sustained cisplatin release for at least several days. The resulting tissue destruction was significantly greater than that with blank capsules. These sustained-release effects should be similar for other drugs and other similar polymers.

Normally, drugs or other agents administered to an animal or human will initially disperse through the body before concentrating in the liver, spleen, kidneys and urinary bladder prior to elimination. It was a surprising discovery that amino acid ester conjugation to polymers affects the character of the capsule causing distribution and uptake of the encapsulated imaging material in an animal to be altered. In a particular example, phenylalanine-conjugated polylactic acid was used to encapsulate meglumine diatrizoate. An animal injected with phenylalanine ester-conjugated encapsulated diatrizoate showed faster liver uptake than animals injected with nonconjugated polymer capsules. In the former case, imaging was possible as early as sixty minutes after injection. Two hours post injection, the nonconjugated microencapsulated material showed both

liver and kidney uptake as well as presence in the systemic circulation. The conjugated microencapsulated material was concentrated mainly in the liver and showed little in the general circulation at two hours post-injection. Both non-conjugated and amino acid-conjugated poly-(D,L)-lactide microencapsulated diatrizoate permitted computed tomography imaging up to three days after administration. Neither material was seen in the liver five days post-administration. *In vitro* mouse liver cell culture studies revealed that the conjugated microcapsules were mainly taken up by hepatocytes whereas the nonconjugated microcapsules were taken up by Kupffer cells. Other amino acids conjugated with polymeric encapsulating material also would be expected to show selective targeting of encapsulated drugs.

Although amino acid conjugated polymers were formulated using phenylalanine ester, other amino acids such as tyrosine, tryptophan, methionine and the like are also contemplated to be useful. A selected amino acid may be conjugated to a polymeric material via an amide bond to link phenylalanine with polylactic acid. This is conveniently performed by carbodiimide coupling procedures well known to those experienced in the art; for example, by reacting with dicyclohexylcarbodiimide in the presence of hydroxysuccinimide. Covalent bonds need not be limited to linkages involving the primary amine of the amino acid but might, for example, utilize a sulfur-carbon bond between a sulfhydryl-containing amino acid and the polymer. Furthermore, depending on the nature of the functional groups on the polymer, other types of linkages could be formed, for example, ether linkages. The use of other conjugates is also envisioned; for example, sugars, amino acids or derivatives of these compounds could also be used to surface-modify a microcapsule polymer.

Surface properties of 100 μm microcapsules may be modified in the same manner as surface properties of the 1 μm microcapsules by conjugating with various amino acids or other surface-modifying materials. In the case of chemoembolization studies, surface modification would likely be important. These particles could be delivered intra-arterially to the organ of interest.

Yet another aspect of the invention relates to microcapsules modified by attachment of selected targeting agents. Attachment is typically covalent and the nature of the chemical bond depends on the particular polymer used to prepare the microcapsule. For example, selected agents with amine functionalities may be reacted with carboxyl moieties on a selected polymer using coupling methods well-known to those of skill in the art. Other modifications include for example, creation of "spacers" on either the target molecule or groups on the microcapsule polymer, although such spacer groups are not necessarily required. In a preferred embodiment, poly benzyl-L-glutamic acid polymer is conjugated with estrone, an estrogen-receptor targeting compound. The conjugated material may then be used for tumor targeting or imaging studies in organs high in estrogen receptors. Such surface modification of microcapsules significantly alters tissue distribution, as shown in the higher uterus-to-muscle ratios achieved with estrone conjugated ^{131}I-labeled microcapsules compared with the labeled microcapsules alone.

A preferred polymer for conjugation of targeting agents is poly-benzyl-L-glutamic acid. When desired agents are attached to the polymer, an important consideration is the percent of targeting material in the conjugated product. While a high amount may appear desirable, it has been found that substitution is

preferably limited to a degree that will permit solubility in a solvent suitable for microcapsule preparation by the disclosed solvent evaporation method. This amount will vary with the nature of the polymer used and with the attached agent. As an example, 12% estrone content in estrone conjugated poly-benzyl-L-glutamic acid microcapsules exhibited good targeting properties while yielding a homogeneous, nonaggregated 1 μm preparation of microcapsules. At high concentrations, an attached targeting agent may adversely affect microcapsule formation.

Generally, it is contemplated that targeting agents will be first conjugated with a selected polymer, then formed into microcapsules. This will not be feasible for some types of targeting agents, such as antibodies or other compounds that might be altered during microcapsule preparation. Such species could be conjugated to surface groups of polymeric material in already formed microcapsules.

While conjugation with estrone has been used to demonstrate the targeting properties of conjugated microcapsules prepared in accordance with the invention, it will be appreciated that enhanced targeting is associated with the microcapsules themselves. Thus, in general, the various polymeric microcapsules disclosed may be surface-conjugated with a wide variety of desirable targeting agents including, but not limited to, steroids, antibodies, particularly monoclonal antibodies or epitopic segments or fragments of antibodies, ricin A conjugated compounds, specific targeting drugs such as Tamoxifin, and the like. Further modifications could be made by attaching targeting agents to microcapsules modified with amino acid groups in accordance with preparations herein disclosed.

Figure 1 shows the *in vitro* profile of Tamoxifen from Tamoxifen microcapsules with Tamoxifen:polymer ratios of 1:1. A statistically significant difference from the corresponding sample after 1 hr of incubation ($p<0.05$ by Student t-test) was determined. Each bar represents the mean ± standard deviation of three samples.

Figure 2 shows the *in vitro* release rate profile of Tamoxifen from Tamoxifen microcapsules with Tamoxifen:polymer ratios of 1:3. A statistically significant difference from the corresponding sample after 1 hr of incubation ($p< 0.05$, Student t-test) was determined. Each bar represents the mean ± standard deviation of three samples.

Figure 3 shows the *in vitro* release rate profile of 5-fluorouracil from 5-fluorouracil microcapsules with 5-fluorouracil:polymer ratios of 1:1. A statistically significant difference from the corresponding sample after 1 hr of incubation time ($p< 0.05$, Student t-test) was determined. Each bar represents the mean ± standard deviation of three samples.

Figure 4 shows the *in vitro* release rate profile of 5-fluorouracil microcapsules with 5-fluorouracil ratios of 1:3. A statistically significant difference from the corresponding sample after 1 hr of incubation ($p< 0.05$, Student t-test) was determined. Each bar represents the mean ± standard deviation of three samples.

Figure 5 is a scanning electron micrograph of PLA microcapsules loaded with Tamoxifen with TX:PLA ratios of 1:1.

Figure 6 is a scanning electron micrograph of PCL microcapsules loaded with 5-fluorouracil:PCL ratios of 1:1.

Figure 7 is a scanning electron micrograph of 1 μm PLA microcapsules (7B) and PLA microcapsules encapsulating meglumine diatrizoate (7A) Drug to polymer ratios were 3:1.

Figure 8 is a microcapsule size distribution curve with data taken from Coulter Counter measurements. Polylactic acid microcapsules were loaded with meglumine diatrizoate.

Figure 9 is a microcapsule size distribution curve prepared from data obtained from Coulter Counter measurements. The mean particle size for the PLA-PHE microcapsules loaded with diatrizoic acid is 3 μm with a range from 2-7 μm.

Figure 10 is a normal mouse hepatocyte culture shown under 40x magnification. All plates were seeded with aliquots from the same cell suspension. 10A shows control (no capsules) hepatocytes; 10B shows hepatocytes incubated for two hours with meglumine diatrizoate-loaded 1 μm polylactide capsules; figure 10C shows hepatocytes incubated for two hours with meglumine diatrizoate-loaded 1 μm phenylalanine ester-conjugated polylactide capsules.

Figure 11 is a normal mouse Kupffer cell culture shown under 40x magnification. All plates were seeded with aliquots from the same cell suspension. 11A shows control hepatocytes; 11B shows Kupffer cells after incubation for two hours with 1 μm polylactide capsules; 11C shows Kupffer after incubation for two hours with 1 μm phenylalanine ester-conjugated polylactide capsules.

Figure 12 shows a computerized tomographic images of two rabbits after intravenous injection with microencapsulated meglumine diatrizoate. A-F show distribution of 1 μm polylactide capsules loaded with meglumine diatrizoate before (A) and immediately post-injection (B), 1 hr. (C), 2 hr. (D), 57 hr. (E), and 120 hr. (F). G-L show distribution of the 1 μm phenylalanine-conjugated polylactide capsules loaded with meglumine diatrizoate before (G) and immediately post-injection (H), 1 hr. (I), 2 hr. (J), 57 hr. (K), and 120 hr. (L).

Figure 13 shows the release of CDDP from 100 μm polylactide capsules as measured in jugular and renal vein plasma in dogs at selected times over a period of 6 hours. The drug was administered intra-arterially into the renal artery.

Figure 14 shows the release of CDDP from 100 μm EHEC capsules as measured in jugular and renal vein plasma from dogs at selected times over a period of 6 hours. The drug was administered intra-arterially into the renal artery.

Figure 15 schmetically illustrates the coupling reaction between estrone and poly-benzyl-L-glutamic acid.

Figure 16 shows an estrogen receptor assay; 16A is a Scatchard plot; 16B shows the saturation curve for estrogen receptor binding in rat uteri.

The invention is a method of preparing micro-encapsulated therapeutic and diagnostic agents in discrete nonaggregated particles suitable for diagnostic radiologic studies and therapeutic use in humans. In particular, the method relates to preparing 1 μm

particles for intravenous and intra-arterial administration as well as 100 μm particles for intra-arterial use. In one aspect of the invention, cells in the body are specifically targeted with drugs microencapsulated in polymeric material whose surface properties are modified by conjugation with an amino acid. The microcapsules may also be conjugated with targeting agents which bind to specific body cell receptors, including steroids, antibodies and the like.

The following examples are intended to illustrate specific embodiments of the present invention. Those skilled in this field will recognize that modifications could be made to the disclosed methods and that other applications would remain within the scope of the present invention.

EXAMPLE 1

100 Micron Microcapsule Preparation of Microencapsulated Meglumine Diatrizoate

Meglumine diatrizoate, 2 g, was dispersed in 40 ml methylene chloride and 1 g poly-(D,L)-lactic acid added to the mixture. Encapsulation was achieved while stirring at 350 rpm in 250 ml 0.9% (w/v) saline solution containing 1.25 g polyvinyl alcohol. The pH of the solution was adjusted below 4 with 1 N HCl. From time to time, formation of microcapsules was determined by examining a drop of the material at 125x magnification under a light microscope. The mixture was stirred for approximately 6 hr until the methylene chloride was completely evaporated. The microcapsules were collected by filtration and washed with distilled water (2 x 100 ml). The microcapsules were air dried at room temperature and then sieved through various meshes,

including stepwise, 600 μm mesh, 600-500 μm mesh, 500-355 μm mesh, 355-212 μm mesh and 106 μm mesh, to give a mixture containing particles of size range 106-212 μm. The weight of the 106-212 μm particles was approximately 70% of the initial total amount of the contrast agent plus polymer. The microcapsules contained 46% (w/w) of meglumine diatrizoate.

EXAMPLE 2

1 Micron Microcapsule Preparation of Microencapsulated Meglumine Diatrizoate

All the following steps were done under aseptic conditions using ultraviolet light with sterile instrumentation.

Meglumine diatrizoate, 1.2 g (Sigma Chemical Company, St Louis, MO), was dissolved in 100 ml water and then 1 ml of Tween 80 was added. The mixture was stirred at 500 rpm and the pH of the solution adjusted below 4 with 1 N HCl. To this mixture was added dropwise 0.5 g poly-(D,L)-lactic acid (MW 30,000-60,000) dissolved in 10 ml acetone. The mixture was stirred at 1500 rpm or sonicated at 20 Khz for 10 min and periodically monitored under a light microscope at 600x magnification until round particles of approximately 1 μm in diameter were observed. The mixture was stirred at 1500 rpm (without sonication) or 500 rpm (with sonication) for an additional 6 hr or until the acetone was completely evaporated. The microcapsules were collected by sieving through a nylon mesh to remove a small amount of aggregated material, approximately 1%. The microcapsule suspension was centrifuged at 24,000 x g and washed 3 times with saline to remove the emulsifier. The microcapsules were resuspended in sterile phosphate

buffered saline. The microcapsules weighed 1.5 g (90% by total initial weight of contrast plus polymer). The microcapsules contained 66% by weight of meglumine diatrizoate. The particles were cultured and found to be sterile. Scanning electron microscopy (SEM) revealed round, uniform particles as shown in Figure 7. The distribution of particles was determined using a Coulter counter, indicating a narrow range of 2-7 μm with 50% having a mean capsular size less than 5 μm, as indicated in Figure 8.

EXAMPLE 3

Conjugation of Amino Acid Ester to Polylactic Acid

To a solution of 2.0 g (0.05 mmol) poly-(D,L)-lactic acid in 10 ml dimethylformamide (DMF) was added 1.2 g (5.5 mmol) of dicyclohexylcarbodiimide and 0.68 g (5.5 mmol) of N-hydroxysuccinimide. After stirring 10 min, 1.2 g (5 mmol) phenylalanine ester dissolved in 5 ml DMF was added. The mixture was stirred overnight. The solid urea was filtered. The filtrate was poured into 100 ml water and the white solid precipitated. The solid was filtered, washed with 100 ml water, air dried and weighed to obtain 2.4 g (75%) of the total chemical yield. Thin layer chromatography indicated a single spot (Rf= 0.3, chloroform/methanol 9:1). The phenylalanine content in the polymer conjugate was 23% as determined by ultraviolet spectroscopy at 254 nm. Similar conditions were used to prepare microcapsules of polylactic acid conjugated with methionine, tyrosine or tryptophan ester.

EXAMPLE 4

Chemoembolization with Microencapsulated Cisplatin

Eighteen adult mongrel dogs were anesthetized with intravenous sodium pentobarbital (Nembutal; Abbott, North Chicago, IL), 30 mg/kg, and an intravenous drip of normal saline was initiated. Through a cutdown, a 5-F polyethylene catheter was introduced into the femoral artery, and the animal was given an intra-arterial bolus of sodium heparin (100 units/kg). The catheter was then advanced into one of the renal arteries. The ipsilateral renal vein was also catheterized via a femoral vein with a 5-F catheter to sample blood for cisplatin (CDDP), while simultaneous systemic venous blood samples were collected through an 18-gauge Cathlon™ catheter inserted in a jugular vein.

Microcapsules with an average size of 106 μm (range 50-350 μm) and containing cisplatin (40-43%) by weight, were formulated as described in Example 1 from lactic acid polymer and ethylhydroxyethyl cellulose polymer. The capsules, in dry form, were sterilized with ethylene oxide. The microcapsules were suspended in a 1:1 solution of radiographic contrast material. Iohexol (Omnipaque, Nycombed, Norway) and normal saline such that the final concentration was 20 mg/ml. The suspension was administered into the renal artery until stasis of flow was observed fluoroscopically. One kidney was embolized in each of three animals with each of the capsular materials containing CDDP, and one kidney from each of five dogs was occluded with each of the capsular materials without CDDP. Renal and systemic venous blood samples were collected in heparinized tubes at 30-minute intervals for 6 hours after embolization. The plasma was analyzed for CDDP using atomic absorption. Drug release

curves were generated from these data. Two such curves are shown in Figures 13 and 14. To evaluate renal and hepatotoxicity, systemic venous blood samples were collected before and at 1, 2, 3, 4, and 6 weeks after embolization to determine blood urea nitrogen (BUN), creatinine, and serum glutamic oxaloacetic transaminase (SGPT) levels.

Renal angiography was performed with Omnipaque before and immediately after embolization, at hourly intervals up to 6 hours after embolization, and 1, 2, 4 and 6 weeks later to document the radiographic changes in the occluded kidneys. After 6 weeks, each animal was killed with an overdose of sodium pentobarbital, and a complete necropsy performed. The gross and microscopic findings in each dog were compared.

Both PLA and EHEC capsules without encapsulated drug produced embolic effects in the kidneys. The polymers loaded with cisplatin damaged kidneys significantly more than polymers alone. PLA capsules loaded with cisplatin had a greater effect on tissue than cisplatin-loaded EHEC capsules. EHEC capsules without CDDP showed slightly more degradation than PLA capsules in these studies.

In vitro drug release data were also determined by incubation of the microcapsules in phosphate buffered saline. The data are shown in Table 1 for release of CDDP from CDDP:PLA microcapsules.

TABLE 1

RELEASE RATE OF CDDP FROM CDDP MICROCAPSULES[1]
(SIZE 100 μm)

Incubation Time (min)	% Released
1	11.6
5	21.3
15	27.4
30	39.5
60	37.7
120	35.0
240	40.4

[1] CDDP:PLA = 1:1

EXAMPLE 5

Bioimaging with Microencapsulated Diatrizoate

1 μm microcapsules loaded with meglumine diatrizoate were prepared as described in Examples 2 and 3 using PLA and PLA conjugated with phenylalanine (PLA-PHE) as the capsular material. Each preparation was injected intravenously into a rabbit and thereafter monitored by computed tomography for organ uptake. The rabbit receiving PLA-PHE showed a faster liver uptake than the rabbit receiving PLA encapsulated diatrizoate. After 2 hr, the PLA-PHE treated rabbit showed liver uptake and little, if any, contrast in the general circulation while the PLA treated rabbit showed both liver uptake and presence in the general circulation. After 48 and 72 hr,

both rabbits showed significant liver uptake. The mean particle size of the PLA-PHE microcapsules loaded with meglumine diatrizoate was determined to be 3 μm, as indicated from a particle size distribution curve obtained using a Coulter Counter, Figure 9.

EXAMPLE 6

In Vitro Release Rates of 100 μm Microcapsules

Microcapsules were prepared as described in Example 1 using the solvent evaporation method with drug:polymer ratios of 1:1 and 1:3 (w/w) and polyvinyl alcohol as emulsifier. The biodegradable polymers used were PCL, PCLD and PLA. The cytotoxic compounds Tamoxifen and 5-fluorouracil were dissolved in methylene chloride, then added with the emulsifier to a water solution with stirring at 400 rpm. After 6 hr, the capsules were washed with water and air dried. Capsules of approximately 100 μm were collected from mesh screens. Assays on the encapsulated drugs were performed by dissolving 5 mg of the microcapsules in 5 ml methanol. The solution was centrifuged and 100 μl of the supernatant diluted with 3 ml methanol and analyzed spectrophotometrically at 238 nm. A standard solubility time curve was produced using the same procedure by adding 2 mg of both TX and 5-FU. The drug content was calculated as a percent of total capsule weight. Triplicate determinations were made.

Dissolution studies were performed on the microencapsulated drugs. Capped test tubes were filled with 5 ml of 0.05 M phosphate buffered saline pH 7.4 and placed in a water bath shaker set at 100 rpm at 37°C. 5 mg of microcapsules were added to each test tube, and sample solutions of 3 ml were collected at different time

intervals after centrifugation. After each determination, the sample solutions were returned to each test tube. The concentrations of the drug released from microcapsules were determined by comparison with the standard drug (2 mg) in the same dissolution solution for the controls and measured spectrophotometrically at 238 nm. Determinations were made in triplicate. A Student's t-test (13) was used to compare the sample after 1 hr of incubation and the corresponding sample at different incubation time intervals ($p < 0.05$ level).

The percent of drug content in the various biodegradable microcapsules is shown in Table 2 below. Scanning electron microscopy showed that all the microcapsules prepared were spherically shaped with smooth outer surfaces (Figures 5 and 6).

TABLE 2

% (W/W) DRUG IN MICROCAPSULES

DRUG	POLYMER	DRUG:POLYMER 1:1	1:3
Tamoxifen	PLA	30.0	22.5
	PCL	30.7	13.0
	PCLD	36.4	14.9
5-fluorouracil	PLA	8.8	8.5
	PCL	9.9	6.6
	PCLD	7.6	7.6

Release rate of TX and 5-FU is shown in Figures 1 and 2. The release rate of TX (1:1 ratio) at 48 hr incubation time decreased in the order: PLA>PCL>PCLD; however, the release rate of 5-FU (1:1 and 1:3 ratios) at 48 hr incubation showed PCL>PCLD>PLA. This study indicates that different polymers alter drug release rate.

EXAMPLE 7

The following example illustrates modification of the phenolic group of estrone to enable coupling with poly-benzyl-L-glutamate. The product illustrates a "spacer" between the estrone 3-position functionality and the conjugating amide bond.

Preparation of 3-Aminoethyl Estrone

Estrone (5.0 g, 18.5 mmole) was dissolved in 80 mL of anhydrous DMF. Sodium hydride (4.4 g, 185 mmole) was slowly added to the solution to generate reactive phenoxide *in situ*. Care was taken to avoid rapid evolution of hydrogen gas. Chloroethylamine (4.3 g, 55 mmole) was added to the solution and the mixture was allowed to react at 60°C for 4 hrs. The product was precipitated with a large volume of water and the precipitate collected. For purification, the crude solid was dissolved in methylene chloride, and washed with water. Evaporation of methylene chloride yielded 3-aminoethyl estrone which after washing with ethyl ether gave 3.0 g (52%) of product m.p. 140°C (decomp.), 3-aminoethyl estrone hydrochloride, m.p. 180°C (decomp.), ^{1}HNMR (ppm): δ3.01 (2,t,$CH_2\underline{CH_2}NH_2$), 2.78 (2,t,$\underline{CH_2}$,CH_2NH_2), 4.00 (2,t,$COCH_2$).

Coupling of 3-Aminoethyl Estrone to Poly(benzyl L-glutamate)(PBLG)

The reaction below was conducted in p-dioxane as solvent. The reaction may also be conducted in dimethyl sulfoxide or dimethyl formamide with comparable success; however these solvents are not so readily removed and are therefore less preferable.

3-Aminoethyl estrone (1.25 g, 4 mmole) was added to a 7 mL dioxane solution of PBLG (0.88 g, 4 mmole). The mixture was allowed to react at 60°C for 2 days. The conjugate formed was collected by precipitating the dioxane solution with water, followed by filtration. For purification, the solid was dissolved in methylene chloride. Insoluble impurities were removed by

filtration. The methylene chloride solution was washed with cold aqueous 0.2N hydrochloric acid solution (x2), water, and saturated NaCl until neutral. Evaporation of methylene chloride yielded 0.4 g product. Elemental analysis for the conjugate, calculated, C: 70.73; H: 7.60; N: 6.60. found, C: 66.70; H: 6.45; N: 6.00. Degree of substitution was calculated to be 12% based on elemental analysis data. ^{1}HNMR (ppm): δ3.70 (2,t,$CH_2\underline{CH_2}NHCO$), 2.86 (2,t,$\underline{CH_2}CH_2NHCO$), 4.04 (2,t,$COCH_2$).

Estrone-conjugated poly-benzyl-L-glutamate was dissolved in p-dioxane and used to prepare 1 μm microcapsules by the method of Example 2.

EXAMPLE 8

This example illustrates determination of binding affinity constants for estradiol in pig uterus.

In Vitro Estrogen Receptor Assay

Affinity for binding the estrogen receptor was determined using a modification of previously reported procedures (14,15). Briefly, uteri (30 g) obtained from domestic swine (30 kg) were homogenized in Tris buffer (10 mM, pH 7.4), (80 ml), which contained EDTA (1.5 mM) and sodium azide (3 mM). The homogenate was centrifuged at 1,000 x g for 1 hr at 4°C. Uteri cytosol was then pretreated with dextran-coated charcoal as described (14). To investigate the nature of estradiol interaction with the estrogen receptor site, a saturation curve was obtained from [^{3}H]estradiol (10^{-5} M to 10^{-10} M) in the presence or absence of excess estradiol (10^{-5} M)(Figure 16). Uteri cytosol was incubated at 4°C for 2 hr with [^{3}H]estradiol (5 nM/tube) and competitor (ranging from 10^{-3} M to 10^{-8} M) or with estradiol (10^{-5} M)(non-specific).

The concentration of test compounds that decreased specific radioligand binding by 50% (IC_{50}) was measured. Protein concentrations were determined according to the method of Lowry *et al.* (16).

Scatchard analysis indicated a single class of binding sites with a mean binding affinity constant kd of 2.2 nm (n=9) and a mean receptor density (B max) of 350 fmol/mg protein, Figure 16. The protein concentration used was 1 mg/ml cytosol. Hill analysis (0.992) indicated that estradiol had competitive reversible binding. The IC_{50} of estrone conjugates to poly-benzyl-L-glutamate was 5×10^{-7} M which is ten-fold lower than the binding affinity for estrone (5×10^{-8} M), Table 3.

Table 3

Comparison of EST-PG and Estrone on Estrogen Receptor Binding in Pig Uterus

	IC_{50}(M)	Equiv. (Wt)
Estrone	5×10^{-8}	0.14 ng
EST-PG[a]	5×10^{-7}	500 ng

[a] EST-PG: Estrone with spacer (ethanolamine) conjugates to polybenzylglutamate (MW 58,000)

[b] Based upon 12.0% of conjugation between estrone and polymer, determined by UV at 282 nm and elemental analysis.

EXAMPLE 9

This example compares tissue distribution of microcapsules containing ^{131}I-iopanoate with estrone conjugated microcapsules containing ^{131}I-iopanoate.

In Vivo Tissue Distribution

Poly-benzyl-L-glutamate microcapsules loaded with ^{131}I-labeled ethyliopanoate were injected into rats (three per group) via the tail-vein (5.7 μCI in 0.3 ml water). Control groups were given only the ^{131}I-labeled iopanoic acid. Rats were sacrificed at 1, 3, 6 and 24 hours post injection. The percent of injected dose per organ or per tissue weight was determined by a COBRA Auto-gamma counter (Packard, Meriden, CT). Results are shown in Tables 4 and 5.

Table 4. Tissue Distribution of ^{131}I-IOPA Loaded Estrone Poly(benzyl L-glutamate) Conjugate Microspheres after I.V. Injection Into Rat (n=3)[1,2].

Organ	1 hr mean (s.d.)	3 hr mean (s.d.)	6 hr mean(s.d)	24 hr mean(s.d.)
blood	1.01(0.12)	0.72(0.08)	0.46(0.01)	0.09(0.03)
lung	1.05(0.15)	0.53(0.03)	0.34(0.02)	0.07(0.03)
liver	1.34(0.18)	0.75(0.09)	0.56(0.03)	0.17(0.02)
kidney	0.52(0.01)	0.62(0.05)	0.26(0.01)	0.06(0.02)
uterus	0.70(0.12)	0.62(0.01)	0.39(0.05)	0.06(0.01)
muscle	0.20(0.01)	0.12(0.01)	0.09(0.01)	0.01(0.01)
fat	0.37(0.01)	0.20(0.02)	0.14(0.01)	0.03(0.01)

[1] IOPA = ethyl iopanoic acid.

[2] Data shown represents percent of injected dose per gram tissue.

Table 5. Tissue Distribution of ^{131}I-IOPA Loaded Poly(benzyl L-glutamate) Conjugate Microspheres after Intravenous Injection Into Rat (n=3)[1,2].

Organ	1 hr mean(s.d.)	3 hr mean(s.d.)	6 hr mean(s.d)	24 hr mean(s.d.)
blood	1.53(0.71)	1.46(0.21)	1.30(0.26)	0.29(0.16)
lung	1.86(0.40)	1.06(0.16)	1.02(0.19)	0.28(0.03)
liver	1.80(0.78)	1.20(0.15)	1.16(0.14)	0.54(0.16)
kidney	0.74(0.28)	0.58(0.09)	0.58(0.05)	0.24(0.08)
uterus	0.85(0.29)	0.76(0.02)	0.73(0.10)	0.14(0.01)
muscle	0.32(0.16)	0.24(0.02)	0.21(0.03)	0.05(0.01)
fat	0.83(0.29)	0.58(0.23)	0.36(0.11)	0.07(0.04)

[1] IOPA = ethyl iopanoic acid.

[2] Data shown represent percentage of injected dose per gram tissue.

Table 6 shows the distribution of ^{131}I-labeled ethyliopanoate in rats in terms of uterus to muscle ratio. After 3 hr, the targeting of the estrone-conjugated labeled microcapsules was significantly greater than targeting by the labeled microcapsules or by labeled ethyliopanoate.

Table 6. Distribution of ^{131}I-Labeled Ethyliopanoate in Rats.

UTERUS TO MUSCLE RATIO

Time (hrs)	1	3	6	24
IOPA[1]	2.92 ± 0.464	3.60 ± 0.346	3.47 ± 0.122	n.d.[3]
PBLG[1]	2.84 ± 0.447	3.23 ± 0.300	3.48 ± 0.369	2.76 ± 0.214
PE[1]	3.50 ± 0.433	5.16 ± 0.592[2]	4.75 ± 0.354[2]	4.25 ± 1.061[2]

[1] IOPA: ethyliopanoate, PBLG: polybenzylglutamate microcapsules loaded with IOPA, PE: microcapsules of estrone and PELG conjugate. Each rate received 5 uCi of radiotracer in saline (0.25 ml).

[2] Significant difference ($p<0.05$) between PE and the corresponding groups by student t-test.

[3] n.d.: not detectable.

The present invention has been described in terms of particular embodiments found by the inventors to comprise preferred modes of practice of the invention. It will be appreciated by those of skill in the art that in light of the present disclosure numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, amino acid modified microcapsules could be attached to specific targeting agents without affecting the intended nature and practice of the invention. All such modifications are intended to be included within the scope of the claims.

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The references listed below are incorporated herein by reference to the extent they supplement, explain, provide a background for or teach methodology, techniques and/or compositions employed herein.

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Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the claims.

CLAIMS:

1. A high yield method of preparing homogeneous, non-aggregated microcapsules about 1 μm in size, comprising the steps:

combining an aqueous solution, a nontoxic emulsifier and a polymer dissolved in an organic solvent;

vigorously agitating the combination for at least five minutes;

and terminating the agitating when an average microcapsule size of less than 5 μm is indicated.

2. A method of preparing homogeneous, nonaggregated microcapsules of about 100 μm in size, comprising the steps:

admixing a polymer in an organic solvent with a solution of a nontoxic emulsifier;

agitating the mixture at a low agitation rate; and

terminating the agitation when the presence of an average microcapsule size of 100 μm is indicated.

3. The method of claim 1 or claim 2 further comprising removing the organic solvent from the combination and collecting the microcapsules.

4. The method of claim 1 wherein the vigorous agitating is by sonication and stirring at about 500 rpm or by stirring alone at a rate of about 1500 rpm.

5. The method of claim 1 or claim 2 wherein the polymer comprises poly-(D,L)-lactic acid, ethylhydroxyethyl cellulose, polycaprolactone, polycaprolactone diol, polylysine, polyglycolic acid, polymaleic acid or poly-benzyl-L-glutamic acid.

6. The method of claim 1 or claim 2 wherein the organic solvent comprises methylene chloride, chloroform, carbon tetrachloride, ethyl acetate or acetone.

7. The method of claim 1 or claim 2 wherein the nontoxic emulsifier comprises Tween-80, polyvinyl alcohol, sodium laurylsulfate, Span 20, Lubrol, Triton™ X-100 or cetylpyridinium chloride.

8. The method of claim 1 or claim 2 further comprising admixing a therapeutic or diagnostic agent with the polymer.

9. The method of claim 8 wherein the therapeutic or diagnostic agent is hydrophilic or hydrophobic.

10. A method of diagnostic imaging, comprising administering to a mammal a pharmaceutically acceptable preparation of a 1 μm microencapsulated imaging agent prepared by the method of claim 1 and detecting

localization of said imaging agent with an imaging device.

11. The method of claim 10 wherein the administering is intravenous or intra-arterial.

12. The method of claim 10 wherein the microencapsulated imaging agent is ferromagnetic or paramagnetic.

13. The method of claim 10 wherein the microencapsulated imaging agent comprises metrizoate, iothalamate, iohexol, ioxaglate, ioxalen or Gd-DTPA.

14. A method of promoting chemoembolization, comprising preparing microencapsulated drug by the method of claim 2 and administering said drug to a mammal in which chemoembolization and sustained drug release during a prescribed period of time are desired.

15. The method of claim 14 wherein the microencapsulated drug comprises cisplatin, 5-fluorouracil or Tamoxifen.

16. A microcapsule prepared by the method of claim 1 or claim 2 wherein an amino acid is attached to the polymer.

17. The microcapsule of claim 16 wherein the amino acid comprises phenylalanine, tyrosine, tryptophan or methionine.

18. The microcapsule of claim 16 wherein the polymer is polylactic acid polymer.

19. The microcapsule of claim 18 wherein phenylalanine is conjugated to the polylactic acid polymer.

20. The microcapsule composition of claim 19 further comprising encapsulation of a diagnostic or therapeutic drug.

21. The microcapsule composition of claim 20 wherein the diagnostic drug comprises meglumine diatrizoate.

22. A microcapsule composition prepared by the method of claim 1 or claim 2 wherein a diagnostic or therapeutic target agent is attached to said microcapsule.

23. The microcapsule composition of claim 22 wherein the therapeutic target agent is directed to estrogen receptors.

24. The microcapsule composition of claim 23 wherein the attached agent comprises estrone.

25. The microcapsule composition of claim 24 wherein the microcapsule is polymerized from poly-benzyl-L-glutamic acid.

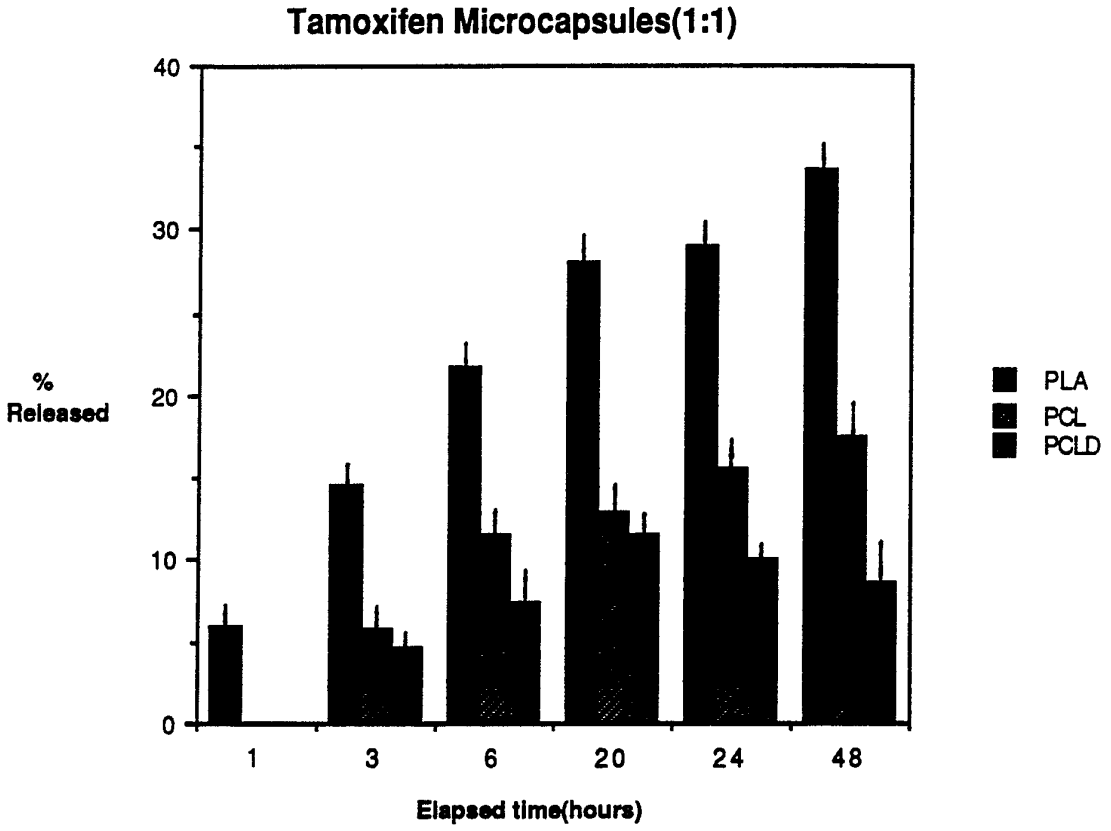

Tamoxifen Microcapsules(1:1)
40
30
20
10
0
% Released
1
3
6
20
24
48
Elapsed time(hours)
PLA
PCL
PCLD

FIGURE 1

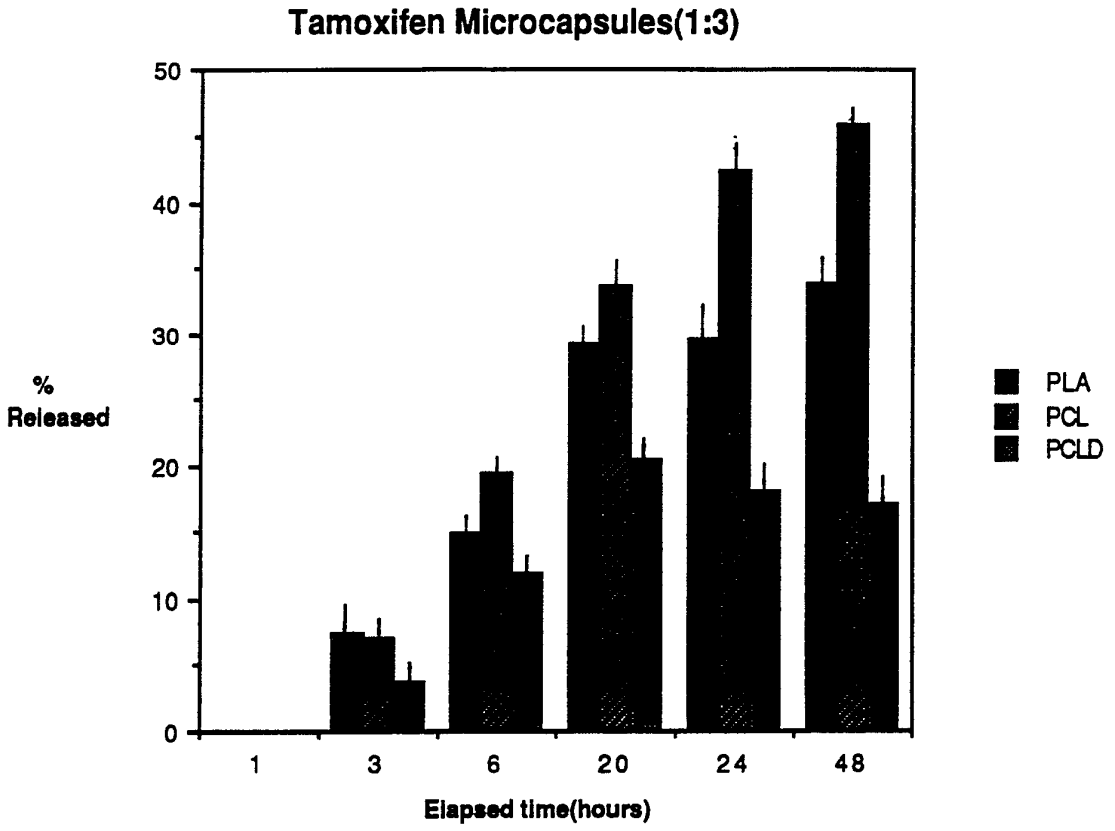

Tamoxifen Microcapsules(1:3)
%
Released
50
40
30
20
10
0
1
3
6
20
24
48
Elapsed time(hours)
PLA
PCL
PCLD

FIGURE 2

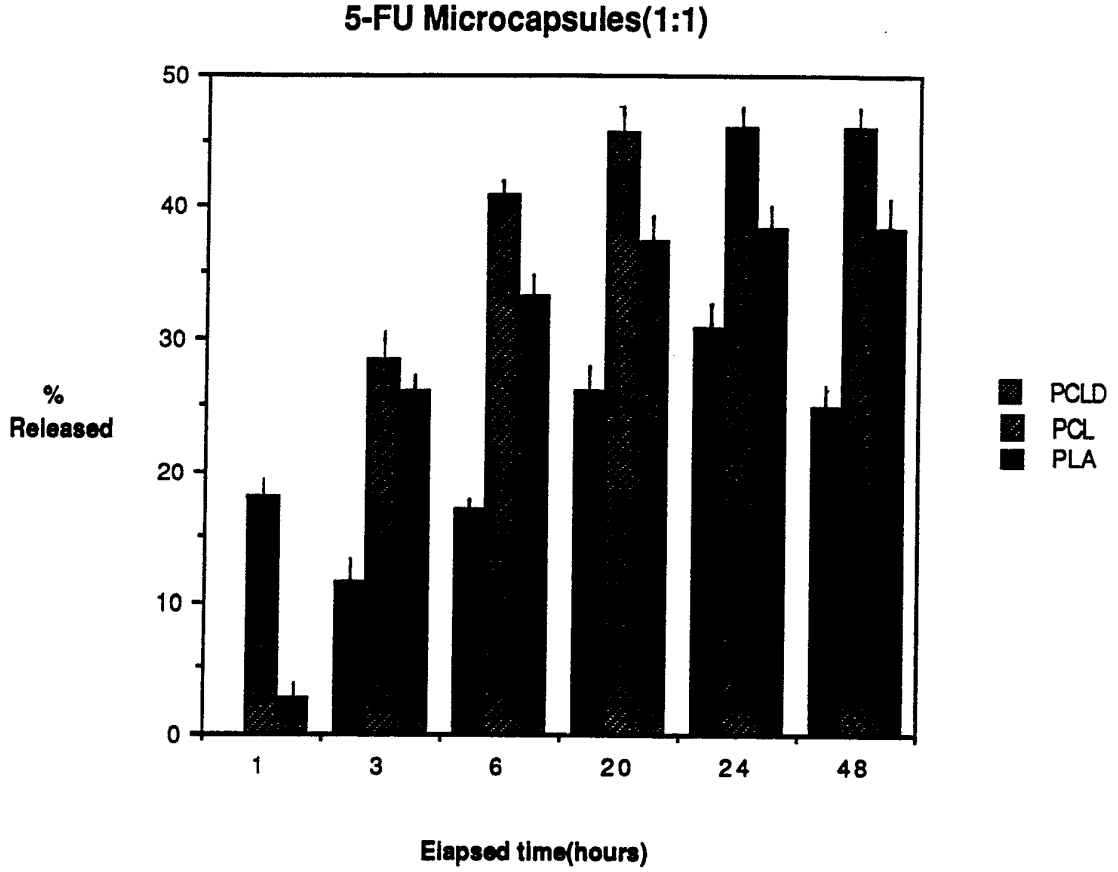

5-FU Microcapsules(1:1)
50
40
30
20
10
0
% Released
1
3
6
20
24
48
Elapsed time(hours)
PCLD
PCL
PLA

FIGURE 3

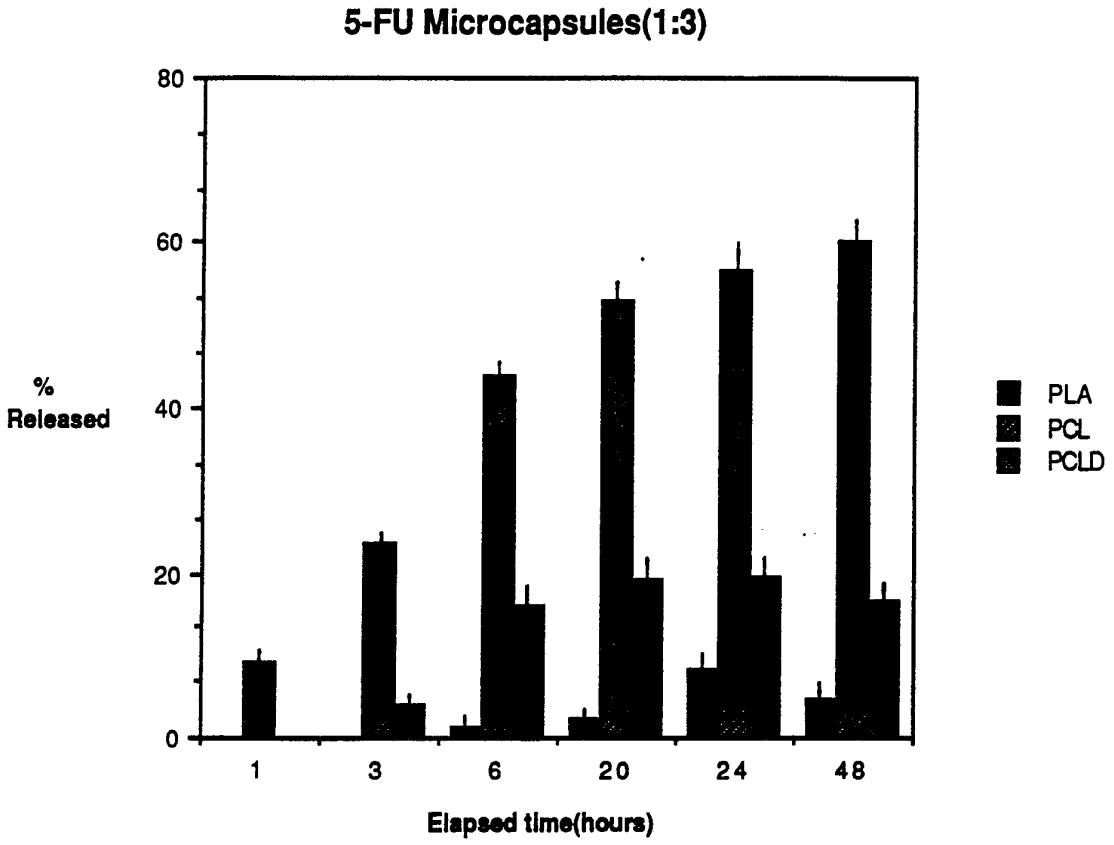

5-FU Microcapsules(1:3)
80
60
40
20
0
% Released
1
3
6
20
24
48
Elapsed time(hours)
PLA
PCL
PCLD

FIGURE 4

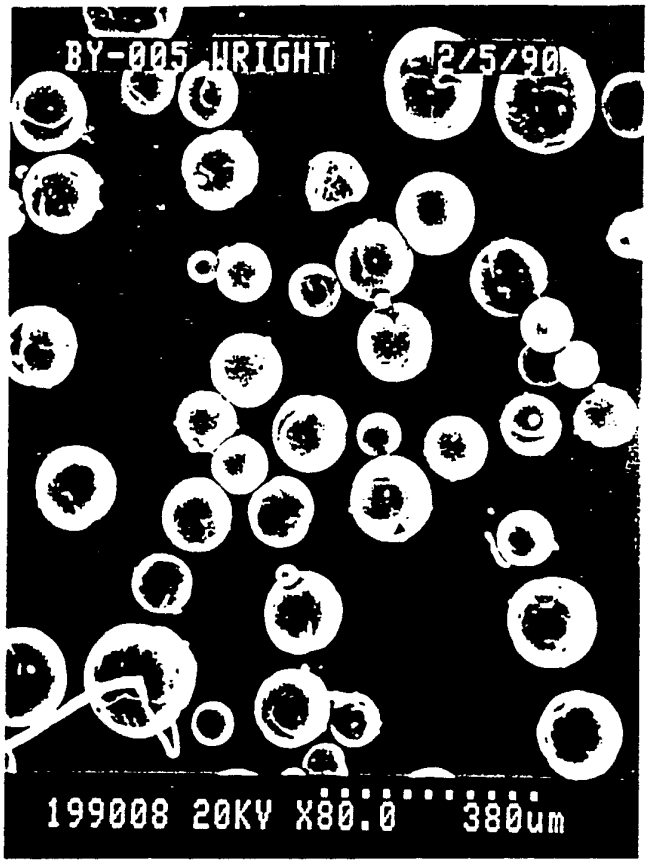


FIGURE 6

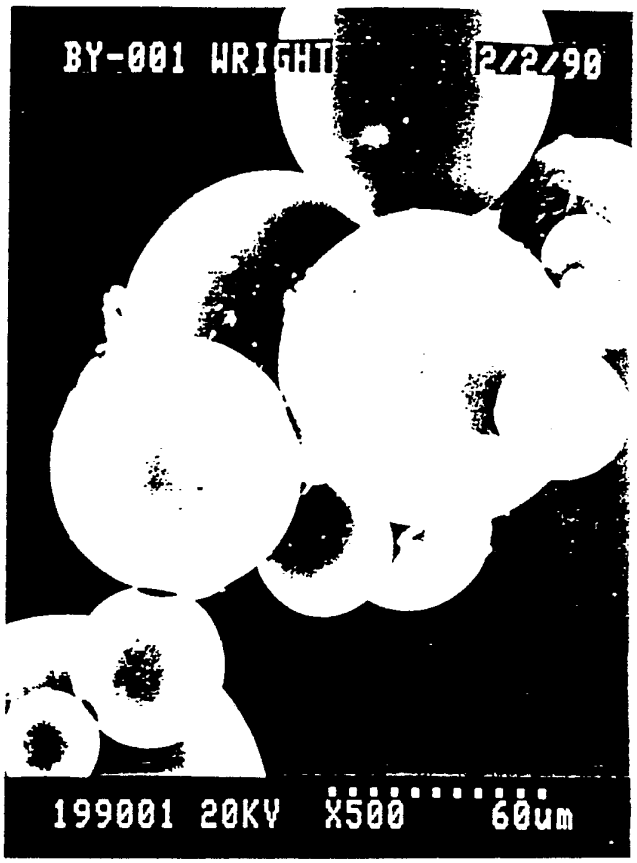
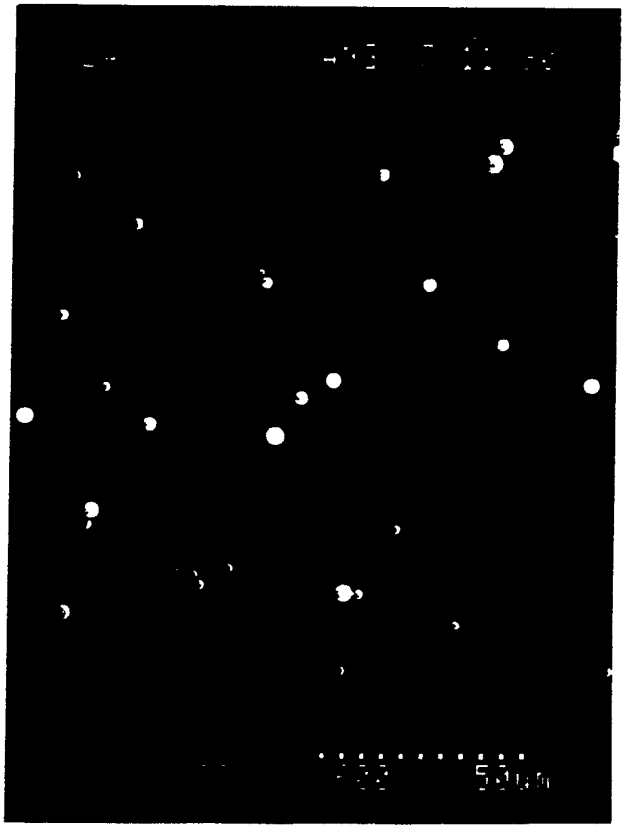


FIGURE 5

7A

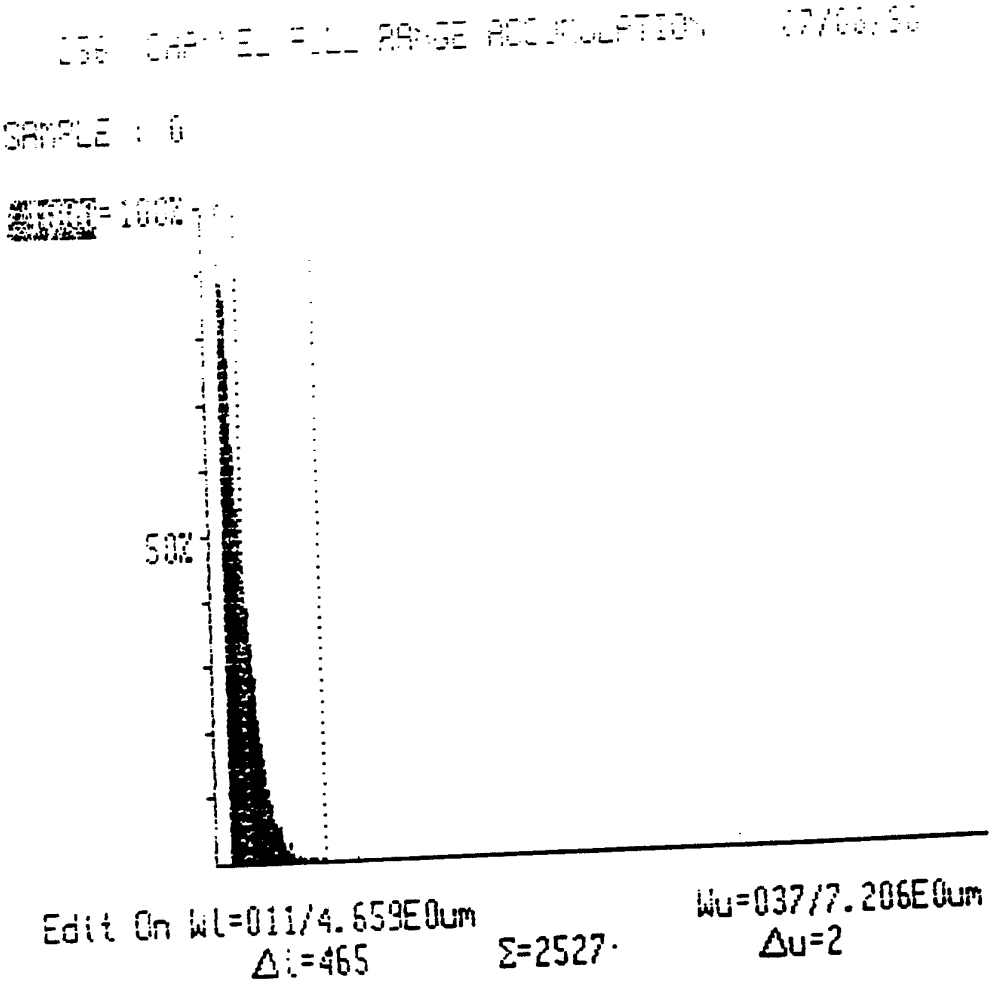
PLA MICROCAPSULES (<10μ) LOADED WITH MEGLUMINE DIATRIZOATE

FIGURE 7



7B

7/15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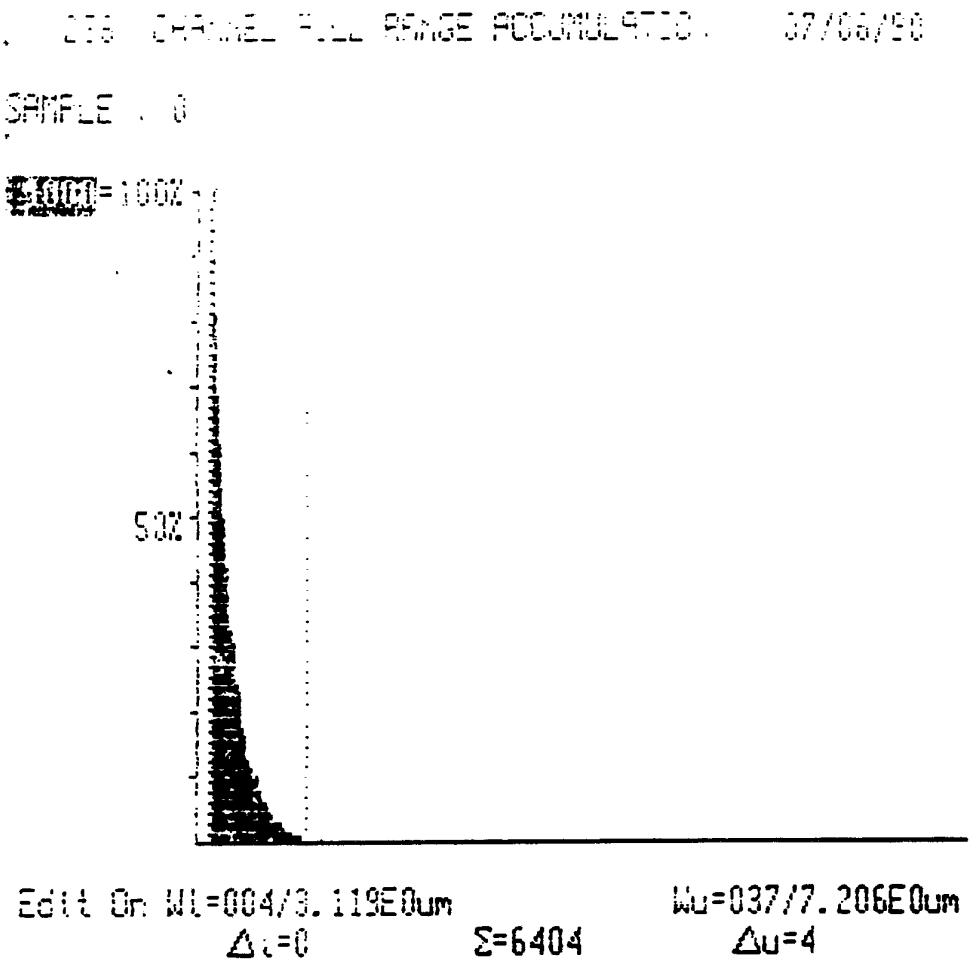
Σ=2527·
Wu=037/7.206E0um
Δu=2

FIGURE 8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SAMPLE . 0
100%
50%
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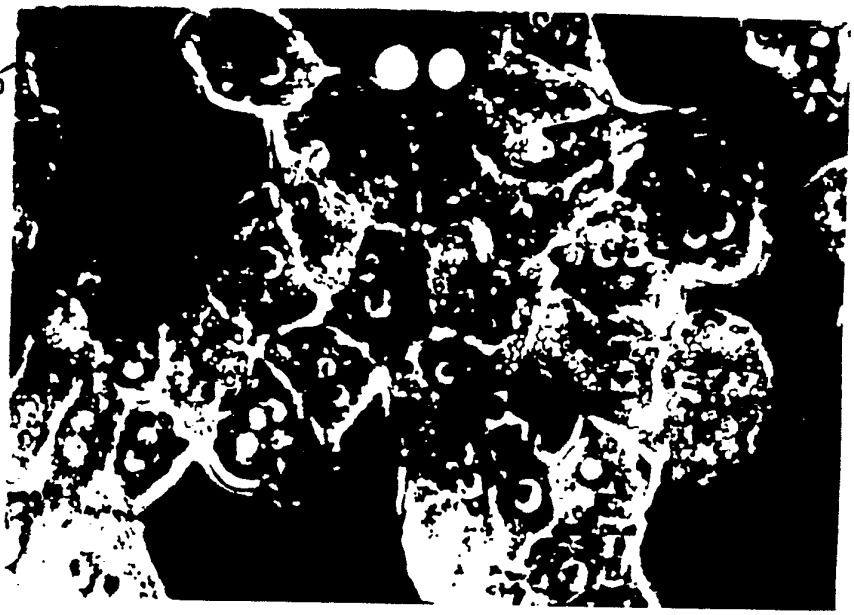
Δu=4

FIGURE 9

9/15

FIGURE 10

10A



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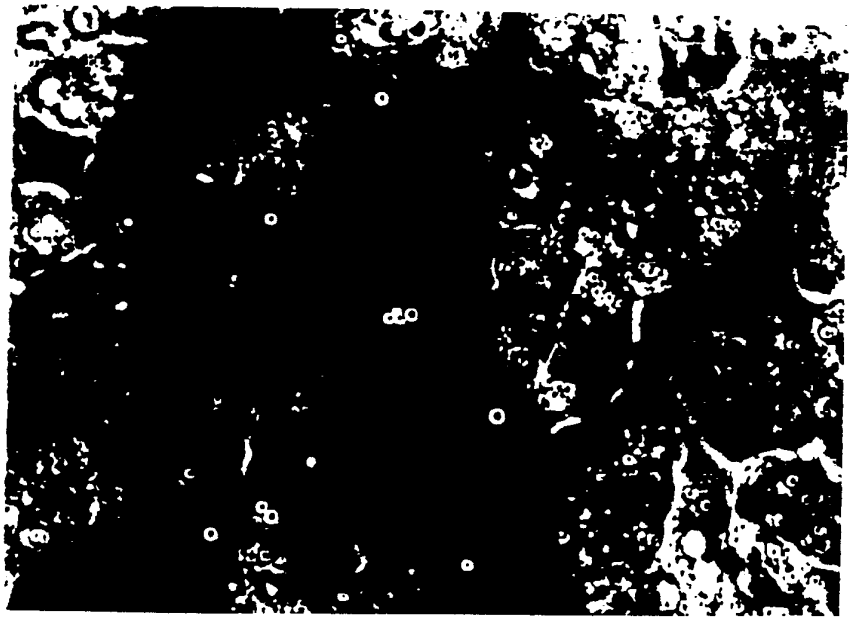

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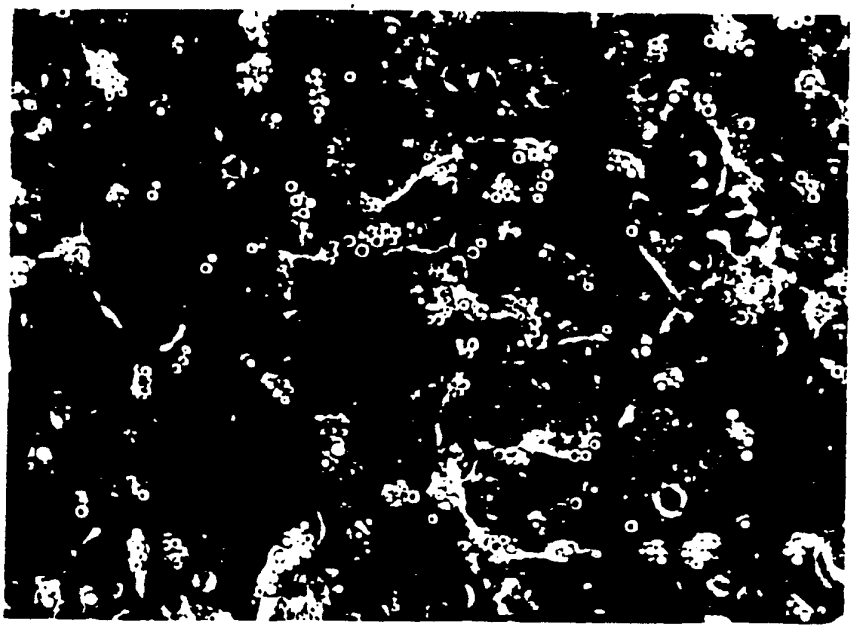
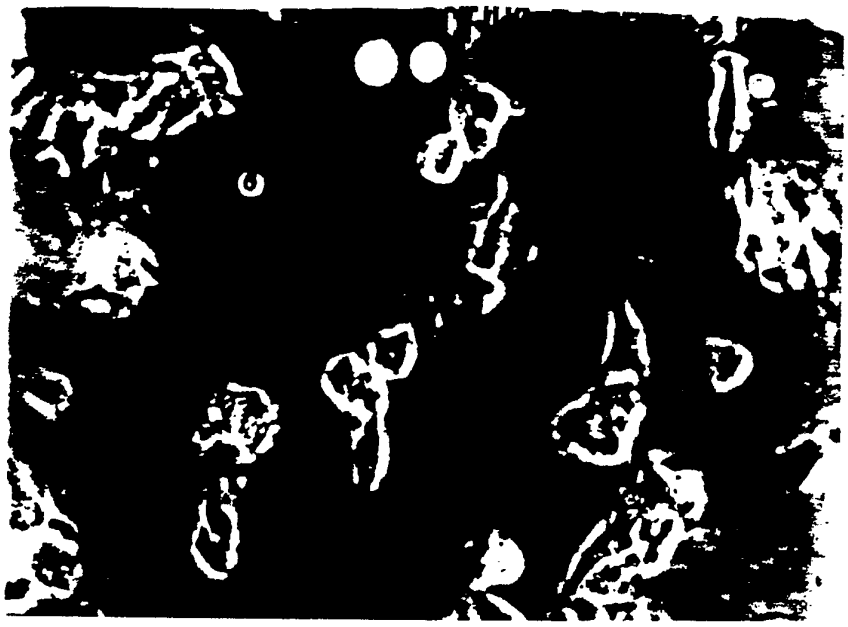


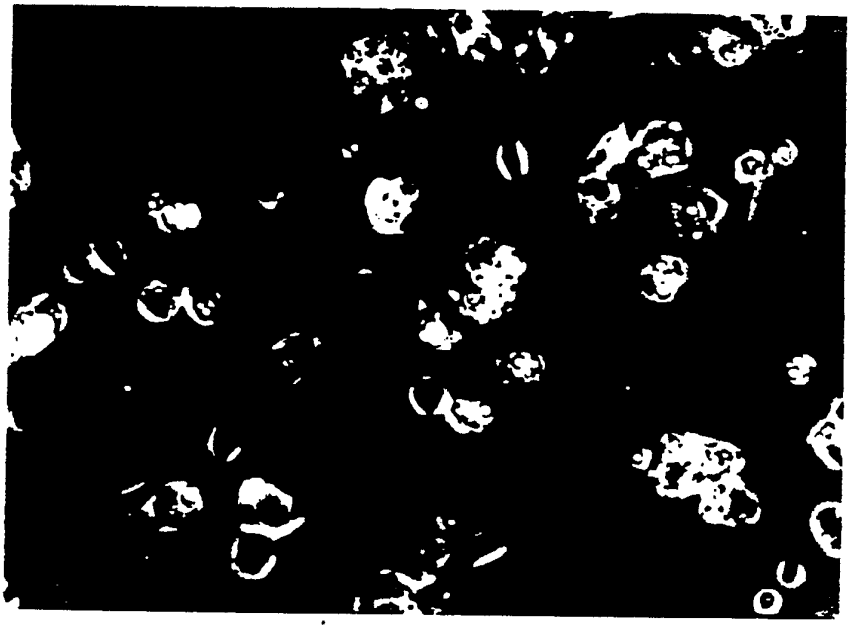
FIGURE 11

10/15

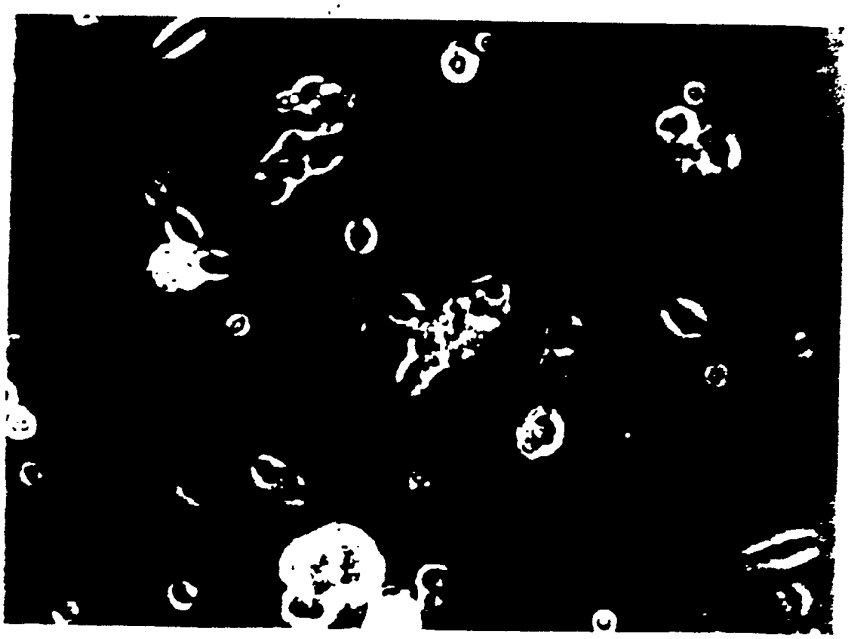
11A



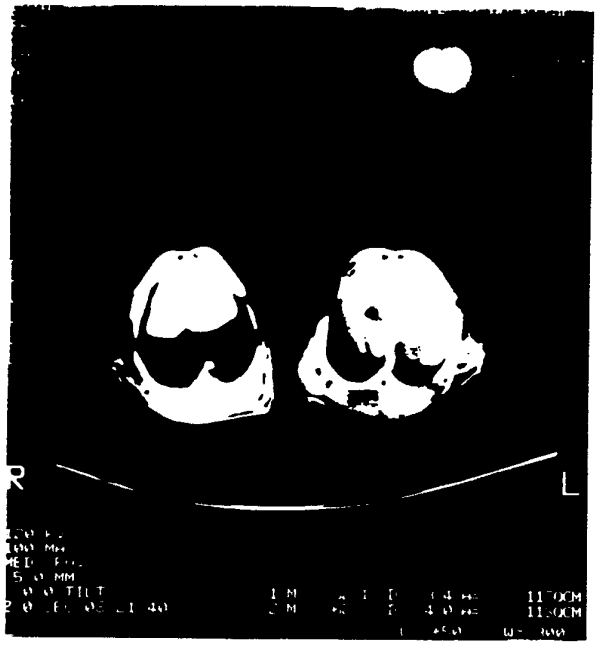
11B



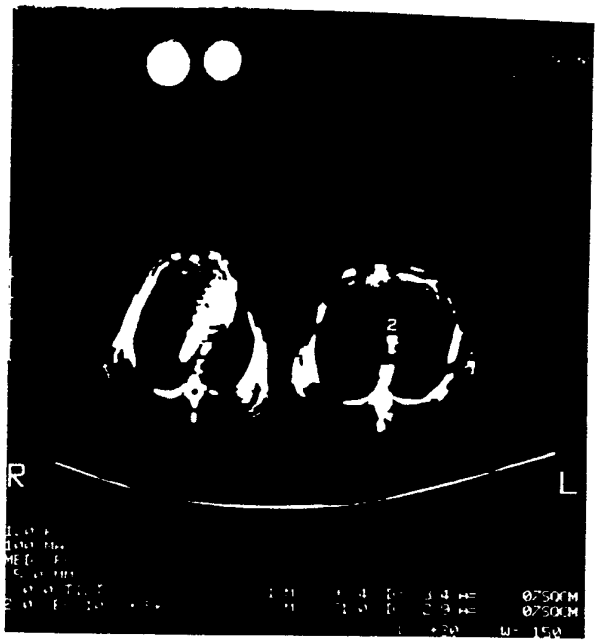
11C



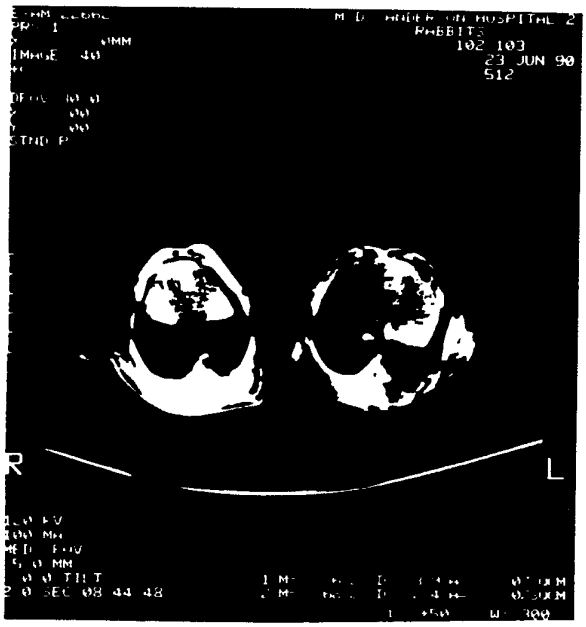
11/15



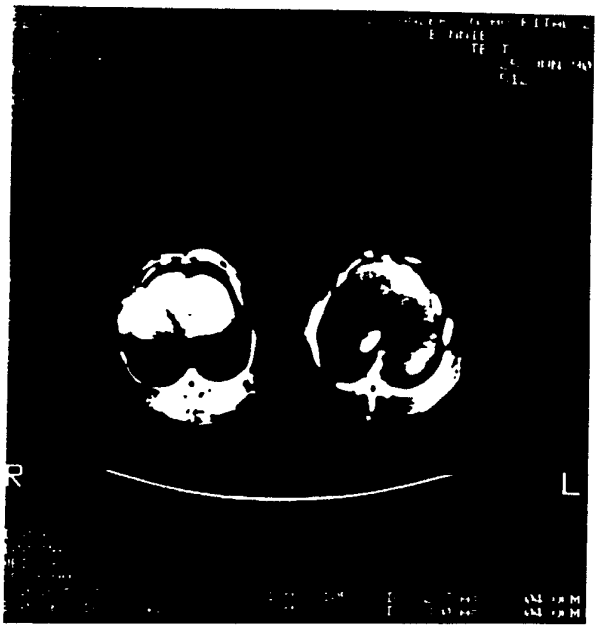
G



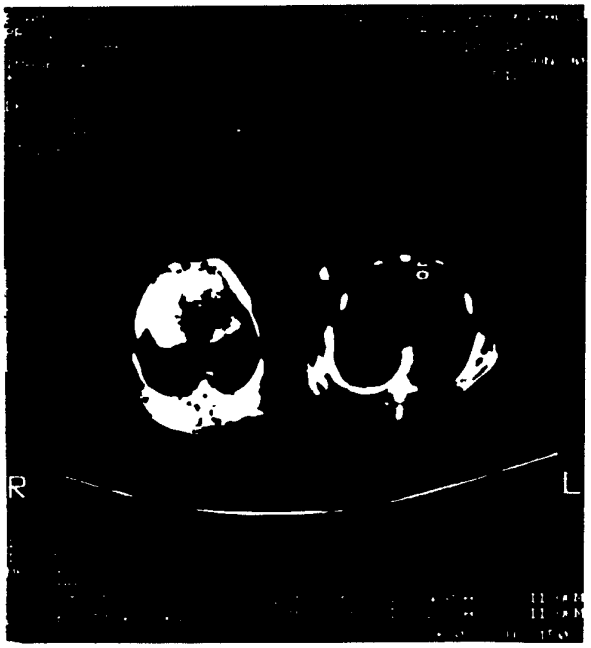
D J



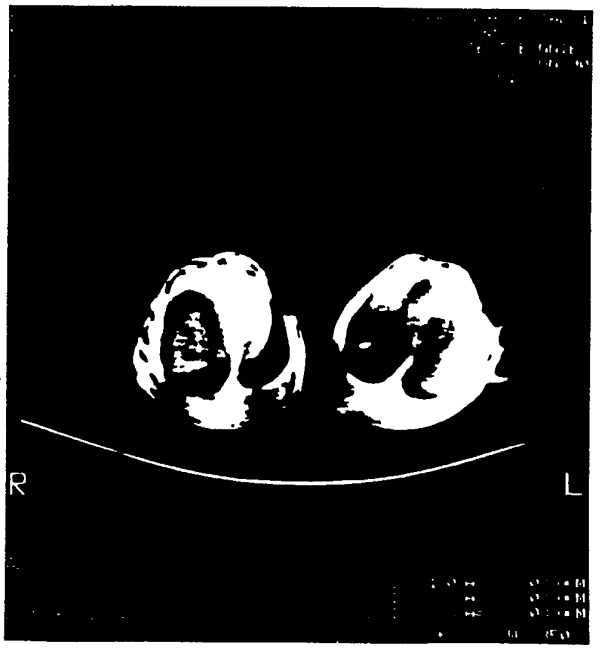
H



E K



I



F

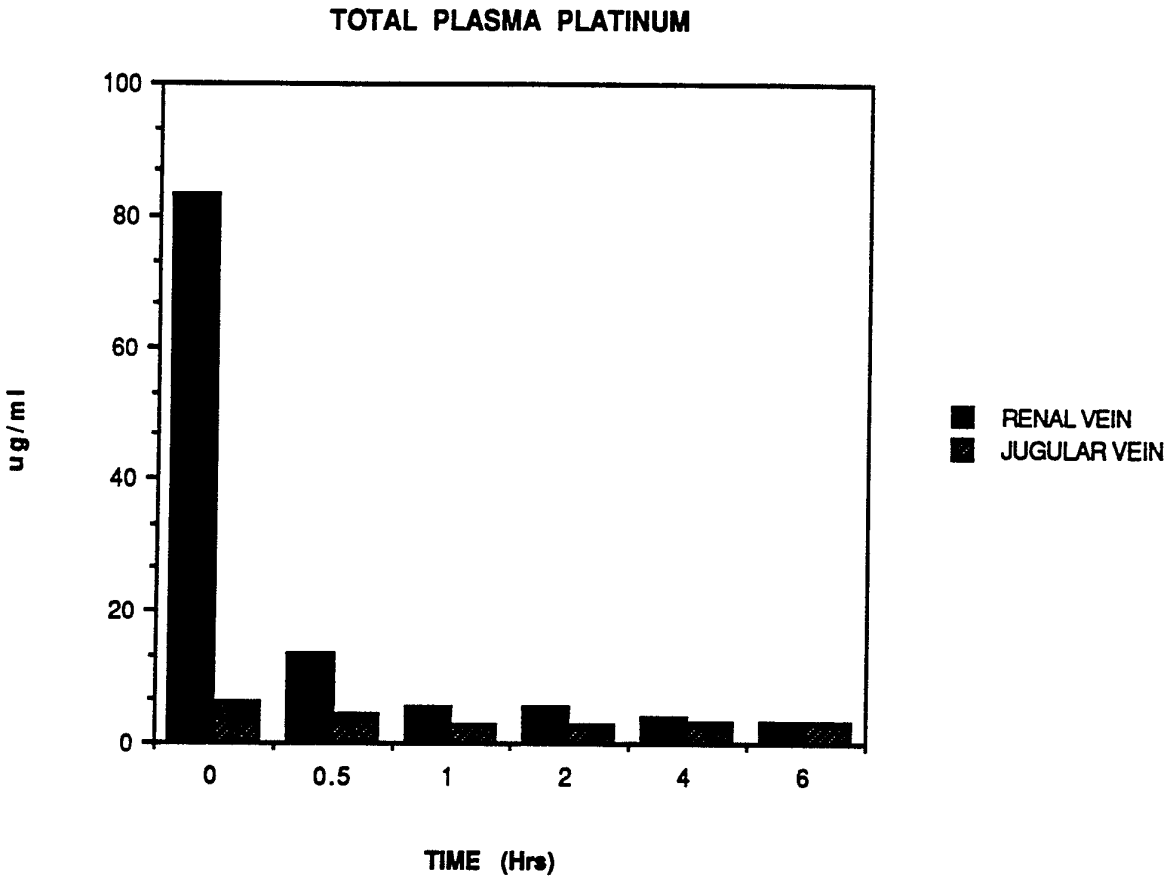

TOTAL PLASMA PLATINUM
100
80
60
40
20
0
ug/ml
0
0.5
1
2
4
6
TIME (Hrs)
RENAL VEIN
JUGULAR VEIN

FIGURE 13

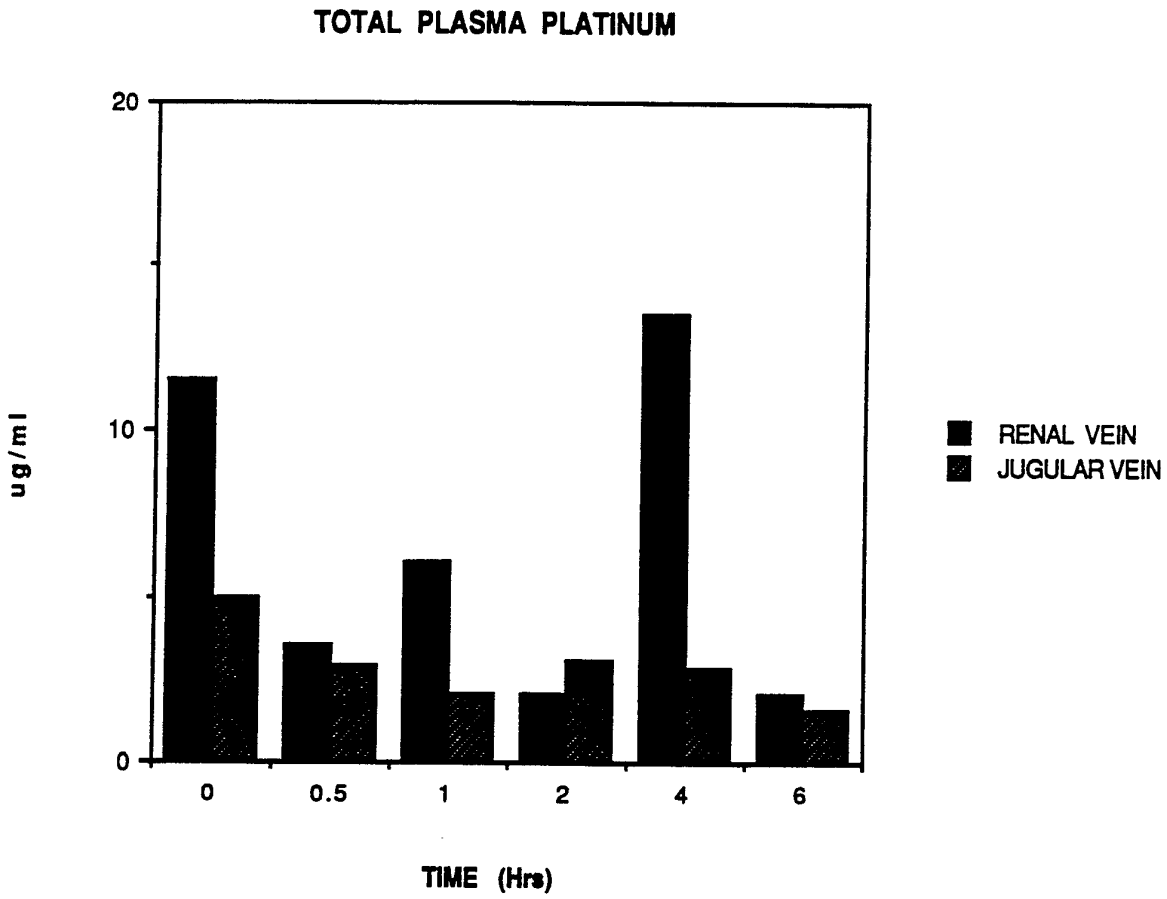

TOTAL PLASMA PLATINUM
20
10
0
ug/ml
0
0.5
1
2
4
6
TIME (Hrs)
RENAL VEIN
JUGULAR VEIN

FIGURE 14

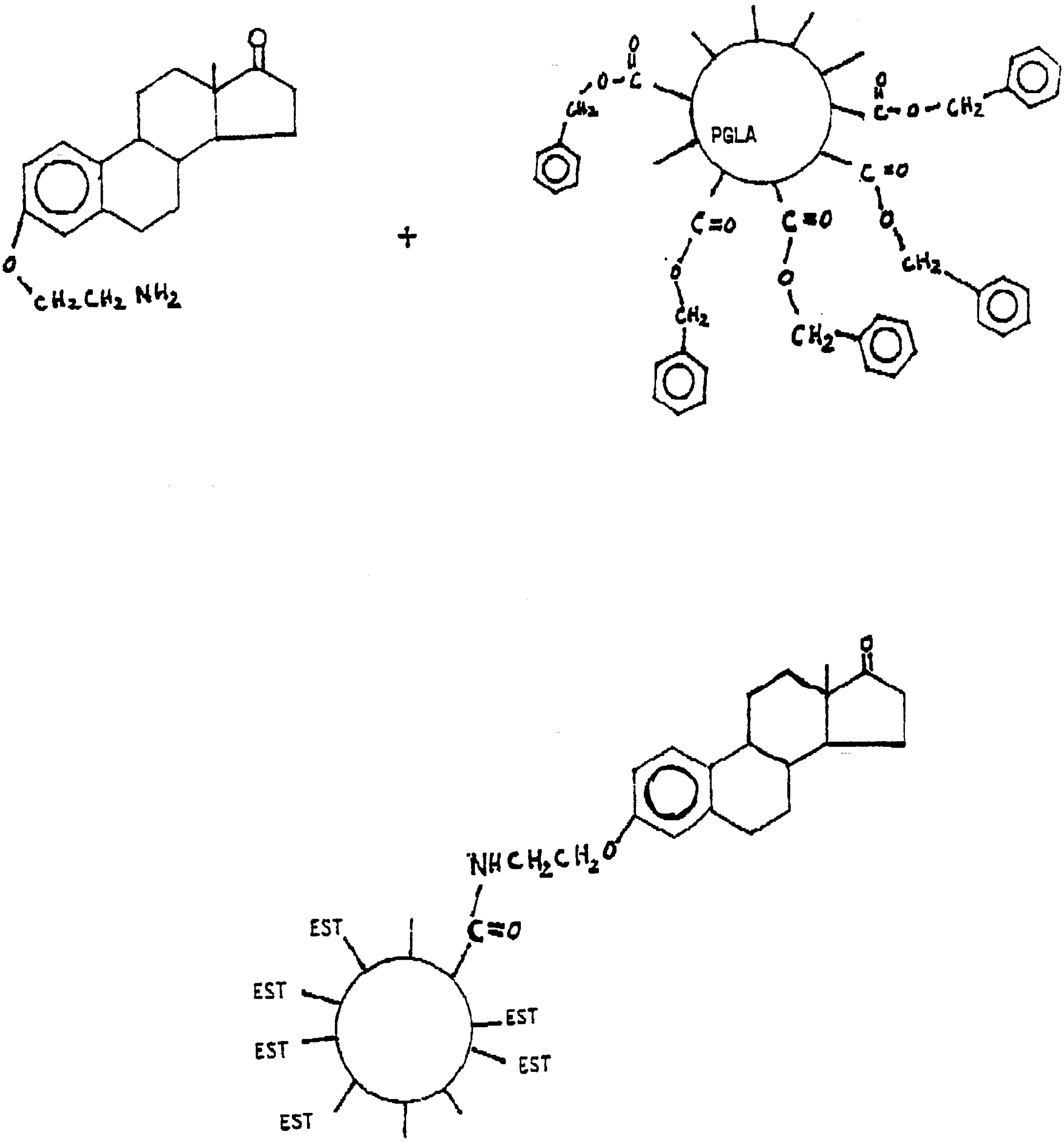
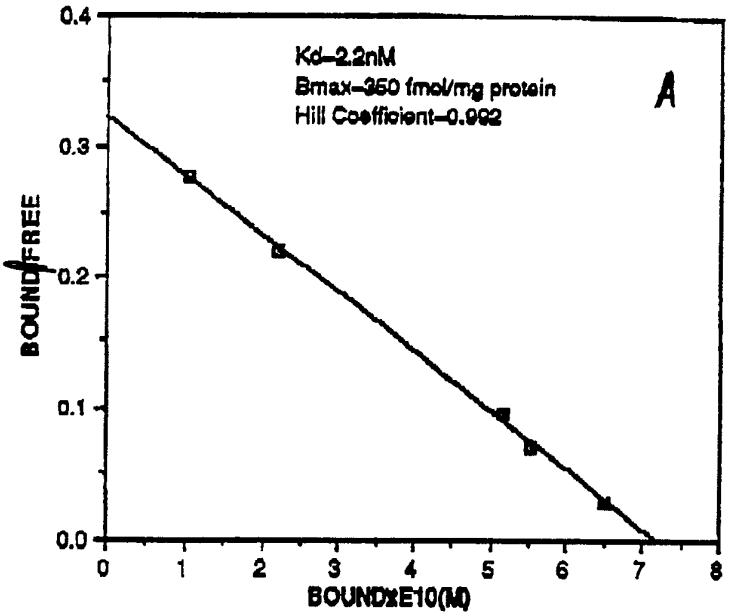


Figure 15


Kd=2.2nM
Bmax=350 fmol/mg protein
Hill Coefficient=0.992
A
BOUND/FREE
BOUNDxE10(M)
0.0
0.1
0.2
0.3
0.4
0
1
2
3
4
5
6
7
8

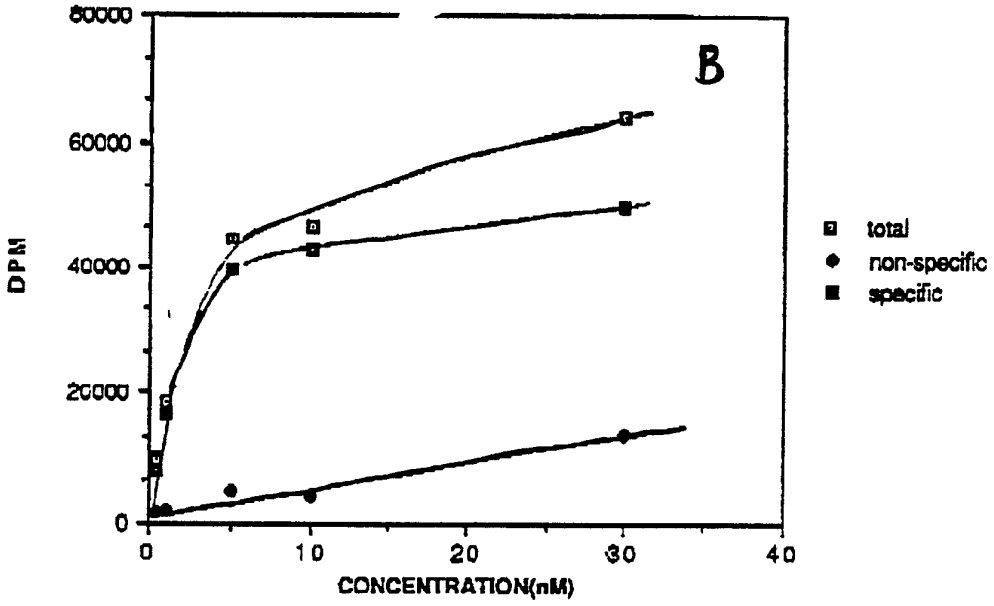

B
DPM
CONCENTRATION(nM)
total
non-specific
specific
0
20000
40000
60000
80000
0
10
20
30
40

Figure 16