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(21) International Application Number: PCT/US90/05679 (22) International Filing Date: 4 October 1990 (04.10.90) (30) Priority data: 418,382 6 October 1989 (06.10.89) US (71) Applicant: THE BETH ISRAEL HOSPITAL ASSOCIATION [US/US]; 330 Brookline Avenue, Boston, MA 02215 (US). (72) Inventor: FOSSEL, Eric, T. ; 66 Priscilla Road, Chestnut Hill, MA 02167 (US). (74) Agent: LORUSSO, Anthony, M.; Lorusso & Loud, 440 Commercial Street, Boston, MA 02167 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: NOVEL OXIDIZED LIPOPROTEINS AND METHODS FOR THEIR PREPARATION (57) Abstract Methods and apparatuses are disclosed for preparing novel oxidized lipoprotein compositions. Peroxides are capable of generating free-radicals which, in turn, can oxidize lipoproteins <i>in vitro</i> or <i>in vivo</i>. It has been discovered that hydrogen peroxide itself or in conjunction with certain enzymes such as peroxidase or lipoxidase can oxidize lipoproteins. Peroxidized low density lipoproteins are the most preferred composition. Oxidized lipoproteins exhibit a cytotoxic effect to which diseased cells which are more susceptible than healthy cells. Thus, oxidized lipoproteins may be useful in treating many disease states. The extent of lipoprotein peroxidation is measured by obtaining a C-13 NMR spectrum of the oxidized lipoprotein solution.		

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NOVEL OXIDIZED LIPOPROTEINS
AND METHODS FOR THEIR PREPARATION

BACKGROUND OF THE INVENTION

Cross-Reference to Related Application

This application is a continuation-in-part of U.S. Patent Application Serial Number 07/418,382 filed October 6, 1989.

Statement Regarding Federally Sponsored Research

Funding for work described herein was provided by the Federal Government under a grant from the Department of Health and Human Services. The Government may have certain rights in this invention.

Prior Art

The treatment of disease using oxidized lipoproteins is described in detail in the parent application cited above which is herein incorporated by reference. Other teachings

by Eric T. Fossel which are incorporated herein by reference are: "Process for the Screening of Cancer Using Nuclear Magnetic Resonance", U.S. Patent No. 4,912,050, March 27, 1990 and "Process for the Detection of Cancer Using Nuclear Magnetic Resonance", U.S. Patent No. 4,918,021, April 17, 1990.

Field of Invention

This invention relates to novel oxidized lipoprotein compositions and especially methods and an apparatus for preparing them.

Most organic compounds, including most tissue constituents, are thermodynamically unstable in the presence of oxygen. Due to the presence of both energy barriers and electron-spin barriers to the direct reaction of ordinary triplet-state oxygen with methylene groups, the reaction is generally very slow. However, certain substances present in the tissues react relatively readily with oxygen. Among these are the unsaturated fatty acids.

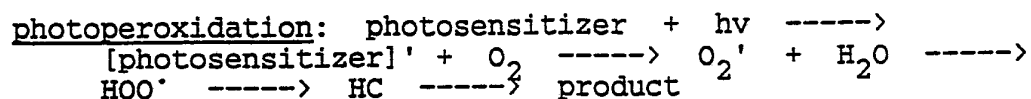
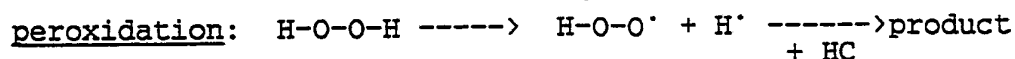
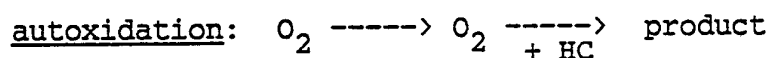
One of the known properties of unsaturated fatty acids, particularly the polyenoic acids, is their susceptibility to oxidation, in particular peroxidation and autoxidation. Autoxidation is a radical chain reaction involving molecular oxygen as a reactant in one of the steps. The term "peroxidation" refers to production of peroxides and their degradation products. In general terms, oxidation involves

one molecule of oxygen whereas peroxidation involves two molecules of oxygen. In accordance with the present invention, another form of oxidation, photoperoxidation is provided. This form of oxidation is mediated by a photosensitizer and ultraviolet light.

A study by Edelson et al., N. Eng. J. of Med., 1987, Vol. 316, pp. 297-303 describes treatment effective for some cutaneous lymphomas and certain leukemias whereby a photosensitizer is added to blood and the blood irradiated with ultraviolet light. In this study, an aromatic compound, 8-methoxypsoralen, was ingested by a patient. Several hours later blood was withdrawn and irradiated with ultraviolet light (type A), and then re-infused into the patient. The Edelson study, however, does not explain the mechanism of this method of treatment, it simply indicates that it is effective against certain forms of cancer.

The present invention provides several methods for oxidizing lipoproteins and photoperoxidation is one such method. It is believed that by adding a photosensitizer such as 8-methoxypsoralen to blood and then irradiating the blood with ultraviolet light produces oxidized lipoproteins. It is further believed, that the oxidized lipoproteins are useful as a therapeutic agent in fighting disease.

Formulas for the three types of oxidation appear below.



Empirically, it was found that the rate of autoxidation is directly proportional to substrate concentration and to the partial pressure of oxygen above 100 mm. The rate also increases with the extent of oxidation, indicating a chain reaction and the autocatalytic nature of the process. The principal initial products are hydroperoxides. FIG. 1 expresses these relationships, where K_0 and λ are empirical constants, RH is unoxidized substrate, p_{O_2} the partial pressure of oxygen and the rate of oxidation is expressed as the derivative with respect to time: d/dt .

To date, most studies dealing with fatty acid oxidation have concentrated on the autoxidation of linoleate. Linoleate, a fatty acid naturally occurring in animals, provides a good model for the mechanism of oxidation.

The initiation reactions, as shown in FIG. 2, are of particular importance since it has been shown that highly purified polyunsaturated fatty acids are stable for long periods of time in the presence of oxygen (Privett and Blank, 1962). Acting as catalysts, traces of peroxides or

transition metals and ultraviolet or ionizing radiation, in addition to several other factors, are known to bring about initiation. Once initiated, the reaction continues by a chain mechanism involving resonance-stabilized free radicals that react readily with oxygen to form peroxy radicals, which can then initiate new chains by the slower abstraction of a hydrogen atom from another molecule of substrate as the peroxy radicals are converted to hydroperoxides.

Hydroperoxides will decompose under certain conditions by homolysis, giving radicals that can initiate new chains. In addition, the hydroperoxides are reasonably strong oxidizing agents and are reduced by thiol-containing proteins, glutathione, cysteine and particularly, by a glutathione-dependent factor in the cytosol which apparently has this function as part of the antioxidant defense of the cell. If not reduced to the alcohol, the hydroperoxide can undergo homolysis, usually catalyzed by transition metal ions, to form a hydroperoxy or alkoxy radical, depending upon the oxidation state of the metal as shown in FIGS. 3 and 4. The alkoxy radical formed, as shown in FIG. 3, reacts with any susceptible molecule in its vicinity, usually by abstraction of a hydrogen atom and creation of another radical likely to initiate further reactions.

Indeed, it is this type of reaction that is responsible for the unpleasant odor and flavor of oxidatively rancid fat, the odor being that of unsaturated aldehydes produced

in this manner from unsaturated fats. For example, hydroperoxides formed by autoxidation of oleic acid would be expected to decompose in this way to give aldehydes as shown in FIG. 5.

Additional reactions that alkoxy radicals may undergo are shown in FIGS. 6A-6D. FIG. 6A shows abstraction of a hydrogen atom, initiating new chains. FIG. 6B shows disproportionation with another radical. FIG. 6C shows a coupling: FIG. 6D depicts addition to double bonds, which can lead to polymeric products of high order of the types commonly found among lipid peroxidation products.

It would seem from the nature of the autoxidation reaction that this reaction would occur most readily in systems such as cell membranes, in which the lipids are in an ordered arrangement that would facilitate the propagation reactions. However, the complexity of such membranes has made the investigation of their peroxidation difficult and has led to the use of several model systems in order to gain insight into the reaction involved.

One such system consists of monomolecular films of unsaturated fatty acids adsorbed on silica gel. Autoxidation of linoleic acid in this way, induced by small amounts of peroxides, leads to disappearance of linoleic acid by apparent first order kinetics. The equation shown in FIG. 1 indicates that autoxidation of polyunsaturated

fatty acids exhibits much more complex kinetics. The monomolecular film thus appears to exhibit a different mechanism of peroxidation and this is confirmed by the finding that major products are the fatty acid epoxides. This reaction is of interest since some of the most potent chemical mutagens and carcinogens are epoxides.

In another model system, the unilamellar liposome, formed by sonication of an unsaturated phosphatidylcholine in aqueous buffered solution, the products of autoxidation are the expected 9- and 13-hydroperoxyoctadecadienoic acids. However, with inclusion of saturated phosphatidylcholine in the liposome, the product mixture becomes much more complex, containing also epoxides, hydroxyepoxides, and di- and trihydroxy acyl moieties (see also Fridovich and Porter, 1981). A simple membrane system from Acholeplasma laidlawii, with a fatty acid composition of the membrane phospholipids consisting of a 50:50 mixture of 18:2 and 16:0, gave very similar fatty acid products, indicating that results with the model systems are indeed representative of those occurring in biomembranes. Lipids, Mead, Plenum Press, New York, p.83 (1986).

Although the prior art is replete with the chemistry for oxidizing fatty acids, including lipoproteins, it is believed that the present invention contains novel methods of oxidation of lipoproteins as well as novel oxidized lipoprotein compositions.

SUMMARY OF THE INVENTION

In accordance with the present invention, it was discovered that peroxide, such as hydrogen peroxide in conjunction with an enzyme, such as horseradish peroxidase, is capable of generating free radicals which in turn can peroxidize lipoproteins, preferably low density lipoproteins. Thus, novel oxidized lipoprotein compositions are provided as well as methods and apparatus for preparing them. Also, the use for such oxidized lipoproteins is indicated.

It is thus an object of this invention to provide a novel oxidized lipoprotein composition which in the most preferred embodiment consists of low density lipoproteins, hydrogen peroxide, horseradish peroxidase and saline solution. Other novel compositions are provided which include flavin, riboflavin, oxidase, lipoxidase, organic peroxide or ditertiarybutyl peroxide as oxidants. Modified lipoproteins can be added to the composition to enhance the effect of the organic peroxides. Also, compositions are provided which include chemotherapeutic agents such as adriamycin and mitomycin-D or photosensitizers such as 8-methoxypsoralen and hematoporphyrins. Additionally, novel lipoproteins compositions are provided which include elemental oxygen or perfluorocarbon fluosal.

Another object of this invention is to provide methods

for preparing oxidized lipoproteins. The preferred method is to add hydrogen peroxide and horseradish peroxidase to a solution of low density lipoproteins and then subject the sample to carbon-13 NMR spectroscopy to determine the degree of peroxidation. The lipoproteins can also be oxidized using other oxidants. Additional methods include oxidizing the lipoproteins by irradiating the sample, to which a photosensitizer has been added, with ultraviolet light or adding certain chemotherapeutic agents to the lipoprotein solution.

It is further object of this invention to provide apparatus for preparing the oxidized lipoproteins. One such apparatus is a peroxidizing module containing an immobilized enzyme into which a pump introduces hydrogen peroxide. Another apparatus is a container, which holds an immobilized enzyme, into which hydrogen peroxide is added. Yet another apparatus is an atrioventricular shunt or arterial bypass which is attached to a patient with a peroxidizing module attached to the shunt.

In a separate patent application, filed on even date with the present application, Eric T. Fossel teaches that oxidized lipoproteins may be helpful in fighting disease states, such as cancer, malaria and viral infections such as acquired immunodeficiency syndrome, which are characterized by diseased cells with an increased number of lipoprotein receptors or an enhanced ability to take up lipoproteins.

The diseased cells have been found to be more susceptible than healthy cells to the cytotoxic effect of oxidized lipoproteins. Thus, therapy with oxidized lipoproteins is believed to have the effect of killing the diseased cells.

Other objects and advantages of the invention will become apparent from the description of the invention which follows, made with reference to the drawings described below.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows an equation that expresses the kinetics of oxidizing linoleic acid;

FIG. 2 shows the initial reactions in the autoxidation of linoleic acid;

FIG. 3 shows a likely decomposition mechanism in which metal ions readily convert hydroperoxides to alkoxy radicals and hydroxyl ions;

FIG. 4 shows the hydroperoxide undergoing homolysis catalyzed by transition metal ions to form a hydroperoxy or alkoxy radical depending on the oxidation state of the metal;

FIG. 5 shows how hydroperoxides formed by autoxidation

of oleic acid are expected to decompose to give aldehydes;

FIG. 6A shows abstraction of a hydrogen atom, initiating new chains;

FIG. 6B shows disproportionation with another radical;

FIG. 6C shows a coupling;

FIG. 6D depicts addition to double bonds, which can lead to polymeric products of high order of the types commonly found among lipid peroxidation products;

FIG. 7 shows the olefinic region of a C-13 spectrum of a normal human plasma sample;

FIG. 8A shows the olefinic region of a 125.8 MHz proton decoupled spectrum from normal human plasma;

FIG. 8B shows the olefinic region of a 125.8 MHz proton decoupled spectrum of the same plasma as in FIG. 8A following the addition of peroxidase (2 mg/ml), and after 3 aliquots of 3% hydrogen peroxide (100 μ l/ml) were added at hourly intervals;

FIG. 9 shows the apparatus of the present invention;

FIG. 10A is a schematic diagram of the mechanism for producing peroxidized low density lipoproteins as carried

out by the human body in response to malignancy;

FIG. 10B is a schematic diagram of the general method for treating cancer in accordance with the claimed invention;

FIG. 11 shows another embodiment of the apparatus of the present invention for oxidizing the lipoproteins in a blood supply and

FIG. 12 shows a further embodiment of the apparatus of the present invention for oxidizing the lipoproteins in a blood supply.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

At the outset, the invention is described in its broadest overall aspects, with a more detailed description following. In its broadest overall aspects, the invention relates to a novel oxidized lipoprotein composition as well as methods and apparatus for preparing the oxidized lipoproteins.

Lipoproteins take various forms in the blood including chylomicrons, chylomicron remnants, very low density lipoproteins, intermediate density lipoproteins, low density lipoproteins, and high density lipoproteins. Certain lipids associate with specific proteins to form lipid:protein

systems in which the specific physical properties of these two classes of biomolecules are blended. There are two major types; transport lipoproteins and membrane systems. In these systems, the lipids and proteins are not covalently joined but are held together largely by hydrophobic interactions between the nonpolar portions of the lipid and the protein components.

The plasma lipoproteins are complexes in which the lipids and proteins occur in a relatively fixed ratio. They carry water-insoluble lipids between various organs via the blood, in a form with a relatively small and constant particle diameter and weight. Human plasma lipoproteins occur in four major classes that differ in density as well as particle size as shown in the table below.

Major Classes of Human Plasma Lipoproteins

	Chylomicrons	Very low density lipoproteins (VLDL)	Low-density lipoproteins (LDL)	High-density lipoproteins (HDL)
Density, g ml ⁻¹	<0.94	0.94-1.006	1.006-1.063	1.063-1.21
Flotation rate, S ₂₀	>400	20-400	0-20	(Sediment)
Particle size, nm	75-1,000	30-50	20-22	7.5-10
Protein, % of dry weight	1-2	10	25	45-55
Triacylglycerols, % of dry weight	80-95	55-65	10	3
Phospholipids, % of dry weight	3-6	15-20	22	30
Cholesterol, free, % of dry weight	1-3	10	8	3
Cholesterol, esterified, % of dry weight	2-4	5	37	15

There are pathways within the body for interconversion among the four major classes.

As shown in the above table, the plasma lipoproteins contain varying proportions of protein and different types of lipid. The very low-density lipoproteins contain four different types of polypeptide chains having distinctive amino acid sequences. The high-density lipoproteins have two different types of polypeptide chains, of molecular weight 17,500 and 28,000. The polypeptide chains of the plasma lipoproteins are believed to be arranged on the surface of the molecules, thus conferring hydrophilic properties. However, in the very low-density lipoproteins and chylomicrons, there is insufficient protein to cover the surface; presumably the polar heads of the phospholipid components also contribute hydrophilic groups on the surface, with the nonpolar triacylglycerols in the interior. Biochemistry, Lehninger, Worth Publishers, Inc., New York, 1975, pp.301.

According to the most preferred embodiment of the present invention, an oxidized lipoprotein composition includes a low density lipoprotein solution, hydrogen peroxide, horseradish peroxidase and saline. In all embodiments described herein, the term "lipoprotein solution" is to be understood as lipoproteins in either saline or buffer. In addition, the lipoprotein solution may consist of LDL, HDL or VLDL whereas a lipoprotein solution containing LDL is the preferred embodiment of this invention.

Another embodiment is a composition of a solution of low density lipoproteins which includes one of the following oxidants: flavin, riboflavin, oxidase, peroxidase, horseradish peroxidase, lipoxidase, peroxide, organic peroxide or ditertiarybutyl peroxide.

The preferred embodiment of the method for preparing oxidized lipoproteins includes adding hydrogen peroxide and horseradish peroxidase to a solution of low density lipoproteins. The lipoproteins oxidized in this manner are then introduced into the patient. To determine and monitor the degree of peroxidation, the sample is subjected to carbon-13 NMR spectroscopy.

Another embodiment of the method of the present invention is oxidizing naturally occurring lipoproteins in a patient. Hydrogen peroxide itself or in conjunction with an enzyme such as peroxidase or lipoxidase is introduced into the patient by means of an atrioventricular shunt (or arterial bypass) or by means of an injection.

Administering non-oxidized but modified lipoproteins should enhance the effect of the organic peroxides in preparing the oxidized lipoproteins. The modified lipoproteins should be administered to the patient or added to the solution prior to oxidation of the lipoproteins. Modified lipoproteins are prepared by enriching the content of natural lipoproteins with triglycerides, such as

trilinoleal triglyceride, phospholipids such as dilinoleal phosphatidylcholine or cholesterol esters such as cholesterol ester of linoleic acid. These substances are believed to be more easily oxidized or result in more cytotoxic peroxidation products. The modified lipoproteins may be included in each embodiment of the present invention.

The lipid peroxidation process of the body may be further augmented by increasing the oxygen level in the blood via inhalation of increased levels of elemental oxygen during breathing. Perfluorocarbon fluosal may also be introduced into a person by means of an intravenous injection to increase the oxygen level in the blood. The oxygen level in the blood should be increased before oxidized lipoproteins are administered or oxidized in the body. Both of these methods of increasing the level of oxygen in the blood may be included in each embodiment of this invention.

In all the embodiments described herein, the blood may be monitored for oxidized lipoprotein level by carbon-13 nuclear magnetic resonance (NMR) spectroscopy. This procedure is taught by Eric T. Fossel in a copending U.S. Patent Application No. 557,529 filed July 24, 1990, where a method for the detection of cancer by measuring lipid peroxidation using NMR is disclosed. It has been discovered that in cancer patients, lipid peroxidation occurs and the ratio of polyunsaturated fatty acids to monounsaturated

fatty acids is different than in healthy subjects. NMR parameters for lipid methyl and methylene groups are determined and compared against a corresponding value for a healthy person.

In all embodiments described herein the lipoproteins may be peroxidized in a sample of whole blood, plasma or serum.

The apparatus of the invention is illustrated in FIG. 9 where the lipoproteins are peroxidized directly by the method of the invention. A peroxidizing module or chamber 50 contains an immobilized enzyme 52, such as peroxidase or lipoxidase, and an inlet 54 from a pump 55 which can very slowly and precisely introduce a flow of hydrogen peroxide into the module. Blood from a patient's artery is introduced through inlet 53 into the peroxidizing module. Blood containing peroxidized lipoproteins is returned to the patient's vein by outlet 51.

FIG. 11 depicts another embodiment of this invention wherein a blood 56 taken from patient 57 is stored in a container 58. A peroxide 59 is added to a separate container 60 and stored there. The peroxide 59 is then added to container 58 thereby causing oxidation of the lipoproteins in the blood. The oxidized lipoprotein-containing blood 61 is then reintroduced to the patient 50.

FIG. 12 illustrates yet another embodiment of the

present invention. Heparinized blood 66 is added to the bottom of a container 68 which holds an immobilized enzyme, such as horseradish peroxidase coated beads 70. Hydrogen peroxide 72 is introduced to the bottom of the container resulting in the formation of oxidized lipoproteins 74 in the blood which exits from the top of the container. The oxidized lipoprotein-containing blood 74 is stored in container 75 and then introduced to the patient 76 when treatment of a disease state is indicated.

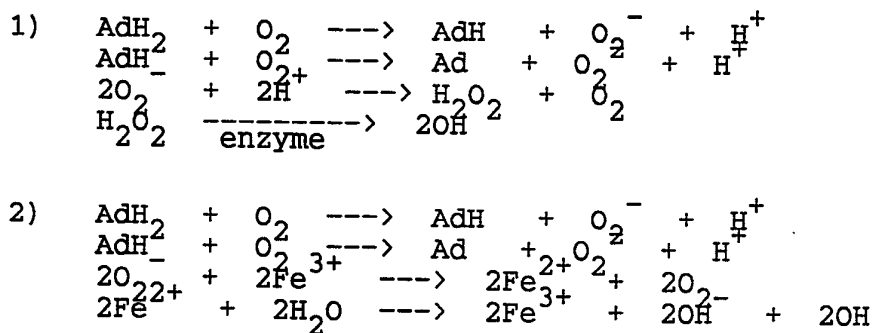
There is an additional method of oxidating lipoproteins, in accordance with the present invention, which consists of adding a photosensitizer to a lipoprotein solution and then irradiating the solution with ultraviolet light. It has been discovered, that by irradiating 8-methoxypsoralen, radical intermediates may be produced resulting in generation of hydroxyl radicals. In turn, these radicals cause oxidation of the lipoproteins which have a therapeutic effect on the diseased cells. Thus, blood is removed from the patient, 8-methoxypsoralen added to the blood and the sample then irradiated with ultraviolet light thereby causing oxidation of the lipoproteins in the blood.

Hematoporphyrins, used in the same manner as the 8-methoxypsoralen described above, are also known to cause death of cancer cells in irradiated blood. In accordance with the present invention, 8-methoxypsoralen as well as

hematoporphyrins are believed to effect peroxidation of lipoproteins.

There is yet another method for effecting oxidation of lipoproteins according to the present invention. This method consists of the addition of a chemotherapeutic agent to a lipoprotein-containing solution.

Adriamycin (Ad) and other molecules of its class have long been used as chemotherapeutic agents effective against certain types of cancer. They also have been suspected of generating free-radicals. The mechanism for such action is unknown, but two possible mechanisms are shown below:



Other metal ions can substitute for Fe^{3+} . In addition, mitomycin-D which is a molecule in the same class, can be used interchangeably with adriamycin as a chemotherapeutic agent. Thus, in accordance with the present invention, it is believed that adriamycin and mitomycin-D generate free radicals which in turn cause oxidation of lipoproteins.

In a separate patent application, filed on even date with the present application, Eric T. Fossel teaches that

oxidized lipoproteins may be helpful in fighting disease states, such as cancer, malaria and viral infections such as acquired immunodeficiency syndrome. These disease states are characterized by diseased cells with an increased number of lipoprotein receptors or an enhanced ability to take up lipoproteins. When low density lipoproteins are oxidized, they have a cytotoxic effect which preferentially kills diseased cells which have an enhanced ability to take-up lipoproteins. As part of its response to disease such as cancer, a human host oxidizes lipoproteins circulating in the blood. The diseased cells have been found to be more susceptible than healthy cells to the cytotoxic effect of oxidized lipoproteins. Thus, therapy with oxidized lipoproteins is believed to have the effect of killing the diseased cells.

FIG. 10A illustrates how the cytotoxicity of peroxidized low density lipoproteins helps fight cancer in humans. In nature, a cancer cell 30 is sensed by a macrophage 32 which secretes tumor necrosis factor (TNF) 34. The TNF 34 induces polymorphonuclear neutrophils (PMN) 31 to undergo a respiratory burst to release superoxide $O_2^{\cdot -}$. The superoxide causes the formation of hydroxyl free-radicals, $\cdot OH$ which in turn oxidize low density lipoproteins 33 to peroxidized low density lipoproteins (p-LDL) 35 while being converted into hydroxide ions, OH^- . The p-LDL 35 then exert their cytotoxic effect on malignant cells 36 leading to cell death. The malignant cells 36 killed by p-LDL 35 may the

same or different than the originally sensed tumor cell 30.

FIG. 10B illustrates the method of the present the invention. Low density lipoproteins 33 will be converted directly to peroxidized low density lipoprotein (p-LDL) by exposure to a peroxidizing agent 40. In one embodiment, the agent 40 is ditertiarybutyl peroxide. In another embodiment, the agent 40 is peroxidase together with a peroxide. The malignant cell 36 is killed by exposure to p-LDL.

The present invention is further illustrated by the following non-limiting examples.

Example 1

An oxidized low density lipoprotein composition is prepared from low density lipoproteins obtained from Sigma Chemical, catalog no. L2139 or prepared by standard methods from fresh human or animal plasma. Lindren FT, Silvers A, Jutagir R, Layshot L, Bradley DD. Lipids 1977; 12:278-282 and Lindren FT, Adamson GL, Jensen LC, Wood PD. Lipids 1975; 10:750-756. Oxidation of the low density lipoprotein is carried out using either soluble horseradish peroxidase (Enzyme Commission Classification No. 1.11.1.7) or immobilized horseradish peroxidase as a catalyst. The immobilized enzyme has the advantage that it can be removed

from the solution of oxidized low density lipoprotein before use. Each ml of low density lipoprotein solution (most preferably 5 mg. protein per ml) is diluted with an equal volume of Dulbecco's phosphate buffered saline and the peroxidase of either form is added to a level of approximately 800-1000 units per ml of solution. Following this, hydrogen peroxide, H_2O_2 , most preferably 0.1 ml of 3% hydrogen peroxide, is added per ml of low density lipoprotein solution. The solution is maintained at room temperature and 0.1 ml of peroxide solution per ml low density lipoprotein solution is added each hour for two hours. The peroxidation level is measured by the ratio of the intensity of the resonances at 128 and 130 ppm in the solution's carbon-13 NMR spectrum. A lower ratio indicates a reduction of the amount of polyunsaturated fatty acid side chains in the lipoprotein lipids. The 128/130 ppm ratio is typically greater than 0.9 before peroxidation and between 0.7 and 0.85 after peroxidation.

Example 2

Peroxidized low density lipoproteins are prepared by treating human low density lipoprotein, most preferably 5mg of protein/ml, (as described in Example 1) with horseradish peroxidase Type II, most preferably 2 mg/ml. This is followed by the addition of approximately 70 to 200 μ liters of hydrogen peroxide, most preferably 3% hydrogen peroxide in one or two equal aliquots, the second addition being made

several hours after the first addition. The peroxidation level is measured by the ratio of the intensity of the resonances at 128 and 130 ppm in the solution's carbon-13 NMR spectrum as described in Example 1.

This technique can be employed with any lipoprotein. It is particularly desirable to peroxidize low density lipoproteins.

FIG. 7 shows the olefinic region of a spectrum from a normal plasma sample. The ratio of the peak at 128-129 to the peak at 130-131 is near one. FIGS. 8A & B show the olefinic region of 125.8 MHz proton decoupled C-13 spectra from normal human plasma and the same plasma following the addition of peroxidase, most preferably 2mg/ml and 3 aliquots of 3% hydrogen peroxide (100 μ l/ml) at hourly intervals. The 128/130 ratio was substantially decreased following treatment of the plasma, indicating that peroxidation has occurred.

Example 3

Oxidized lipoproteins were prepared by reacting 8-methoxypsoralen was reacted with LDL. LDL was isolated from fresh human plasma and then dialyzed overnight to remove EDTA. Lipid concentration was monitored using C-13 NMR spectroscopy and the LDL preparation was then stored at 4° C and used within two weeks. The photosensitized

reactions were carried out using 540 μ l (4-5 mg protein/ml) and 60 μ l of 8-methoxypsoralen stock solution. The final concentration of 8-methoxypsoralen was 200 ng/ml. The reaction was allowed to equilibrate for 30 minutes with stirring before irradiation at 27-30^o C. Six Sylvania FR15T12 PUVA lamps irradiated the samples for up to six hours and any reduction in volume was replenished every 30 minutes during irradiation. C-13 NMR spectra of all samples were obtained before and after the addition of 8-methoxypsoralen and the irradiation. These spectra were used to monitor the photosensitized reactions of the lipids of LDL and characterize the chemical changes induced by these reactions.

The control plasma showed no change in the 128/130 ppm C-13 ratio following 30 minutes of ultraviolet A irradiation (the ratio was 0.98 before and 0.97 afterwards). However, the 8-methoxypsoralen containing plasma showed a reduction of the ratio from a pre-irradiation value of 0.98 to 0.77 following 30 minutes of light treatment. Thus, the results show that free-radical induced oxidation appears to occur and may be responsible for the therapeutic effect observed by Edelson et al.

Example 4

Oxidized lipoproteins were prepared by the addition of adriamycin to six aliquots of normal plasma and bubbling

intermittently with 95% O₂:5% CO₂. An equal amount of adriamycin was also added to six additional aliquots of plasma and bubbled similarly with 95% N₂:5% CO₂. The ratio of the 128/130 ppm resonances changed as shown in the Table below. The lower ratio, as compared with the control sample, indicates that peroxidation has occurred. Adriamycin mediated lipid peroxidation occurred in the presence of oxygen but not in its absence.

	<u>128/130 ppm</u> <u>intensity</u>
Control plasma	0.96 +/- 0.06 (n = 6)
Control plasma + Adriamycin + O ₂	0.72 +/- 0.09 (n = 6)
Control plasma + Adriamycin + N ₂	0.94 +/- 0.05 (n = 6)

Example 5

Ditertiarybutyl peroxide is administered to a person by i.v. (intravenous) injection. The level of oxidized lipoprotein is monitored by nuclear magnetic resonance and the ditertiarybutyl peroxide dose adjusted accordingly.

The patient's blood oxygen supply may also be augmented by inhalation of elemental oxygen or by i.v. injection of perfluorocarbon fluosal before administering the ditertiarybutyl peroxide.

Additionally, the patient's supply of lipoproteins may be augmented by intravenous injection of lipoproteins enriched with triglycerides, phospholipids, or cholesterol esters before administering the ditertiarybutyl peroxide.

The ditertiarybutyl peroxide of this procedure may be replaced with any of the following in its proper dose: riboflavin, peroxidase, lipoxidase, or other flavins, peroxides, organic peroxides or oxidases.

Example 6

An atrioventricular shunt or arterial bypass is attached to a person. An extracorporeal peroxidizing module is attached to the AV shunt or arterial bypass. It has an inlet fluid connection from a pump which introduces hydrogen peroxide into the module which contains peroxidase or lipoxidase which peroxidizes the plasma lipoproteins in the presence of the hydrogen peroxide.

A supply of blood is secured. The blood supply source may be a donor, a blood bank, or any other blood supply source.

The lipoproteins of the blood supply are then oxidized by adding an oxidant to the blood, thus producing of oxidized lipoproteins.

The oxygen available in the blood may be further

increased by adding elemental oxygen or perfluorocarbon fluosal to the blood.

The lipoprotein content of the blood can also be augmented by adding lipoproteins enriched with triglycerides, phospholipids, or cholesterol esters.

While the foregoing invention has been described with reference to its preferred embodiments, various alterations and modifications will occur to those skilled in the art. All such alternations and modifications are intended to fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for preparing oxidized lipoproteins comprising:
 - (a) providing a sample of bodily fluid;
 - (b) introducing the sample into a container holding an immobilized enzyme and
 - (c) introducing a peroxide to the sample in said container thereby producing oxidized lipoproteins.
2. The method of claim 1 wherein said sample comprises whole blood.
3. The method of claim 1 wherein said sample comprises plasma.
4. The method of claim 1 wherein said sample comprises serum.
5. The method of claim 1 wherein said sample comprises high density lipoprotein.
6. The method of claim 1 wherein said sample comprises purified low density lipoprotein.
7. The method of claim 1 wherein said sample comprises very low density lipoprotein.

8. The method of claim 1 wherein said enzyme is horseradish peroxidase.

9. The method of claim 1 wherein said peroxide is hydrogen peroxide.

10. The method of claim 1 wherein said sample comprises heparinized blood.

11. The method of claim 1 wherein said immobilized enzyme comprises horseradish peroxidase coated beads.

12. The method of claim 1 wherein a carbon-13 NMR spectrum of the oxidized low density lipoprotein solution is obtained to determine the extent of lipid peroxidation as measured by the 128/130 ppm ratio.

13. The method of claim 1 further comprising administering non-oxidized, modified lipoproteins which have been enriched with specific triglycerides, phospholipids or cholesterol esters.

14. The method of claim 1 further comprising increasing the oxygen available in the blood by adding elemental oxygen or perfluorocarbon fluosal.

15. A method of preparing oxidized lipoproteins comprising preparing a solution of low density lipoproteins

and oxidizing the lipoproteins by adding an oxidant selected from the group consisting of a flavin, riboflavin, an oxidase, a peroxidase, horseradish peroxidase, a lipoxidase, a peroxide, an organic peroxide or ditertiarybutyl peroxide.

16. The method of claim 15 wherein said lipoprotein solution contains low density lipoproteins in saline.

17. The method of claim 15 wherein said lipoprotein solution contains low density lipoproteins in buffer.

18. A method for preparing oxidized lipoproteins comprising:

(a) administering an oxidant to a person by means of an intravenous injection in an amount sufficient to effect oxidation;

(b) monitoring the level of lipoprotein oxidation by carbon-13 nuclear magnetic resonance and

(c) adjusting the dose of the oxidant accordingly.

19. The method of claim 18 wherein the oxidant is selected from the group consisting of riboflavin, peroxidase, lipoxidase, flavin, peroxide, organic peroxide and oxidase.

20. The method of claim 18 further comprising increasing the oxygen available in the blood by inhalation of elemental oxygen or injection of perfluorocarbon

fluosal.

21. The method of claim 18 further comprising injecting modified lipoproteins enriched with triglycerides, phospholipids or cholesterol esters.

22. A method for preparing oxidized lipoproteins comprising:

- (a) providing a sample of bodily fluid;
- (b) adding a photosensitizer and
- (c) irradiating the mixture with ultraviolet light thereby producing oxidized lipoproteins.

23. The method of claim 22 wherein said sample comprises whole blood.

24. The method of claim 22 wherein said sample comprises plasma.

25. The method of claim 22 wherein said sample comprises serum.

26. The method of claim 22 wherein said sample comprises high density lipoprotein.

27. The method of claim 22 wherein said sample comprises purified low density lipoprotein.

28. The method of claim 22 wherein said sample comprises very low density lipoprotein.

29. The method of claim 22 wherein the photosensitizer is a hematoporphyrin.

30. The method of claim 22 wherein the photosensitizer is 8-methoxypsoralen.

31. The method of claim 22 wherein a carbon-13 NMR spectrum of the oxidized low density lipoprotein solution is obtained to determine the extent of lipid peroxidation as measured by the 128/130 ppm ratio.

32. A method for preparing oxidized lipoproteins comprising:

- (a) providing a sample of fresh human plasma;
- (b) isolating low density lipoproteins from the plasma;
- (c) adding 8-methoxypsoralen;
- (d) stirring the mixture;
- (e) irradiating the mixture with ultraviolet light for up to six hours thereby producing oxidized lipoproteins and
- (f) obtaining a carbon-13 NMR spectrum of the oxidized low density lipoprotein solution to determine the extent of lipid peroxidation as measured by the 128/130 ppm ratio.

33. A method for preparing oxidized lipoproteins comprising:

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- (a) providing a sample of plasma;
- (b) introducing a chemotherapeutic agent to the sample and
- (c) bubbling the mixture intermittently with a mixture of O_2 and CO_2 thereby producing oxidized lipoproteins.

34. The method of claim 33 wherein said chemotherapeutic agent is adriamycin.

35. The method of claim 33 wherein said chemotherapeutic agent is mitomycin-D.

36. The method of claim 33 wherein a carbon-13 NMR spectrum of the oxidized low density lipoprotein solution is obtained to determine the extent of lipid peroxidation as measured by the 128/130 ppm ratio.

37. A method for preparing oxidized low density lipoproteins comprising:

- (a) providing a solution of low density lipoproteins;
- (b) subjecting the low density lipoprotein solution to a peroxide in the presence of an enzyme catalyst capable of catalyzing the oxidation of low density lipoproteins;
- (c) obtaining a carbon-13 NMR spectrum of the oxidized low density lipoprotein solution to determine the extent of lipid peroxidation as measured by the 128/130 ppm ratio and
- (d) repeating steps (b) and (c) until the desired quantity of oxidized low density lipoproteins is obtained.

38. A method for preparing oxidized low density lipoproteins comprising:

(a) preparing a solution of low density lipoprotein solution from fresh human or animal plasma or commercially prepared plasma;

(b) diluting each ml of said low density lipoprotein solution with an equal volume of phosphate buffered saline;

(c) adding horseradish peroxidase;

(d) maintaining the solution at room temperature;

(e) adding hydrogen peroxide lipoprotein each hour for two hours;

(f) obtaining a carbon-13 NMR spectrum of the oxidized low density lipoprotein solution to determine the extent of lipid peroxidation as measured by the 128/130 ppm ratio and

(g) storing the preparation at 4° C until used.

39. An oxidized low density lipoprotein composition comprising:

(a) low density lipoprotein solution;

(b) immobilized enzyme;

(c) saline solution and

(d) peroxide.

40. An oxidized low density lipoprotein composition comprising:

(a) human low density lipoprotein with horseradish peroxidase Type II and

(b) 3% hydrogen peroxide in one or two aliquots.

41. An oxidized low density lipoprotein composition comprising:

- (a) a solution of low density lipoprotein solution from fresh human or animal plasma or commercially prepared plasma;
- (b) phosphate buffered saline;
- (c) horseradish peroxidase and
- (d) hydrogen peroxide.

42. An apparatus for oxidizing lipoproteins comprising:

- (a) a peroxidizing module containing an immobilized enzyme;
- (b) an inlet from a pump and
- (c) a pump which can precisely introduce a flow of hydrogen peroxide into the module.

43. An apparatus for oxidizing lipoproteins comprising:

- (a) an atrioventricular shunt or arterial bypass which is attached to a person;
- (b) an extracorporeal peroxidizing module attached to said shunt or bypass which contains an immobilized enzyme;
- (c) an inlet fluid connection from a pump to said module and
- (d) a pump which introduces hydrogen peroxide into the module.

44. The apparatus of claim 43 wherein the said enzyme is lipoxidase or peroxidase.

45. An apparatus for oxidizing lipoproteins comprising:

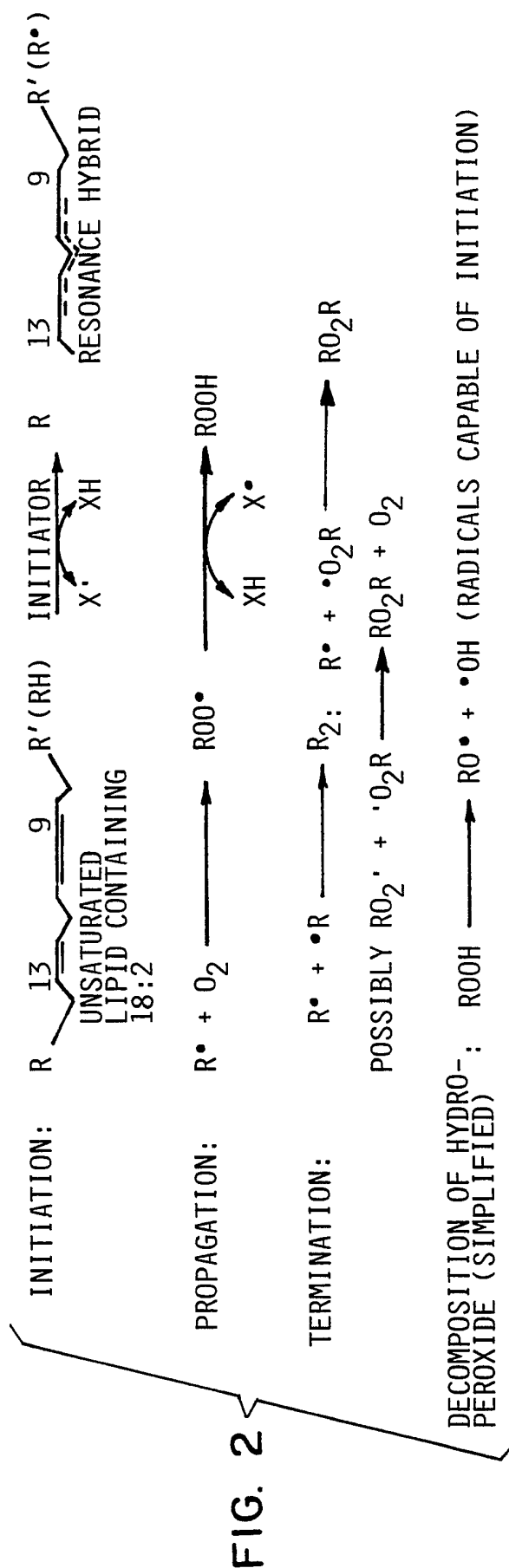
- (a) a container with heparinized blood holding an immobilized enzyme;
- (b) means for introducing hydrogen peroxide to the bottom of the container and
- (c) means for exiting the blood containing oxidized lipoproteins from the top of the container.

46. The apparatus of claim 45 wherein the said immobilized enzyme comprises horseradish peroxidase coated beads.

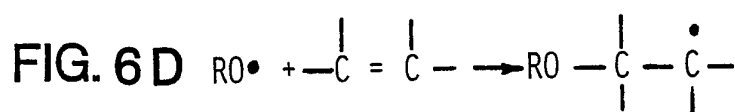
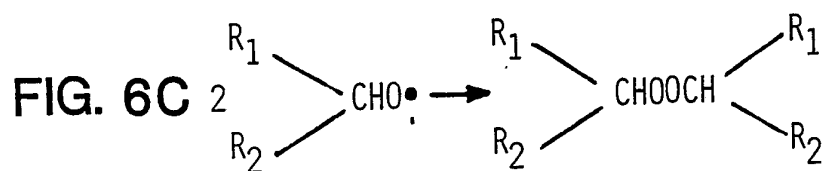
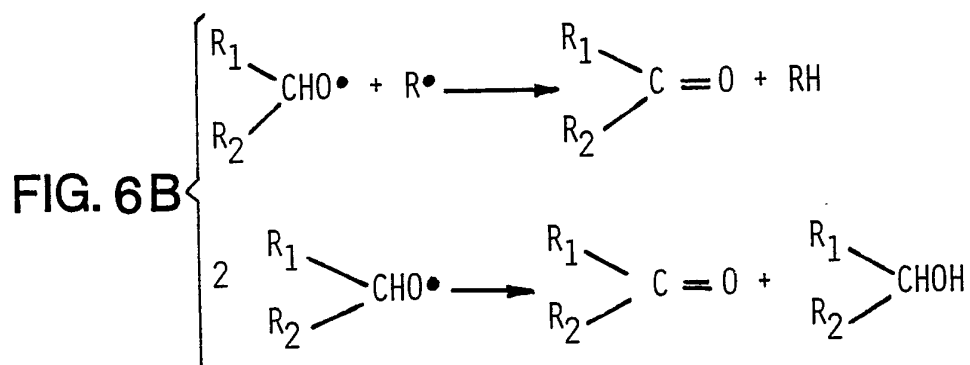
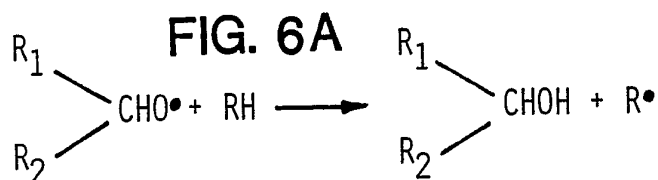
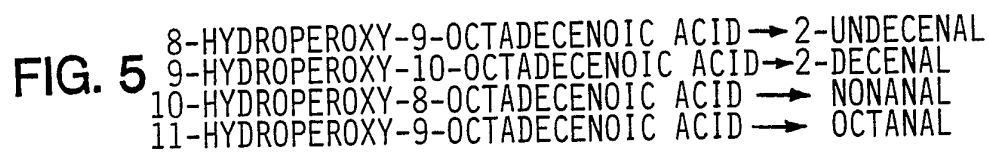
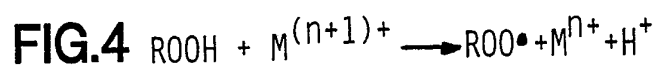
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FIG. 1

$$\frac{d O_2}{dt} = \frac{K_0 (RH) (ROOH)}{1 + \frac{\lambda (RH)}{p O_2}}$$



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FIG. 7

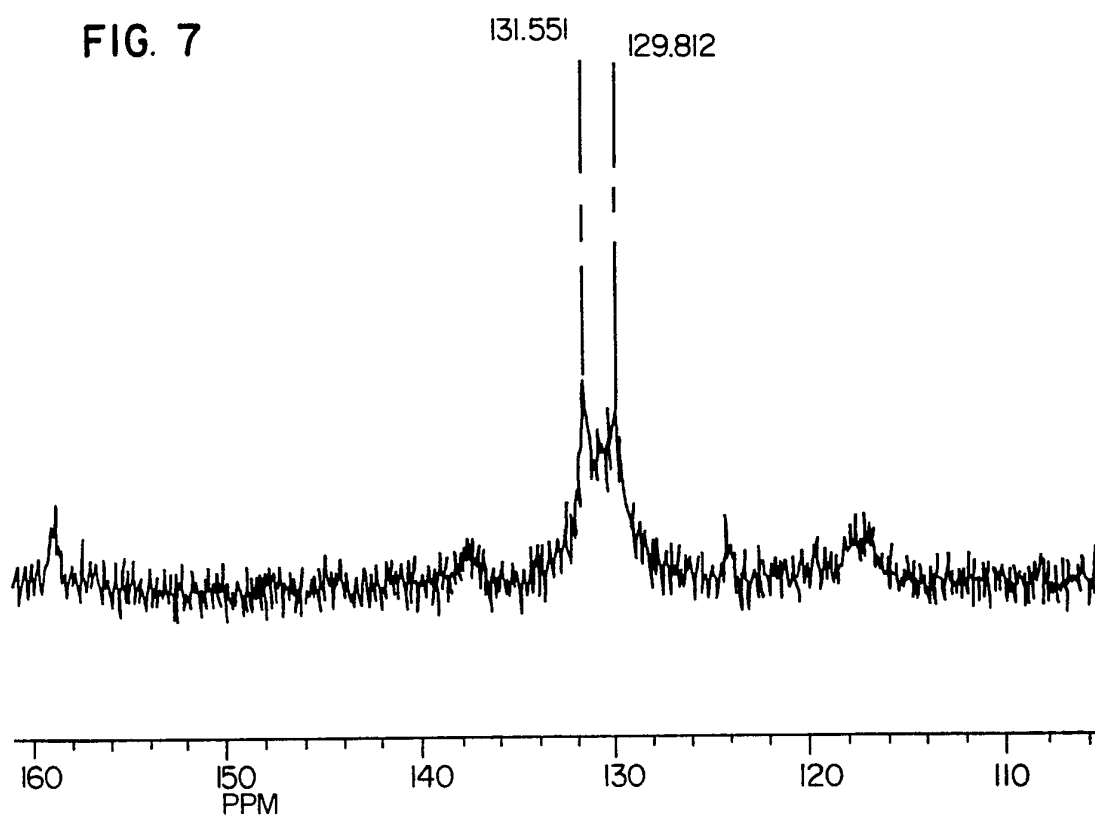
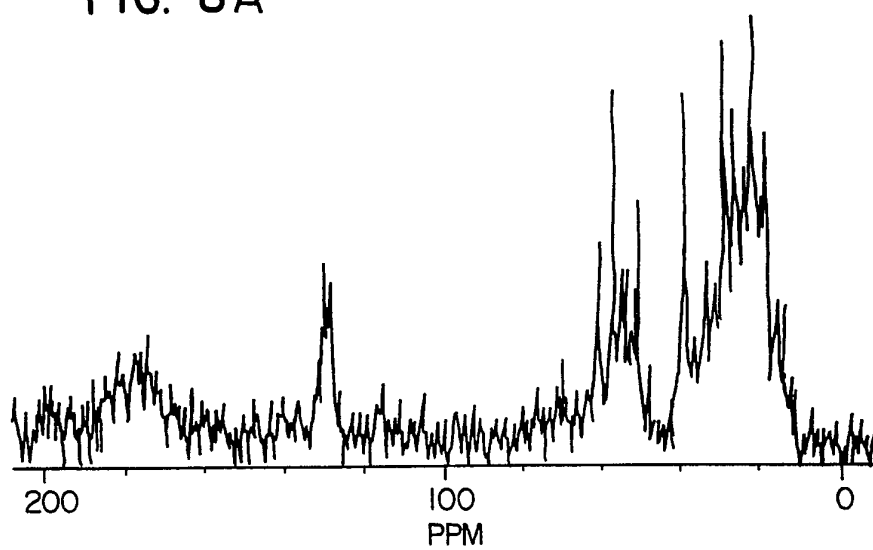


FIG. 8A



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FIG. 8B

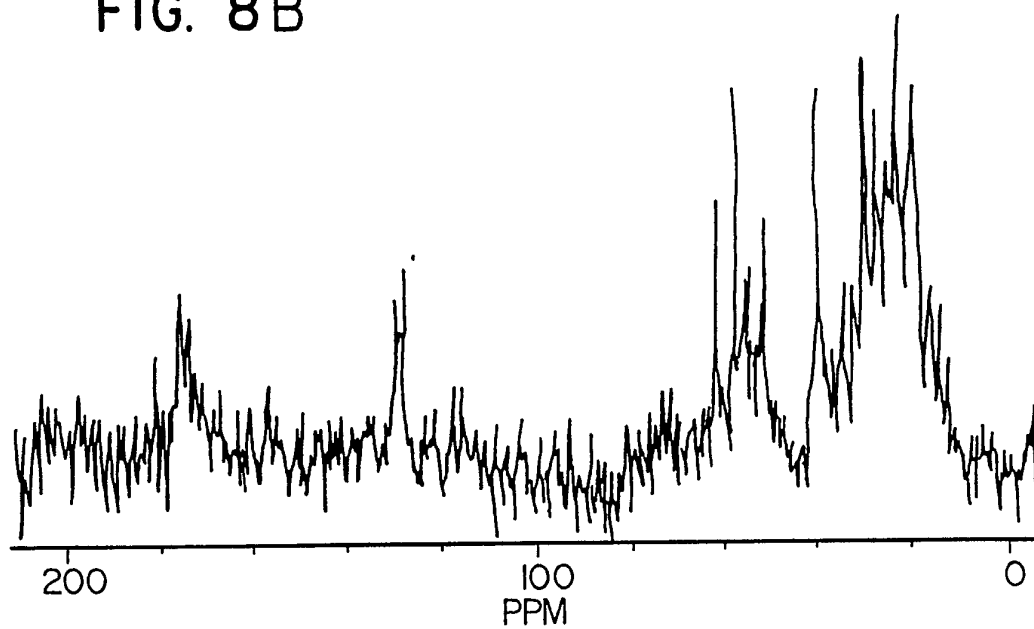
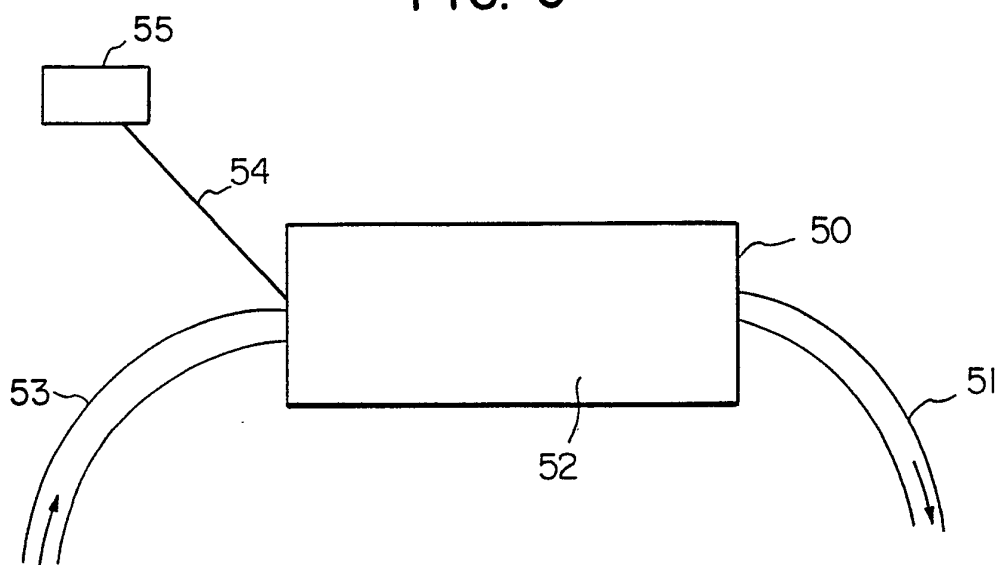


FIG. 9



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FIG. 10A

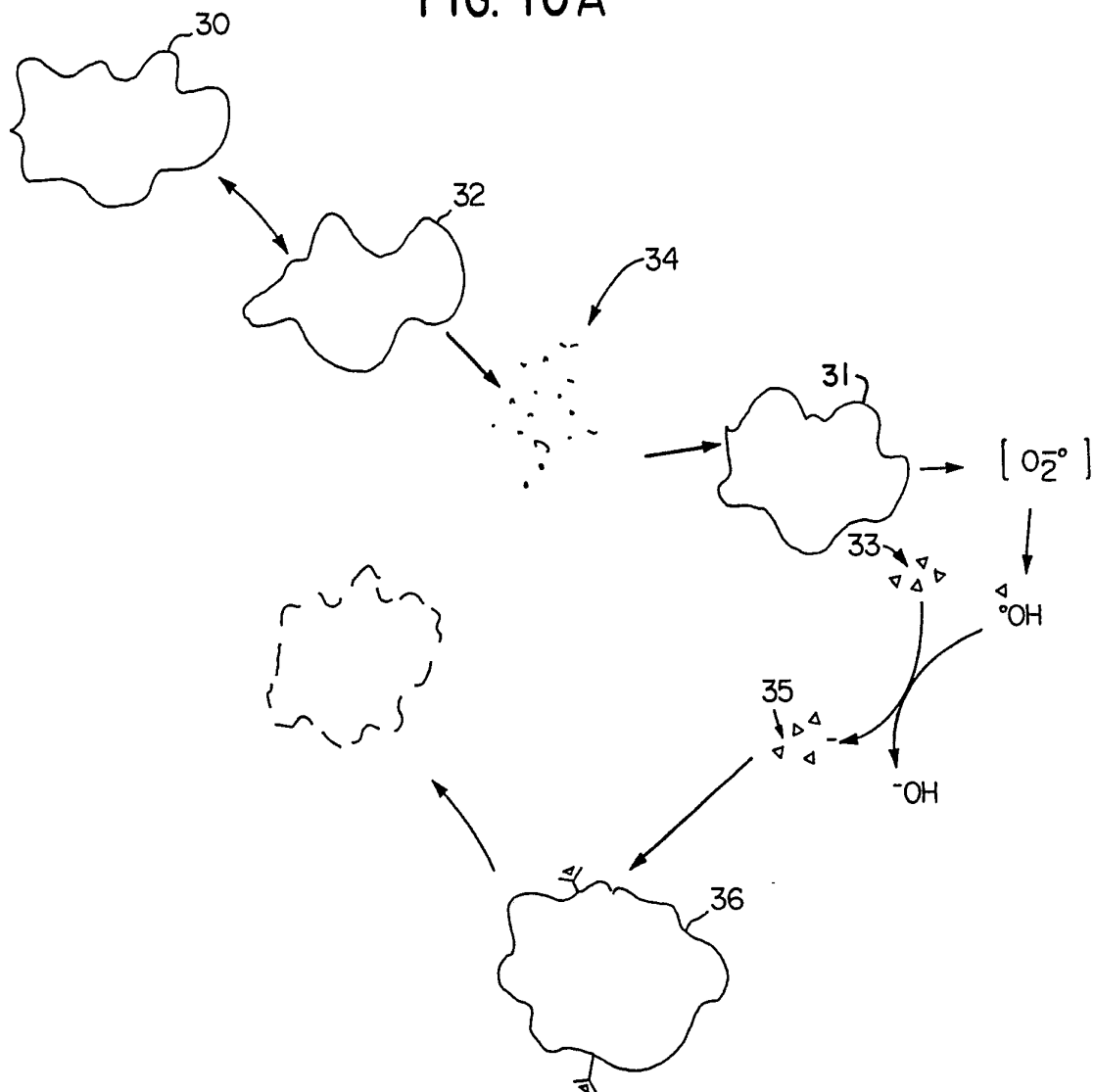
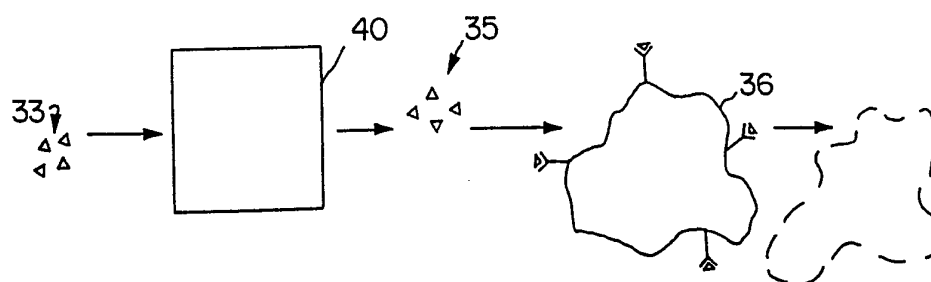


FIG. 10B



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FIG. 11

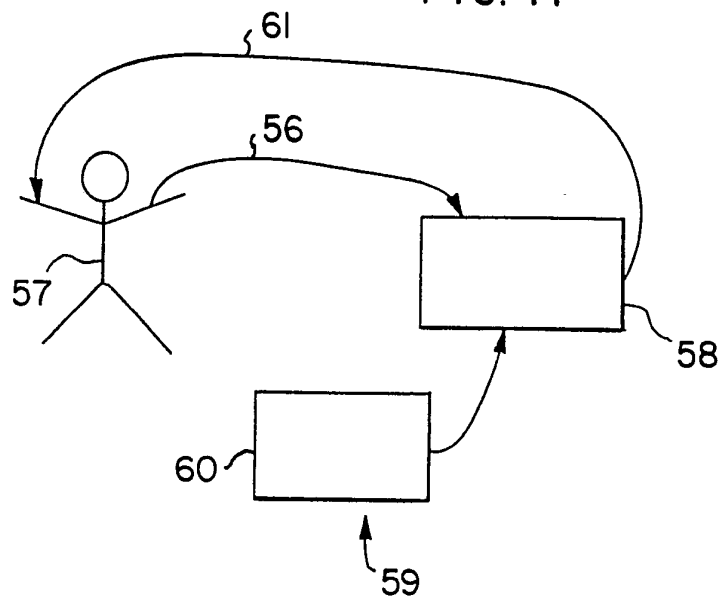
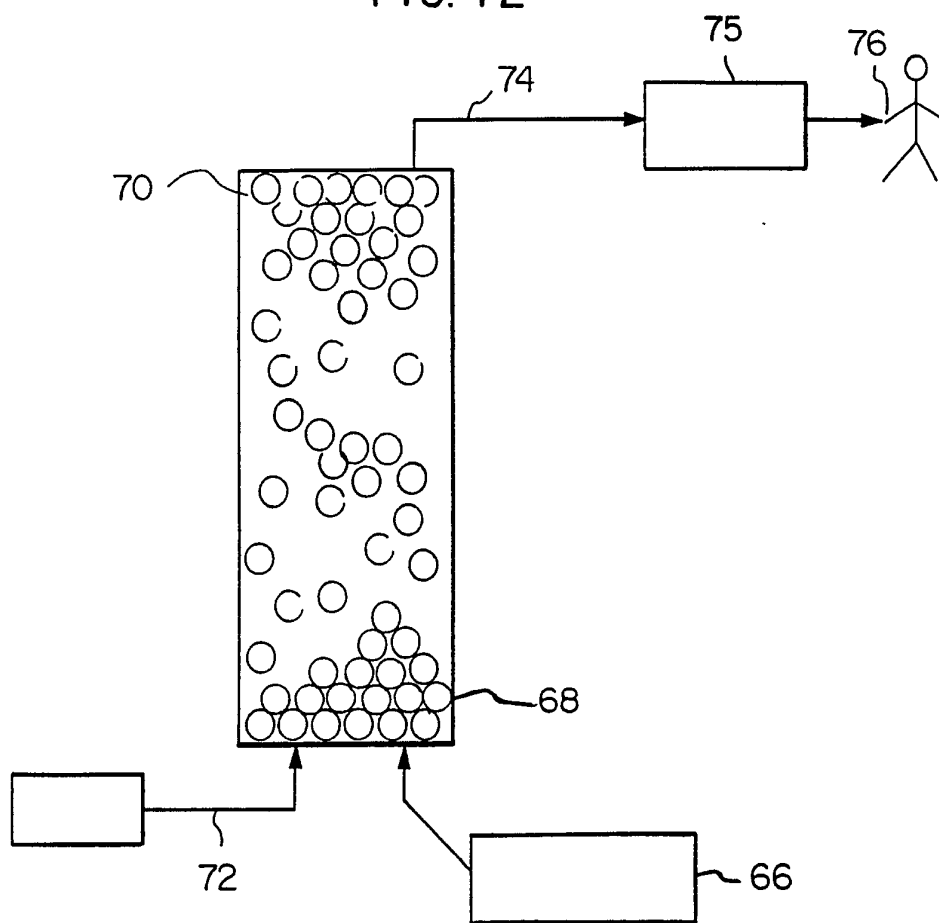


FIG. 12

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