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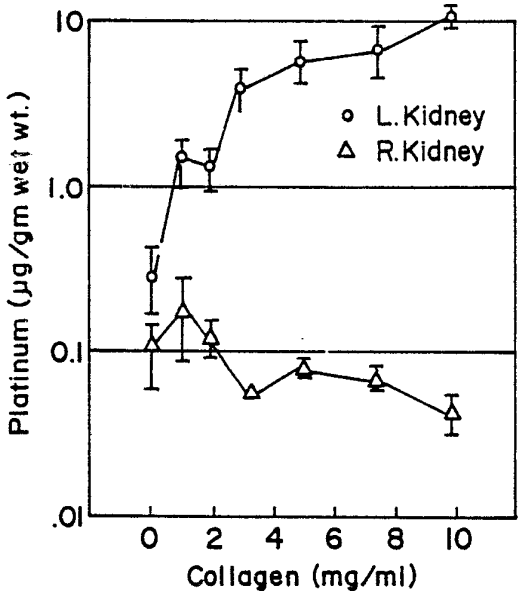
(54) Title: VASO-OCCLUSIVE COLLAGEN COMPOSITION AND METHOD

(57) Abstract

A method and composition of achieving persistent occlusion of blood vessels, for a period of at least about two weeks. The method includes (a) providing a vaso-occlusive material composed of a suspension of atelopeptide collagen fibers which are cross-linked under conditions which raise the melting temperature of the suspension at least about 10°C, and (b) introducing the material into the blood vessels to be occluded. Also disclosed are novel methods which employ vaso-occlusion for enhancing hyperthermia and drug treatments, particularly of solid-tumor tissue.



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VASO-OCCLUSIVE COLLAGEN COMPOSITION AND METHOD

1. Field of the Invention

The present invention relates to vaso-occlusive collagen compositions, and to therapeutic methods based on vaso-occlusion at a selected tissue target site.

2. References

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3. Background of the Invention

Endovascular occlusive therapy has been used in treating a variety of conditions, such as controlling internal bleeding and occluding blood supply to tumors (Athanasoulis, Wallace, Reuter). It is well known that collagen is a hemostatic agent and there are several reports describing the use of a microfibrillar collagen

as a transcatheter embolic agent. The use of microfibrillar collagen as an embolic agent and its use in the preoperative embolization of hypervascular head and neck neoplasms has been reviewed (Kumar). The microfibrillar collagen used was a powdery substance that was mixed with an aqueous contrast material to form a slurrylike suspension. Even though suspensions of microfibrillar collagen have better flow characteristics and vascular bed penetration than alternative agents such as IVALON foam or gelfoam sponge, they are nonetheless nonhomogeneous and relatively difficult to administer by means of small diameter catheters. Also, there are reports that microfibrillar collagen is inflammatory and causes vasculitis when injected into blood vessels (Kaufman).

U.S. Patents Nos. 3,949,073 and 4,140,537 describe an atelopeptide collagen suspension prepared by solubilizing collagen, treating it with enzyme to remove telopeptide groups, purifying the resulting atelopeptide collagen, and partially reconstituting it to form a fibrillar suspension. A commercial embodiment of this atelopeptide collagen is sold under the trademark ZYDERM®, and is used for augmenting soft tissue. The fibrillar collagen suspension is less immunogenic than undigested, unpurified collagen. Also, the suspension is substantially free of particulate or clumped matter, so it can be easily delivered from a small diameter catheter without spiking, at a controlled flow rate.

The atelopeptide collagen suspension just mentioned has been tested as a vaso-occlusion material in a variety of model systems in which the distribution and persistence of the material can be evaluated. The suspension typically contained a contrast agent, such as diatrizoate meglumine, which allowed the distribution

of vaso-occlusion to be monitored fluoroscopically. When administered by catheter into a localized tissue region, the material was found to localize, by flow-direction infusion, in vessels having lumen diameters predominantly in the 20-250 micron size range. Since many of the arterial vessels in this size range are distal to the collateral vessels in the vascular bed, the material was quite effective in reducing blood flow into the tissue area, by preventing both direct and collateral circulation. Despite these advantages, the atelopeptide material showed rather poor persistence, typically being cleared from the infused vessels within a few days when formulated with a contrast agent.

4. Summary of the Invention

It is a general object of the invention to provide a vaso-occlusive collagen composition which has the advantages of the above-described atelopeptide collagen material, in terms of favorable immunogenic and flow characteristics, and ability to produce occlusion distal to collateral circulation, but which is persistent in vessels for selected periods of at least about two weeks and up to six months or more.

Another object of the invention is to provide a method for producing persistent vaso-occlusion with a cross-linked collagen material, where the persistence time of the material can be selectively varied according to the degree of cross-linking.

One specific object of the invention is to provide a composition and method for producing persistent occlusion of the small arterial vessels supplying a tumor region in a tissue, and a novel method for treating solid tumors which combines vaso-occlusion

and hyperthermia, to produce selective heating effects in the vaso-occluded tumor area.

Another specific object of the invention is to provide a composition for producing persistent occlusion of large arteries and veins, and a composition for use in delivering a drug to a selected vascular site, such as a tumor site.

In one aspect, the invention includes a method of achieving persistent occlusion of blood vessels for a period of at least about two weeks. The method employs a vaso-occlusive material composed of a suspension of atelopeptide collagen fibers which are cross-linked under conditions which raise the melting point of the suspension at least about 10°C, and preferably 15°C or more. The cross-linked collagen material may be combined with a radio-opaque contrast agent, such as diatrizoate meglumine, without appreciable loss of persistence when injected. The material is introduced typically by catheter injection into the vessel(s) to be occluded.

The method may be used to achieve vaso-occlusion for a selected period of between about two weeks and up to six months or more. Greater persistence times are achieved by cross-linking the collagen fibers under conditions which lead to increased cross-linking, as measured, for example, by the percent of total lysine residues which are cross-linked, between about 15-85 percent. Alternatively, the embolic material may be infused repeatedly at periodic intervals of two weeks or more.

If injected in relatively dilute form, the cross-linked material is adapted to be carried by flow-directed infusion into relatively small (20-250 micron) vessels fed by an arterial segment which is

accessible by catheter. Alternatively, the material can be injected in a pastelike form, for occluding larger arterial vessel or for administration in a retrograde fashion into veins. The material may be used to arrest hemorrhaging, to seal off tissue regions prior to surgery, to block blood flow to a tumor region, to starve a tumor region, and/or as an adjunct to hyperthermic tumor treatment, and in treating arteriovenous malformations.

In another aspect, the invention includes an improved method for treating an internal tissue, particularly a solid tumor tissue, by hyperthermia. In the method, a vaso-occlusive material is introduced at the target site, to produce localized vaso-occlusion, and this region is heated to raise the temperature of the region to at least about 42° C, and preferably between about 44° and 46° C. According to an important feature of the invention, the heating is effective to produce a temperature differential, between occluded and nonoccluded tissue, of at least about 2° C. The heating is applied for a time sufficient to produce selected tissue necrosis in the occluded tissue. The vaso-occlusive material preferably is persistent at the occluded site for 2 weeks or more, allowing for multiple heat treatments over the period of occlusion. At the same time, the persistent vaso-occlusive material contributes to selective tumor destruction through hypoxic effects.

Another aspect of the invention is an improved method for administering a drug at a localized target tissue site, by injecting a mixture of collagen vaso-occlusive agent and drug to the vessel(s) supplying the target site. The drug administered in this form shows a severalfold increase in accumulation at the

target site, when compared with drug administered by intravenous administration, intraarterial administration, or by intraarterial administration with proximal balloon occlusion.

The invention also includes a nonimmunogenic vaso-occlusive composition having a biological persistence of greater than about two weeks. The composition includes a vaso-occlusive material composed of atelopeptide collagen fibers which are cross-linked under conditions which raise the melting temperature of the suspension at least about 10°C. One novel composition includes the collagen suspension in combination with a contrast agent, for use in vaso-occlusion applications where it is desired to monitor the distribution of vaso-occlusiive material at a target site. Another novel composition includes the collagen suspension in combination with a pharmacological agent, such as a therapeutic drug, for use in localized drug delivery at the site of vaso-occlusion.

These and other objects and features of the invention will become more fully apparent from the following detailed description of the invention, when read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1A shows a calorimetric melting curve for fibrillar collagen cross-linked with 0.0075% glutaraldehyde;

Figure 1B is a plot of melting temperatures of cross-linked collagen as a function of percent glutaraldehyde used in the cross-linking reaction;

Figure 2 is a plot of free lysine content of samples cross-linked with varying concentration of glutaraldehyde;

Figure 3 shows the time course of digestion of noncross-linked (triangles) and cross-linked (circles) fibrillar collagen by bacterial collagenase, over a two-hour period;

Figure 4A is a plot of the log of viscosity, as a function of shear rate for noncross-linked fibrillar collagen at 35 (solid circles), 50 (+), and 65 (open circles) mg/ml;

Figure 4B is a plot of concentration-dependent viscosities like that of Figure 4A, for collagen cross-linked with 0.0075% glutaraldehyde;

Figure 5 is a simplified view of a tissue region containing a network of branch vessels fed by a supply artery, here shown containing a double balloon catheter used for injecting vaso-occlusive collagen into the region;

Figures 6A-6C are graphic representations of photomicrographs showing in cross-section vessels which have been vaso-occluded according to the method of the invention, examined 1 week (A), 1 month (B), and 2 months (C) after vaso-occlusion;

Figure 7 shows changes in liver function, as reflected by changes in alkaline phosphatase, bilirubin, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), as a function of time after collagen embolization of the hepatic artery;

Figure 8 shows tissue deposition of cisplatin in kidney, left and right liver, muscle, and plasma two hours after drug administration by (a) intravenous (iv) administration, (b) intraarterial infusion into the common hepatic artery, (c) intraarterial infusion into

the hepatic artery with proximal balloon occlusion, and (d) collagen chemoembolization of the hepatic artery;

Figure 9 shows tissue deposition of cisplatin in left and right kidney cortex, left and right liver, and lung two hours after drug administration by (a) intravenous administration, (b) intraarterial infusion into the left kidney, and (c) chemoembolization of the left kidney; and

Figure 10 shows the uptake of cisplatin in left and right kidneys for drug administration by collagen chemoembolization in the left kidney, as a function of collagen concentration in the embolic material.

Detailed Description of the Invention

I. Vaso-Occlusive Composition

A. Preparing an Atelopeptide Collagen Suspension

The collagen suspension used in forming the cross-linked vaso-occlusive material of the invention is prepared from a aqueous solution of atelopeptide collagen, such as that described in U.S. Patent No. 4,140,537, and incorporated herein by reference. The collagen is preferably derived from cutaneous mammalian sources, such as bovine or porcine corium.

To prepare the collagen solution, acid-treated corium is digested with a proteolytic enzyme, such as pepsin or papain, that attacks the telopeptide but not the helical portion of collagen. The digestion is carried out typically for 2-14 days at a pH between 1.5 and 5. After denaturing the enzyme and filtering the fiber solution, the filtrate may be fractionated by ion-exchange chromatography to produce a substantially pure atelopeptide collagen fiber solution.

The collagen solution is reconstituted to form a suspension of collagen fibers by incubating the solution at a pH preferably between about 5 and 8 under hypotonic conditions. Within this range, pHs below about 7 favor formation of fine, soft fibers whereas pHs above 7 favor formation of coarser fibers, which may be more difficult to inject. Reconstitution to fibrillar collagen generally occurs within about 1-18 hours under the above incubation conditions. The preparative method described in Example 1A below is illustrative.

The fibrillar suspension may be further treated by forcing the suspension through a screen of defined pore size after or during the reconstitution step. This procedure, called "screening", breaks up fibrillar aggregates present in the reconstituted fiber suspension and gives a more uniform fiber size distribution. A preferred screening protocol is to pass the fiber suspension through a 60 mesh stainless steel screen at about 5°C and at a flow rate of about 7-7.5 1/min. The suspension is recirculated through the screen repeatedly, with about 50 or more passes through the screen being optimum. After the screening, the fibrillar suspension is incubated in the reconstitution medium for an additional 6 to 15 hr. The screening procedure is detailed in U.S. patent application for "Mechanically Sheared Collagen Implant Material and Method", U.S. Serial No. 715,098, filed 22 March 1985.

The screening conditions just described produce fiber sizes with an average size of about 70 microns in length and 5 micron in fiber width. Very little change in fiber size is observed when the screened fibers are cross-linked under mild cross-linking conditions.

B. Collagen Cross-Linking

According to an important aspect of the invention, it has been found that the biological persistence of atelopeptide collagen fibers can be extended from a period of only a few days to at least about two weeks by cross-linking the reconstituted collagen fibers under defined reaction conditions; and further, that the biological persistence can be selectively increased, for periods of up to six months or more, by increasing the extent of cross-linking.

The concentration of collagen in the cross-linking reaction is preferably adjusted to 0.1 to 10 mg protein/ml, and usually between about 1-5 mg/ml. At relatively high collagen concentrations, more interfibrillar cross-linking occurs, at a given concentration of cross-linking agent, leading to larger and more heterogeneous fiber sizes. The larger sizes may be suitable in preparing a cross-linked material for use in occluding large vessels. At lower collagen concentrations, and particularly when the concentration of the cross-linking agent is also low, fiber size may not increase significantly over that of the original collagen suspension, indicating that much of the cross-linking may be intrafibrillar. Cross-linked collagen material having smaller fiber sizes are generally preferred in producing vaso-occlusion of small vessels, e.g., having lumen sizes between about 20-250 microns.

The cross-linking agent is preferably an aldehyde -- such as formaldehyde, glutaraldehyde, acetalaldehyde, glycoxal pyruvic aldehyde, and dialdehyde starch -- which is capable of reacting with and cross-linking free amine groups in collagen. Glutaraldehyde is a preferred cross-linking reaction.

The concentration of cross-linking agent is adjusted to produce a selected degree of cross-linking needed to achieve the desired biological persistence of the material. When glutaraldehyde is used, relatively light cross-linking is achieved at concentrations between about 0.005% to 0.01%. The cross-linking method detailed in Example 1B is illustrative.

Where relatively larger-size and/or more persistent material is required, cross-linking at concentrations up to 2% glutaraldehyde or greater may be suitable. The extent of cross-linking can be gauged by one of a number of changes in the characteristics of the fibrillar material, as will be discussed below. A preferred method for gauging the extent of cross-linking, under relatively light cross-linking conditions, is by melting point calorimetry. For cross-linking under relatively heavy conditions, the percent lysine residues present in the collagen is a preferred test for degree of cross-linking.

In melting point calorimetry, the collagen sample is placed in a conventional calorimeter, and enthalpy transitions are recorded. Figure 1A (taken from McPherson), shows a scan of a collagen sample cross-linked with 0.0075% glutaraldehyde and suspended in phosphate-buffered saline (PBS) to about 1 mg/ml. The reference sample (dotted line) contained PBS. Enthalpy transitions were recorded between 10°C and 100°C, at a scan rate of 10°C/minute. As seen, the collagen shows a melting transition at about 74°C.

A plot of melting temperature as a function of glutaraldehyde concentration is presented in Figure 1B. At glutaraldehyde concentrations up to about 0.01%, there is a strong melting temperature dependence on cross-linker concentration, indication that significant

changes in the collagen structure due to cross-linking are produced by cross-linking at glutaraldehyde concentrations up to 0.01%. The melting temperature changes observed on cross-linking at glutaraldehyde concentrations up to about 0.01% are accompanied by significant changes in the stability of the material, as measured by solubilization by an ionic detergent and resistance to proteolysis, as will be considered below. That is, the melting temperature changes correlate with and thus provide a measure of the increased stability of the material. In practicing the invention, the cross-linking is carried out under conditions which produce an increase in melting temperature of at least about 10°C, and preferably 15°C or more.

At glutaraldehyde concentrations above about 0.01% (or concentrations of other cross-linking agents which produce a comparable degree of cross-linking), the extent of cross-linking can be more accurately gauged by the number of lysine residues present in the cross-linked material. This assay is based on the gradual loss of free lysine residues in the collagen, as more of the free amine groups become involved in cross-linking. To measure lysine content, collagen samples are reduced, hydrolyzed, and analyzed for amino acid composition according to standard methods. The number of lysine residues is typically expressed in terms of number of residues per thousand, based on a comparison of the lysine/alanine ratios of noncross-linked and cross-linked collagen samples. Figure 2 (McPherson) shows the lysine content of collagen fibrillar samples cross-linked with increasing concentrations of glutaraldehyde, up to 1%. The lysine content in noncross-linked material is 28 residues per thousand. Between 0.01% and 1% glutaraldehyde, there is

a fairly linear relationship between the log glutaraldehyde concentration and the disappearance of lysine residues. At 1% glutaraldehyde, about 85 percent of the total lysine residues have been destroyed. Presumably, the remaining free lysine groups are sterically shielded or otherwise relatively unavailable for cross-linking.

The cross-linking reactions may be quenched, after a specified reaction time, by adding an excess free amine, such as lysine, to the reaction mixture. After the cross-linking reaction has been terminated, the cross-linked atelopeptide collagen product may be washed with an aqueous buffer solution to remove unreacted aldehyde, aldehyde polymers, and, if quenching is employed, unreacted quenching agent and aldehyde-quenching agent adducts. A sodium phosphate-sodium chloride buffer solution, pH 6.9 to 7.4, is preferred.

C. Physical Characteristics

In addition to above-noted changes in melting temperature and lysine content, the cross-linked material shows significant changes in a number of other physical/chemical properties which are related to biological stability, size, and/or injectability. The changes which reflect increased stability include solubility of the material at 45°C and in the presence of a strong ionic detergent, and susceptibility of the cross-linked material to degradation by proteolytic enzymes. Significantly, these stability changes, like the changes in melting temperature, are most dramatic in material cross-linked at glutaraldehyde concentrations less than or equal to 0.01%.

The neutral solubility of various collagen samples was determined by heating the samples to 45°C in phosphate-buffered saline (PBS) for 30 minutes, centrifuging the sample to remove insoluble material, and determining the amount of collagen in the supernatant by a conventional hydroxyproline assay (Jamill). The noncross-linked sample was completely solubilized at 45°C. It is noted that this melting temperature is roughly 10°C less than the 55°C melting point observed for noncross-linked material by calorimetric measurement (Figure 1B). This difference is due in part to the relatively rapid heating rate (10°C/minute) used in the calorimetric measurement, which caused the effective melting temperature to lag the actual calorimeter temperature by several degrees, and is also due to the fact that over an extended heating period, the fibrillar material can progressively melt or dissociate the collagen fibers at 45°C, whereas rapid melting requires, as observed in the calorimeter measurement, higher temperature.

It has also been observed that in the presence of the contrast agent diatrizoate meglumine, noncross-linked fibrillar collagen melts at around physiological temperatures, the lowered melting temperature presumably resulting from a destabilizing effect of the contrast agent on fibrillar organization. This effect may account, at least in part, for the poor biological persistence of noncross-linked material containing contrast agent.

Cross-linked fibrillar collagen, whether cross-linked with 0.01%, 0.1%, or 1.0% glutaraldehyde, showed less than 0.5% solubilization at 45°C (Delustro). This result is consistent with the above calorimetry melting temperature data, showing that

cross-linking with 0.01% glutaraldehyde increases the melting temperature of the material by up to 20°C. The increased biologic persistence of lightly cross-linked material containing contrast agent, seen below, also suggests that the cross-linked structure may be less susceptible to the dissociative effects of contrast agent. That is, the extent to which contrast agent lowers the melting temperature of cross-linked material would be expected to be less than that seen for noncross-linked material.

Analysis of the cross-linked material by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) also shows that cross-linking at low glutaraldehyde concentrations is effective to produce a significant increase in fiber cohesiveness. In this study, samples cross-linked with either 0%, 0.001%, 0.0025%, 0.005%, or 0.01% glutaraldehyde were solubilized in SDS with heating before fractionation by SDS-PAGE. The noncross-linked sample and that cross-linked with 0.001% glutaraldehyde both showed strongly stained bands at positions corresponding to α1 and α2 collagen subunits. The material cross-linked with 0.0025% glutaraldehyde showed a single weakly stained band at the position corresponding to α1, and the two remaining samples (cross-linked at 0.005% and 0.01% glutaraldehyde) showed no detectable soluble bands in the gel (McPherson). The results demonstrate extensive cross-linking of the subunits making up the collagen fibers, even at 0.005% glutaraldehyde.

Another measure of the stability changes which occur on mild cross-linking is resistance to degradation by proteolytic enzymes, such as trypsin and collagenase. This effect was demonstrated in a protease

sensitivity assay in which a fibrillar collagen preparation, at a concentration of 1 mg/ml in PBS was reacted with either trypsin or collagenase, at an enzyme substrate mass ratio of 1:500. Following incubation at 37°C for various times, the reaction was stopped by the addition of trichloroacetic acid (TCA). After centrifuging the samples to remove precipitated protein, the supernatant fraction was assayed for TCA-soluble peptides by a fluorescamine assay (Udenfriend). Figure 3 shows the amount of collagen release produced by collagen, expressed as a percentage of total degradable collagen, over a two hour incubation period for noncross-linked material (triangles) and collagen cross-linked with 0.075% glutaraldehyde for 16 hours (circles). As seen, cross-linking substantially prevented the progressive protease degradation observed with noncross-linked material. Similar results were also observed for trypsin treatment of the two collagen preparations (McPherson).

The fiber size characteristics of the cross-linked material have also been investigated. Electron microscopy of collagen material cross-linked with 0.007% glutaraldehyde shows mean fiber sizes about 5 micrometers (standard deviation of about 2) times 72 microns (standard deviation of about 40). Electron microscopy of fibrillar collagen treated with 0.01%, 0.1%, or 1.0% glutaraldehyde revealed only modest differences in fiber organization, with increasing cross-linking (McPherson). These differences include a slight increase in fiber diameter, at higher glutaraldehyde concentration. In addition, at the highest glutaraldehyde concentration (1.0%), the formation of fibrillar lattices was observed, presumably due to interfibrillar cross-linking.

In addition to stability and size characteristics, flow characteristics, such as viscosity, also should be considered in preparing cross-linked vaso-occlusive collagen. In particular, where the cross-linked collagen is to be administered in relatively dilute form through a small-bore catheter, the material should (a) resist spiking and clogging, and (b) have a viscosity which allows flow through the catheter at moderate extrusion pressures. Spiking -- referring to high pressure transients which are encountered during extrusion -- and clogging are due to macroscopic fibrillar networks that can form during material flow through a small-bore tube. In general, these problems become more serious at higher protein concentrations and with a greater degree of cross-linking. One important advantage of screened cross-linked material, as brought out in the above-cited patent application, is that spiking and clogging are substantially eliminated, at least in lightly cross-linked material.

Cross-linking also increases the viscosity of a collagen fiber suspension, although the increases are well within the acceptable limits for delivery by small-diameter catheters. To illustrate, the viscosities of fibrillar collagen before and after cross-linking with 0.0075% glutaraldehyde were measured by conventional viscometer methods (McPherson). The viscosity versus shear rate for noncross-linked and cross-linked collagen are shown in Figures 4A and 4B, respectively, with each figure showing plots measured at three different protein concentrations, as indicated in the drawing descriptions. In both preparations, viscosities were dependent on shear rates -- characteristic of non-Newtonian polymer fluids -- and

both preparations exhibited shear-thinning, i.e., showed reduced viscosity with increasing shear rate. The latter property is due to increasing alignment and less interfibrillar interference at higher shear rates. Both preparations also showed increasing viscosities with increasing protein concentrations, reflecting the increasing interference of fibrillar particles with each other during flow. At comparable protein concentrations, the cross-linked collagen was 2-3 times more viscous than the noncross-linked material, in the shear rate range indicated in the figures.

D. Collagen/Contrast Agent Composition

In one embodiment of the invention, the vaso-occlusive composition includes a mixture of the cross-linked collagen material and a radio-opaque contrast agent which allows the material to be monitored fluoroscopically after embolization. Preferred contrast agents include various iodine-containing organic compounds such as diatrizoate meglumine, diatrizoate sodium, ipodamide meglumine, iothalamate meglumine, iothalamate sodium, metrizoic acid, methiodal sodium, metrizamide, iohexol, iopamidol, and ioxaglate. Tantalum powder and barium sulfate may be used in the invention. Many of these agents are supplied commercially in sterilized solution or suspension form, at a concentration of between about 20-80 weight percent contrast agent, for use in x-ray fluoroscopy.

The concentration of cross-linked collagen in the composition is selected to produce desired viscosity (flow) characteristics for use in embolization, and sufficient contrast agent to allow the composition to permit the composition to be visualized under radiographic (X-ray) examination. In a preferred method

for preparing the composition, a concentrated form of the collagen material, e.g., at a collagen concentration of between about 30-65 mg/ml collagen, is diluted with a suspension or solution of contrast agent to produce the desired final collagen concentration. If necessary, a suitable physiological buffer, such as a concentrated citrate, bicarbonate, or phosphate buffer, can be added to stabilize the pH within a physiologically acceptable range.

The final concentration of the collagen is adjusted to produce flow characteristics which are compatible with the intended use. Where the cross-linked material is to be used for occluding small branch arteries, e.g., in the 20-250 micron size range, the material is prepared at a concentration which allows flow-directed deposition of the material from a supply artery into the smaller branch arteries. Collagen concentrations of between about 0.5-15 mg/ml, and preferably between about 1 and 5 mg/ml, are suitable for this application. However, it is noted that even relatively thick collagen compositions have given vaso-occlusive effects which indicate directed flow from the site of infusion into smaller branch vessels. For occluding large vessels and/or vessels in which the material is introduced in a retrograde fashion, against the flow of blood in the vessel, or into a large arterial vessel, a thick, pastelike composition is prepared. Here collagen concentrations of greater than about 15 mg/ml and as high as 40 mg/ml are suitable. In high-concentration formulations, it may be necessary, after addition of a contrast agent, to remove excess water from the suspension, and this can be done by known methods, such as centrifugation or ultrafiltration.

An important feature of the collagen/contrast agent composition just described is its ability retain its reconstituted fibrillar form in vivo. It is known that contrast agents can strongly solvate collagen fibers, lowering the fiber melting point by reducing intermolecular cooperativity. Introducing cross-links into the collagen fiber increases its melting point, as seen above, allowing the collagen to remain active (in fibrillar form) in the presence of contrast agent at physiological temperatures.

E. Collagen/Drug Composition

The invention also includes a collagen/drug composition designed for localizing a therapeutic agent at a target site, by drug release from a vaso-occlusion mass at the target site. The composition is formed by mixing the cross-linked collagen suspension from above with the selected drug or drug agents, at a ratio which gives a desired dose of drug in a volume of collagen which is needed to produce vaso-occlusion at the target site. Preferably, the drug is added to the above collagen/contrast agent composition, allowing the distribution of the drug-containing collagen material to be monitored fluoroscopically. In one preferred embodiment, a cross-linked collagen which has been diluted with contrast agent, as above, to produce a desired suspension viscosity, is mixed with a given amount of drug, in a relatively small solution volume, to yield a composition having the selected viscosity and collagen-to-drug ratio.

Where the composition is used for tumor therapy, the drug is a selected antitumor agent, such as adriamycin, cisplatin, 5-fluorouracil, or a steroid

hormone, such as tamoxifin. Methods of tumor treatment using such compositions are detailed below.

Alternatively, the drug may be an antimicrobial agent, such as one of a number of known antibacterial drugs or drugs directed against various parasitic organisms which are known to localize in specific internal organs, particularly the liver.

The therapeutic agent may include a peptide or protein agent, such as a peptide hormone, or an immunostimulator, such as interleukin and interferon. The peptide composition, when infused into a suitable vascular site, is be useful in certain types of hormone or antitumor therapy where peptide localization near an occludable vascular site is advantageous.

II. Vaso-Occlusion Methods

A. Small-Vessel Vaso-Occlusion

In one general treatment method, the cross-linked collagen composition is used for occluding small arterial vessels in a selected tissue region. The size of vessels occluded is preferably in the 20-250 micron range, so that occlusion occurs distal to the collateral circulation in the tissue region. The occluded tissue region is then cut off from direct blood supply, and also from collateral blood supply which feeds the tissue collaterally through small branch arteries. This vaso-occlusive method has a number of applications. One application is based on the use of small-vessel vaso-occlusion to produce ischemic tissue-necrosis effects in solid tumors, by persistent vaso-occlusion in the small vessels supplying the tumor. Two additional applications, involving a combination of vaso-occlusion and hyperthermia or

chemotherapy, are discussed separately below. Other applications are for treating hemorrhaging in injured tissue or sealing off a tissue region prior to surgery. These applications follow the general strategy and collagen delivery techniques now to be described.

Figure 5 shows anatomical features of a tissue 10 containing a localized region 12 whose small arterial supply vessels are to be occluded. It is understood that region 12 may be a localized tumor region, an impaired region where hemorrhaging is occurring, or other tissue region at which persistent vaso-occlusion is desired. The upper portion of the localized region is defined by the dashed line seen at 14, and the lower portion, by an arterial vessel 16 which supplies the tumor with blood, in the direction of arrow 16a. The tissue surrounding the localized region is indicated generally at 18.

As shown in the figure, the tissue, including region 12, is supplied from a network of small blood vessels which branch from vessel 16. The network, which is shown in simplified form in the figure, includes (a) primary vessels, such as vessels 20, 22 branching from vessel 16; (b) secondary vessels, such as vessels 24, 26, 28 branching from the primary vessels; and (c) tertiary vessels, such as vessels 30, 32, 34 branching from the secondary vessels. The blood supply network also includes a capillary bed (not shown) supplied by the smallest vessels. Vessel 16, which forms the trunk of the blood-supply network just described, has a typical lumen diameter between about 1 to 5 mm. The secondary and tertiary vessels characteristically have diameters ranging between about 10 to 500 microns, and the primary vessels, intermediate sizes, i.e., between about 200 and 1,000 microns.

It is observed from the figure that the secondary and tertiary vessels contain extensive interconnections, distal to their respective primary vessels. These interconnections form a collateral supply system which allows blood to be supplied to the smaller vessels from primary vessels other than those directly supplying blood from vessel 16. For example, tertiary vessel 30 within the tumor region may be supplied blood collaterally from primary vessel 22 disposed within normal tissue. Usually, the collateral system becomes an important supply source for the tissue only after primary vessels are blocked or otherwise damaged.

In practicing the vaso-occlusive method, cross-linked fibrillar collagen suspension prepared as above is injected by a catheter into vessel 16, where the material is carried by blood flow into the small tumor branch arteries fed by by the vessel. Where, as here, the sizes of branch arteries to be occluded are in the 20-250 micron range, the collagen suspension is preferably formed to have final cross-linked fibers also in this size range. For producing biological persistence of a few months or longer, the material should be cross-linked under conditions which produced moderate-to-heavy cross-linking, such as at glutaraldehyde concentrations of between 0.01% to 1%, and under these conditions, the final fiber sizes may be larger than the smallest vessel sizes. This problem can be minimized, as noted above, by initially screening the reconstituted collagen fibers before cross-linking.

The collagen suspension is formulated to a final concentration of preferably between about 0.5 and 15 mg collagen/ml, and more preferably between 1 and 5 mg/ml collagen, which allows the material to be

administered readily from a small-bore catheter, and to be carried by blood flow into the small branch vessels. The infused material preferably includes contrast agent, for fluoroscopic monitoring of collagen distribution at the target site.

A preferred type of catheter for use in delivering the collagen material is a double-balloon catheter of the type disclosed in co-owned U.S. Patent Application for "Catheter Device", Serial No. 803,806, filed December 2, 1985. This catheter, which is shown at 40 in Figure 1, has a pair of balloons 42, 44 which are independently inflatable, in situ, by pneumatic supply tubes 46, 48, respectively. The collagen suspension is supplied to the vessel through a third tube 50 which terminates adjacent the upstream balloon and which encases tubes 46, 48, up to the position of balloon 44. Tube 46 is carried slidably within tubes 50 and affixed to a metal guide wire (not shown), allowing downstream balloon 42 to be moved axially with respect to the upstream balloon, with the catheter placed in the vessel, to produce a desired spacing between the two balloons.

In operation, catheter 40 is threaded along the vessel of interest, and may be used initially, in a conventional manner, for delivering a radio-opaque agent to the vessel, for purposes of localizing the tumor and/or its supply vessel. The catheter is then manipulated to place the upstream and downstream balloons adjacent the upstream and downstream ends of the vessel segment feeding region 12. This arrangement is illustrated in Figure 1, showing balloons 42, 44 positioned adjacent the opposite ends of region 12. The balloons are then inflated, constricting the vessel adjacent opposite sides of the tumor, and the

vaso-occlusive material injected under pressure into the vessel segment. It can be appreciated that the catheter allows the occlusive material to be injected into the localized region under pressure, and in a highly localized manner.

In a second general anatomical configuration (not illustrated), the tissue region to be treated is supplied by an arterial vessel which feeds into--i.e., terminates within--the region. With this configuration, the occlusive material can be injected into the tissue site selectively using a single-balloon catheter to occlude the upstream side of the injection site, or may be injected under low pressure without vessel occlusion.

The volume of vaso-occlusive material which is injected into the tissue site will vary according to (a) the concentration of occlusive material, (b) size of the region 12, and (c) the extent to which the injected material can be localized at the target site. In a typical vaso-occlusion procedure, for occluding a tissue region having a volume between about 5 and 1000 cc, and using a collagen material at a concentration of about 0.5 to 5.0 mg protein/ml, the total volume of material injected is between about 1 and 200 ml. More generally, the injecting step may be carried out by following the infusion of contrast/collagen material into the region, by real-time fluoroscopy, and injecting material until a selected-size region becomes occluded. Adequate occlusion is indicated by progressive slowing of normal flow until complete cessation and ultimately, reversal of direction of normal flow is obtained.

The vaso-occlusive method just described is illustrated in Example 2 below, in which the degree and persistence of cross-linked collagen in the vascular bed supplied by the femoral artery in dogs was examined.

The collagen suspension used was cross-linked with 0.0075% glutaraldehyde, diluted with contrast agent to about 15 mg/ml, and infused by catheter into the arterial site. At two days, two weeks, and two months after administration, tissue sections from the vaso-occluded areas were removed and examined microscopically. The sections showed substantial vaso-occlusion in the vessels, even after two months. By contrast, three other collagen compositions prepared by prior art methods showed complete or nearly complete loss of occlusive material after two weeks for two of the compositions, and after two months for the third composition.

The histological appearance of vaso-occluded vessels, over a two month period, is illustrated in Figures 6A-6C, which are representations of actual photomicrographs of hepatic vessels, ranging roughly between 75 and 150 microns in size, occluded with a collagen fiber suspension cross-linked with 0.0075% glutaraldehyde, and examined one week (6A), one months (6B), and two months (6C) after vaso-occlusion. The blood vessel in each figure is indicated at 52. As seen in Figure 6A, the vessel is initially completely blocked with the vaso-occlusive mass, shown here by a fibrillar network pattern. After one month, the vessel shows evidence of recanalization, as endothelial cells lining the vessel, indicated at 50, begin to engulf the collagen mass. This process of recanalization is further established after two months (Figure 6C), although more than half the cross-sectional area of the lumen remains occluded.

The effects of persistent vaso-occlusion in liver have also been examined, to demonstrate the feasibility of treating liver tumors by persistent

arterial vaso-occlusion. The liver is somewhat unique as a tumor site in that normal liver tissue receives about 70% of its oxygen needs from the portal venous blood supply, but hepatic tumors themselves are generally supplied entirely by arterial vessels. This offers the potential of treating liver tumors by generalized arterial vaso-occlusion, with tumor tissue showing greater ischemic damage because of the greater dependence on arterial blood supply. Here it is noted that generalized arterial vaso-occlusion of an entire lobe or segment of liver is often the only practical method for targeting the liver, due to the difficulty of identifying specific tumor supply vasculature in the liver.

Heretofore, various strategies for producing arterial occlusion sufficient to achieve selective tumor necrosis in liver have been proposed. Hepatic tolerance to arterial occlusion by ligation or to embolization with nonbiodegradable particles suggest a preestablished network of intrahepatic collateral arterial channels. That is, collateral arterial circulation is sufficient, along with normal portal blood supply, to prevent liver necrosis. Complete arterial occlusion, however, leads to liver necrosis. This has been demonstrated surgically (Mays, Popper), with polymerized liquid silicone (Doppman), with repetitive occlusion using 150 and 250 micron nonbiodegradable particles (Stridbeck, 1984), and with 150-250 micron particles coupled with proximal occlusion (Stridbeck, 1985). These studies, taken together, establish that portal venous blood supply is not sufficient to support hepatic oxygen needs.

In the specific liver embolization method described in Example 3, animal livers were embolized via the hepatic artery with the above collagen/contrast

agent composition, by infusing material into the artery until no significant antegrade flow of contrast material was observed. Histological examination of livers from animals immediately after vaso-occlusion showed collagen material in vessels between about 20-250 microns. There was no evidence of thrombosis in the embolized vessels.

Changes in serum transaminases, bilirubin, and alkaline phosphatase over a 28 day period following embolization are shown in Figure 7. The changes seen are consistent with severe, but reversible ischemic hepatic injury. Recovery of hepatic function began after 48-72 hours, and was complete within one week to one month. During the early period of recovery, recanalization of the vessels was apparent, as a vascular space is formed around the collagen by migration of endothelial cells. The correlation between histological and biochemical changes suggests that recanalization, and not collateral arterial supply, is the dominant factor in the recovery of hepatic function after embolization with collagen.

Long-term liver-function histological studies showed that the collagen is resorbed from the hepatic arterial vascular space over a three month period, and that the liver is restored anatomically and functionally to its preembolized state. Resorption takes place without morphological evidence of inflammation or macrophage activity.

As part of the study, several animals were repeatedly embolized at two-week intervals. The procedure was well tolerated, and only produced definite hematological and liver function abnormalities at high collagen doses.

The study indicates that the vaso-occlusive method of the invention, when applied to generalized

arterial vaso-occlusion in liver, produces effective arterial blockage, but allows sufficient recovery by recanalization, to prevent long-term liver damage and necrosis. At the same time, the occlusion of vessels in the 25-250 micron size range is effective in blocking, at least partially, collateral arterial circulation, so that arterial circulation through the occluded region of the liver is restricted. This is in contrast with prior art approaches, which either allowed substantial collateral circulation in the occluded region, or as in the case of injected silicone and other irreversible occlusive agents, produced significant liver necrosis related to long-term ischemic effects.

B. Hyperthermic Tumor Treatment

It has been discovered, according to one aspect of the invention, that localized small-vessel vaso-occlusion provides a significant and unexpected enhancement in differential heating achievable in hyperthermic treatment of tumors. The desirability of enhancing temperature differential effects in hyperthermic cancer treatment is related to the greater rate of tissue destruction which occurs at increasing tissue temperatures above about 42°C. The general rule is that each degree increase in tissue temperature approximately halves the time required to produce a given amount of tissue damage. Thus if a given amount of tissue damage results from heating the tissue at 42°C for two hours, the same amount of damage is produced in an hour at 43°C, and in one-half hour at 44°C. The present invention, in contrast to earlier vaso-occlusive methods for enhancing hyperthermic effects, such by arterial clamping, provides typical temperature differentials of at least about 2° C between occluded

and adjacent nonoccluded tissue regions, and therefore allows for significant localized tumor necrosis effects for a heating period which does not seriously destroy adjacent nonoccluded tissue.

In practicing this aspect of the invention, conventional radiographic or surgical methods are used to locate the target tumor region and, preferably, a arterial vessel which supplies this region. In a typical method, a catheter designed to release a (radio-opaque) contrast material into the blood is threaded through the arterial system toward the suspected tumor site. The region of interest is monitored fluoroscopically as the contrast material is released. From the observed rates of flow of material through the vessels, and the patterns of accumulation of material in the tissue, the tumor(s) can be localized, and the major vessel(s) supplying the tumor identified.

As discussed above, the tumor may be supplied from a segment of an arterial vessel (the configuration shown in Figure 5), by an end-artery system, or may be localizable only generally, as with many liver tumors. In the first case, the vaso-occlusive agent is administered preferably as described above, using a proximal or double-balloon catheter to direct the embolic material into the vessel(s) supplying the target region. Similarly, for an end-artery system, simple catheter arterial infusion, or infusion with proximal balloon occlusion, is suitable. Methods for producing generalized vaso-occlusion, for example by infusing collagen material into the hepatic artery, are described above.

According to an important feature of the method, the vascular occlusion produced by the injected collagen material is confined substantially to the

secondary and tertiary vessels in the tumor, including vessels or vessel portions which are distal to collateral vessels which may also supply the tumor tissue. The occlusive material thus acts to restrict blood flow to the tumor from both direct and collateral sources, as discussed above in Section IIA.

After embolization of the target tissue, the occluded region is heated under conditions which produce selective tissue necrosis. A variety of known methods for heating tissue may be used. These include microwave and ultrasonic heating, and where the tumor region lies close to the body surface, dielectric heating or direct contact with a heating pad. Methods for operating and controlling such heating devices, to achieve focused heating in a selected tumor region, are known.

The tumor is preferably heated to a temperature of between about 42° to 46°C. The heating temperature will typically be one at which a significant temperature differential between occluded tumor tissue and nonoccluded tissue is achieved. The tumor temperature is preferably raised until a desired temperature differential of preferably between about 2° and 4°C is attained. Because of the localized nature of the vaso-occlusion, and the occlusion in small vessels which are distal to collateral circulation, heat differentials of 2° C or more are generally attainable. The temperature of tumor and adjacent, nonoccluded tissue can be measured in a conventional manner, for example, by placing thermocouples, thermistors or other types of temperature probes at or near the tumor site. The probes may be positioned conveniently by catheter placement in many cases.

The tumor is heated at the above temperature and/or temperature differential for a period calculated

to produce selective tumor destruction. The extent of tissue destruction generally increases proportionally with increased heating time above about 42°C, and, as noted above, this time becomes proportionally less as the tissue temperature is raised. The amount of tissue destruction produced under defined temperature and time conditions can be measured directly, such as by determining the metabolic activity or histological features of biopsied material after treatment. Preferably, to avoid surgical invasion, the heating is applied for a period which has been determined from an earlier experimental or clinical study to cause significant tissue damage. The heating period will generally range between a minimum time, at which only minor tissue damage in the adjacent, nonoccluded tissue occurs and a maximum time, at which tissue destruction in the occluded tumor begins to plateau. Typically, the tumor is heated for between about 2-8 hr at a tumor temperature of about 42°C, and for proportionately shorter time periods at temperatures above 42°C. It can be appreciated that, with a temperature differential of 2°C or greater, the heating time can be adjusted to produce at least about four times more tissue necrosis in the occluded tumor tissue than in the adjacent, nonoccluded tissue. If necessary, the occluded tumor may be heat-treated in the above manner at selected intervals during the several weeks or months of persistence of the occlusive material. The use of hyperthermia in treating various spontaneous tumors is described in Example 4 below.

C. Chemoembolization

Experiments carried out in support of the invention, and reported in part in Examples 5 and 6,

demonstrate that the vaso-occlusive method of the invention can be used for localizing a drug agent selectively in a vaso-occluded target site. The method is referred to herein as chemoembolization.

A collagen/drug composition suitable for the chemoembolization method is described in Section IE above. A preferred composition contains cross-linked collagen diluted to a desired concentration with a cross-linking agent and mixed with a selected concentration of drug. Usually the contrast agent and drug are admixed with a thick collagen suspension shortly before administration. The concentration of drug in the final composition mixture is calculated to provide a desired drug dosage when a given amount of collagen (that needed to produce effective vaso-occlusion) is infused into the target site. The required amount of collagen is typically estimated from experience and/or by extrapolating animal vaso-occlusion data to human subjects.

Alternatively, an initial vaso-occlusive dose of drug-free collagen can be infused into the target site, followed by the desired dose of drug, either administered in pure form or in combination with additional collagen. It is also noted that since long-term vaso-occlusion may not be required for drug localization, the collagen material may not need to be chemically cross-linked to provide long-term occlusion. It will be understood further that the presence of contrast agent, although convenient for determining the distribution of the collagen material <u>in situ</u>, is not required for achieving drug localization according to the method.

The study reported in Example 5 illustrates the use of chemoembolization in liver where. Briefly, a

composition containing cisplatin, cross-linked collagen and contrast agent was infused into the hepatic artery over a 30 minute period. For purposes of comparison, the same dosage of cisplatin was administered iv, by intra-arterial infusion (free drug), and intra-arterial infusion of free drug with proximal balloon occlusion. The levels of cisplatin present in the kidney, right and left liver, muscle and plasma two hours after drug administration are shown in Figure 8 for the four routes of administration. As seen, drug localization by chemoembolization was about 2.5 higher in the right lever than by the other three routes of drug delivery, and comparable to the other routes in nonhepatic tissue. Interestingly, intra-arterial infusion, even with proximal balloon occlusion, did not enhance drug localization in the liver, suggesting that balloon occlusion was secondary to continued collateral blood flow. The results demonstrate that significantly enhanced enhanced drug targeting effect can be achieved by chemoembolization even in the liver, where the normal oxygen needs of the tissue are supplied by portal vein and collateral blood flow, as well as direct arterial flow.

To demonstrate the effectiveness of the method in an end-artery system, a cisplatin/collagen composition was infused into a left kidney region supplied by a single arterial supply vessel. Drug levels in the right and left kidneys, right and left liver and lung were compared about two and a half hours after drug infusion. For comparison, the same amount of drug was also administered iv and by intraarterial (left kidney) infusion. The results, which are reported in Example 6, are shown in Figure 9. As seen, drug localization was highly specific for the site of

vaso-occlusion, and as in the liver system, much greater for chemoembolization than intra-arterial infusion into the same vessel site.

Figure 10 shows drug localization in right and left kidneys as a function of collagen concentration in the collagen/drug composition. As seen, increasing the collagen concentration increased the drug level in the occluded left kidney, and decreased the drug level in the nonoccluded kidney. At the highest collagen concentration, the drug level in the occluded kidney was over two orders of magnitude greater than in the nonoccluded kidney, demonstrating the high degree of drug localization which can be achieved in an end-artery system.

From the foregoing, it can be appreciated how various objects and features of the invention are met. The vaso-occlusive method of the invention permits vaso-occlusive treatment to be extended to periods of up to several weeks or months, and with periodic repeated infusion, for longer periods. As a result, a variety of treatment strategies involving long-term occlusion are now possible. These include long-term occlusion of a localized tumor region, to starve the tumor of oxygen and nutrients and to allow repeated treatments, such as by hyperthermia, in conjunction with vaso-occlusion, and treatment of aneurisms and varicose vein, by long-term blockage of large blood vessels.

The cross-linking conditions used in preparing the vaso-occlusive material are readily manipulated to produce desired biological persistence, size, and viscosity characteristics. In particular, the biological persistence of the material can be varied selectively according to the extent of cross-linking,

and such can be readily monitored, under light-to-moderate cross-linking conditions, by changes in the thermal melting temperature of the material, and under moderate-to-heavy conditions, by changes in the free lysine content of the material.

The material can be mixed with a conventional contrast agent, such a diatrizoate meglumine, to form a stable suspension, and without apparent loss of biological persistence. Further, the advantageous properties of atelopeptide collagen for use in vaso-occlusion -- including its favorable immunogenic and flow characteristics -- are preserved.

When combined with hyperthermic tumor treatment, small-vessel vaso-occlusion provides significantly enhanced differential heating and tumor necrosis effects, with respect to prior art hyperthermia methods. Vaso-occlusion also provides an effective method for localizing a therapeutic drug or radioimaging compound at the site of the vaso-occlusion, particularly when the collagen/drug material is applied to an end-artery system. The results presented herein show that drug localization can be enhanced two orders of magnitude over that seen with iv or conventional intra-arterial drug infusion.

The following examples illustrate various collagen vaso-occlusive compositions and their use in long-term small-vessel vaso-occlusion, hyperthermia and drug delivery. The examples are in no way intended to limit the scope of the invention.

Example 1
Preparation of Vaso-Occlusive Material

A. Reconstituted, Noncross-linked Collagen Suspension

Bovine hide was softened and depilated by treatment with an aqueous acetic acid solution. The hide was then comminuted and dispersed in aqueous HCl, pH 2, at a concentration of 10-30 g/l. A freshly prepared pepsin solution (0.5 g in 10 ml 0.01 M HCl) was added to the dispersion at 0.1% by weight based on total protein, and the mixture was allowed to incubate for about 100-300 hours at 15 - 20 C. Following pepsin treatment, NaOH was added to raise the pH of the incubation medium to above 7.0 to denature the pepsin, and thereby terminate the reaction. The solution was then purified and brought to a final concentration of 3 mg/ml in dilute aqueous HCl, pH 1-4.

The collagen fibril solution was reconstituted by adding 0.2 M Na_2HPO_4 to neutralize the solution. The solubilized collagen fibrils were allowed to aggregate for two hours at 15°-22° C, to form the fiber suspension. The material was mechanically screened by repeated passage through a 60 mesh wire screen, according to the procedure described in Section IA.

B. Cross-linked Collagen Suspension

To 165 ml of the reconstituted collagen suspension, at a concentration of about 3 mg/ml, was added 18.3 ml of 0.075% glutaraldehyde at pH 3. The glutaraldehyde solution was added gradually with stirring to obtain a final concentration of 0.0075%. After a reaction period of 16 hours, the cross-linked collagen was washed three times with approximately 100 volumes of buffer, 0.02M Na_2PO_4, 0.13 NaCl, pH 7.4,

with centrifugation at 17,000 x g for 5-7 minutes between each wash. The material was resuspended to a final suspension containing about 30 mg/ml collagen in 0.9% NaCl, 25 mM PO_4, pH 7.2.

Example 2

Vaso-Occlusive Persistence of Various Collagen Materials

The biological persistence of cross-linked atelopeptide collagen fibers was compared with that of three other collagen compositions. All four compositions contained about 15-20 mg/ml collagen in about 60% diatriazole meglumine in phosphate buffered saline (PBS). The compositions are:

A. Noncross-linked collagen prepared as described in Example 1A, concentrated, then diluted with the contrast agent to about 15 mg/ml;

B. Cross-linked collagen, prepared as described in Example 1B, and diluted with the contrast agent to about 15 mg/ml;

C. Bone collagen, prepared by fragmentation, decalcification, and trypsin hydrolysis, concentrated, and diluted with the contrast agent to about 20 mg/ml; and

D. XRT, X-irradiated bone collagen, prepared as in C, but irradiated to 5,000 rads.

Twenty-four mongrel dogs were divided into three groups according to the time at which they were reexamined following intraarterial embolization with one of the four collagen compositions. The animals in Group I were examined at two days following embolization; those in Group II were examined two weeks following embolization; and those in Group III were examined two months after receiving the collagen.

All animals were anesthetized using Nembutal induction followed by endotracheal intubation and Halothane anesthesia. In order to carry out vaso-occlusion, or embolization, a catheter was inserted percutaneously into one of the femoral arteries and then either the left or right internal iliac artery was selectively catheterized. After a control angiogram was performed, one of the collagen compositions was injected. In order to minimize the morbidity associated with the procedure, there was no attempt made to totally obliterate the vascular bed of the vessels being embolized. The distribution of the material could be directly observed and the effect of the embolization was periodically observed by test injections of contrast medium. An average of 3 to 4 cc of one of the compositions was used for each animal. Immediately following embolization, a repeat angiogram was performed.

At 2 day, 2 week, or 2 month intervals, a follow-up angiogram was performed and the animal was sacrificed and tissue samples were taken from the region of arteries which had been occluded. Sections from the bladder base, the prostate, and, for control proposes, the testis ipsilateral to the side of embolization were taken from each animal. Following fixation in a 4% formalin solution, these were sectioned and stained by conventional methods.

The angiographic studies were reviewed for evidence of immediate and delayed effects of vaso-occlusion. Regardless of the composition used, all studies done immediately after embolization demonstrated evidence of arterial blockage. However, for similar amounts of compositions, much more effect was noted in the studies done following embolization with Composition B (glutaraldehyde cross-linked) than was the case with

any of the other materials. All angiographic studies done at a 2 week or 2 month interval showed less change than did those done immediately after embolization. Of the studies done at a two month interval, only the animals embolized with Composition B showed definite persistent evidence of blockage of arteries large enough to be seen angiographically. Some angiograms done at a 2 week or a 2 month interval on animals embolized with the other compositions showed indirect evidence of very small arterial blockage, i.e., asymmetries of flow and arterial size, but did not demonstrate persistence of the changes in the larger arteries that were seen on the immediate post-embolization studies.

The tissue sections were reviewed with special attention to the persistence of any collagen within vascular structures as well as for the presence of an inflammatory response both within a vessel or in a perivascular distribution. In none of the sections was collagen identified in a venous structure. No focal inflammatory response was noted either in or around any artery which contained intraluminal collagen.

No persistent collagen was identified in the histological sections taken at a 2 day interval after embolization with Composition A, and there was also none present in the sections taken after a 2 day interval from one of the two animals embolized with Composition C. Otherwise persistent intraluminal collagen was found in all sections taken at two days. In the sections taken at a two week interval, only those from the two animals embolized with composition B and one of the animals embolized with composition D showed vaso-occlusion persistence. Sections taken at a 2 months interval showed no persistence of intraluminal collagen in any of the animals embolized with

Compositions A or D. A small amount of intraarterial collagen was present in sections from both of the animals embolized with Composition C and a large amount was present in the arteries of the two animals which had been embolized with Composition B. These results are summarized in Table I below.

TABLE I

Composition	Persistence 2 days	2 weeks	2 months
A	--	–	---
B	++	++	++
C	+-	–	-+*
D	++	+--	--

*Slight

The plus (+) and minus (-) signs indicate individual animals which showed persistence (+) or absence (-) of intraluminal collagen.

Example 3

Effect of Complete Embolization

Cross-linked collagen prepared as in Example IA (30 mg/ml in 0.9% NaCl, 25 mM PO_4, pH 7.2) was suspended in 60% lothalamate meglumine at a ratio of 1:10 or 1:30 (final concentration of collagen was 3.0 or 1.0 mg/ml).

Mongrel dogs weighing from 20 to 40 kilograms were divided into three groups for embolization studies. Prior to embolization, blood was obtained for complete blood cell count, platelet count, albumin, alkaline phosphatase (para-nitrophenyl method), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), lactate dehydrogenase

(LDH), total bilirubin, uric acid, creatinine, blood urea nitrogen and antibodies to collagen.

The hepatic artery was catheterized through a percutaneous femoral approach using standard 5.0 and 6.3 Fr polyethylene angiographic catheters (Seldinger). The hepatic artery was fluoroscopically visualized with 60% iothalamate meglumine and flow patterns recorded. Bolus injections of 5 ml were delivered at approximately one minute intervals into the common hepatic artery branches until no significant antegrade flow of contrast media within the hepatic artery was observed.

Gross necropsy was performed on all animals. Tissue samples from liver, right kidney, lung, duodenum, and pancreas were immediately placed in buffered formalin. Tissue sections for microscopic study were stained with hematoxylin and eosin and Masson's trichrome.

A. Group I: Acute

Two mongrel dogs underwent hepatic arterial embolization with sacrifice immediately following the procedure. Collagen was administerd at 3 mg/ml in 60% iothalamate meglumine.

Group I animals were sacrificed immediately after embolization. Gross appearance of tissues was unremarkable. Histologic evaluation of liver revealed collagen in vessels from 20 micrometers to 250 micrometers. The collagen was admixed with red blood cells and was also visualized in vessels in the duodenum, pancreas and gallbladder.

B. Group II: Chronic, Single Dose

Twelve mongrel dogs were preassigned to groups for repeat angiography and sacrifice at 1 week (1 dog),

2 weeks (1 dog), 1 month (2 dogs), 2 months (3 dogs), and 3 months (2 dogs) after embolization. Three animals died prematurely and were replaced during the course of the study. Collagen was administered at 3 mg/ml in 60% lothalamate meglumine. Sequential blood specimens were obtained for all continuing animals.

Group II animals were to be followed for viable times after single dose embolization. Seven of the 12 animals did well clinically and were followed as scheduled. Lethargy and loss of appetite with occasional emesis were noted on day 1. Weight returned to normal within 10-12 days. A mild febrile response occurred on days 1 to 3.

Five of the 12 chronic dogs died as a result of the procedure or became significantly ill. In all of these dogs there was evidence at necropsy of reflux embolization into tissues in the distribution of the gastroduodenal artery including the pancreas, duodenum and stomach. Gall bladder infarction was seen in two animals. No evidence of embolization was seen in spleen, lung, or kidney. The incidence of clinically apparent reflux was a function of the total dose of collagen. The seven animals doing well averaged 2.67 mg/kg (range 1.1-4.47) collagen. The five experiencing reflux infarction received an average of 4.32 mg/kg (range 2.81-5.53) collagen. Gross necropsy of animals receiving from 1.10 to 2.81 mg/kg collagen was unremarkable. Necropsies of those animals receiving greater than 2.81 mg/kg collagen revelated various abnormalities including liver fibrosis and splenomegaly, ascites, and infarction of gallbladder, duodenum, and pancreas.

Histologic evaluation of liver demonstrated collagen in vessels from 20 micrometers to 250

micrometers. Collagen was consistently identified in liver tissue from animals sacrificed between 1 week to 2 months after embolization. The collagen fibers formed a dense intravascular plug, similar to that seen in Figure 6A. Recanalization of the occluded vessels was seen as early as 1 week and continued for 2 months after embolization. Endothelial cells extended as a single layer over the collagen surface, and vascular channels developed within this endothelial cuff between vessel wall and collagen, similar to what is shown in Figures 6B and 6C. No collagen was seen in the livers of animals sacrificed at 3 months although small amounts were visualized in the duodenum with no evidence of recanalization. General microscopic anatomy of hepatic structures including bile ducts was normal but no post-mortem cholangiograms were performed.

Hemoglobin remained relatively stable. Leukocytosis was noted during the first 2 weeks after embolization. Mean platelet counts were sharply reduced to 50% of baseline at 24 and 48 hours after embolization. Recovery was noted at 1 week with overshoot at 2 weeks. No evidence for disseminated intravascular coagulopathy was found in 3 of 3 studied dogs. Fibrinogen and protein levels were within the lower limits of normal levels, and the protamine test remained negative.

As seen from the data in Figure 7, liver function tests (SGPT, SGOT, bilirubin, alkaline phosphatase) for all animals demonstrated peak elevation within 1 week with a gradual decline to preOembolization levels within 12 weeks. Pretreatment and time of sacrifice serum samples were assayed for antibodies to bovine collagen. None were detected.

C. Group III: Chronic, Repetitive Dose

Repetitive hepatic embolizations were performed at two week intervals on four animals. In an effort to protect the animals from inadvertent embolization of adjacent structures, several procedural changes were introduced before attempting Group III repetitive embolization studies: (a) the collagen concentration was reduced from 3 mg/ml to 1 mg/ml; (b) earliest signs of flow arrest were more closely monitored fluoroscopically; and (c) at completion of embolization, blood was aspirated to clear residual collagen from the lumen of the large access branch.

These animals were embolized with cross-linked collagen (Example 1A) suspended in 60% iothalamate meglumine at a ratio of 1:30 (final concentration of collagen, 1.0 mg/ml). Sequential blood specimens were obtained for all continuing animals. The duration of study was to be determined by animal's tolerance and observed cumulative effect. One dog was embolized three times, and three dogs were embolized five times. Two subject dogs were sacrificed 3 days after the last embolization, one at 1 week, and one at 4 months after the last embolization. Duration of the study ranged from 6 weeks to 5.5 months.

Eighteen embolization procedures in 4 animals were performed. Each animal was embolized from 3 to 5 times. Two animals received fixed doses of collagen (average dose 1.48 mg/kg). They were sacrificed at 3 days and at 4 months after their last embolization. Two animals were embolized to stagnation of flow, receiving an average of 3.18 mg/kg each session. Nitroglycerin 100 mg (5 mg/ml in normal saline) was infused immediately prior to the first embolization. Since nitroglycerin had no apparent impact upon the vascular

capacity for embolization, it was not used in subsequent embolizations. These animals were sacrificed at 3 days and 1 week following their last embolization. These procedures were well tolerated. There was occasional emesis during the 6 to 8 hours after embolization. Occasional temperature elevations not exceeding 1°C were noted on days 1 to 3. Weights remained stable. Only minor hematologic and liver function changes were noted in these animals. In particular, those receiving the larger dose of collagen demonstrated more definite hematologic and liver function abnormalities. Sequential liver function tests suggested improved tolerance with repetitive embolization.

Gross necropsies were all unremarkable. Sections of liver revealed collagen in vessels from 20 to 250 micrometers. The total collagen present appeared greater than in single embolized Group I animals. Recanalization with partial clearance of collagen in vessels was seen in all slides. In contrast with singly embolized animals, persistence of some collagen was noted at 4 months. Collagen was also visualized in arteries within the duodenum in three of three examined animals.

Example 4

Hyperthermia Treatment of Spontaneous Tumors

A group of dogs having spontaneous tumors of the extremities (2 animals), nose (1 animal), and chest wall (1 animal) were examined for tumor response to hyperthermia combined with vaso-occlusion at the tumor site. In each animal, the tumor and major arterial vessel supplying it were identified by angiography. The collagen material was the same as that used in Example 2.

The tumor regions in each animal were heated by ultrasound for a period of four minutes, with temperature monitoring at multiple locations within the tumor and the surrounding tissue. The tumor region was then selectively occluded by injecting cross-linked collagen into the tumor's major supply artery, and heated a second time under identical conditions. Comparison of pre- and post-occlusion heating showed that (a) heating was more rapid in vaso-occluded material, comparing both tumor tissue before and after occlusion and tumor and surrounding tissue after occlusion; (b) differential heating between tumor and surrounding tissue was enhanced substantially after vaso-occlusion; and (c) the temperature drop in vaso-occluded tissue after heating was slower than in nonoccluded tissue. One of the animals was treated by heating the occluded tumor at 46°C for 30 minutes. Follow-up showed no tumor regrowth.

Example 5

Chemoembolization in Liver

Cisplatin was obtained from Bristol-Myers (Syracuse, NY). Cross-linked collagen material was prepared as in Example 1A, and diluted to about 15 mg/ml collagen with 60% diatrizoate meglumine.

Mongrel dogs were divided into four groups. Group 1 received 1 mg cisplatin/kg body weight by intravenous injection (iv). Group 2 received the same cisplatin dosage by intraarterial catheter infusion in the common hepatic artery, and group 3 also the same dose by intraarterial administration, but with restricted flow due to temporary balloon occlusion in the common hepatic artery. The fourth group received the same dose of cisplatin administered in the

cross-linked collagen material within the common hepatic artery. Infusion durations were 30 minutes for each animal. In some animals, hepatic blood flow was monitored by indocyanin green dye.

The animals were sacrificed 2 hours after drug administration, and platinum levels in the left and right liver, kidney, lung, heart muscle, diaphragm, plasma, plasma filtrate and muscle were determined by atomic absorption spectroscopy. Values were computed as µg Pt/mg wet weight of tissue. Tissue levels were normalized within each animal to platinum levels in the left kidney.

The data, presented as the average value of the animals in each group, are given in Figure 8. Compared with iv drug administration (the left bar in each group), intraarterial infusion (the two middle bars in each group) resulted in an approximately 20 % increase in normal hepatic tissue levels. Occlusion infusion at a restricted rate into the common hepatic artery was not effective in increasing local tissue deposition beyond that seen with nonoccluded intraarterial infusion. Concurrent estimates of relative hepatic blood flow by indocyaninin green also showed no effect of balloon-occlusion upon clearance of the dye.

Chemoembolization with the collagen material resulted in an approximately 2.5 fold increase in platinum levels selectively in normal hepatic tissue. Since portal blood flow through the liver was unimpaired in both the balloon-occlusion and chemoembolization groups, it is assumed that the lack of effectiveness of balloon occlusion is not related to the continued flow of the hepatic arterial collateral supply.

Example 6

Chemoembolization in Kidneys

Cisplatin and cisplatin/collagen infusion material were as in Example 5, except that collagen concentration was 18 mg/ml. New Zealand white rabbits were divided into three groups. Group 1 received 1 mg cisplatin/kg body weight by iv injection. Group 2 received the same cisplatin dosage by intraarterial catheter infusion into the main artery supplying the left kidney. The third group received the same dose of cisplatin administered in the cross-linked collagen material in the main artery supplying the left kidney. Infusion durations were 6 minutes for each animal.

The animals were sacrificed 2 hours after drug administration, and platinum levels in the left and right kidney, left and right liver, and lung were determined by atomic absorption spectroscopy. Values were computed as µg Pt/mg wet weight of tissue. Tissue levels were normalized were normalized within each animal to platinum levels in the left kidney.

The data, presented as the average value of the animals in each group, are given in Figure 9. Compared with iv drug administration (the left bar in each group), intraarterial infusion (the middle bars in each group) resulted in a relatively small absolute increase in left kidney tissue levels. Chemoembolization with the collagen material in this end-artery system resulted in an approximately 100 fold increase in platinum levels selectively in normal kidney tissue.

In a second experiment, the accumulation of cisplatin in chemoembolization, as a function of collagen concentration was examined. Collagen compositions containing increasing collagen concentrations between about 1 and 10 mg/ml and drug in

mixed with contrast agent were infused as above in the left kidney of the animals. About 2 hours after chemoembolization, cisplatin levels in left and right kidneys were measured. The data, which are seen in Figure 10, show a steady increase drug accumulation into the infused kidney and a gradual decrease in drug levels in the noninfused kidney, at higher collagen concentrations, when compared with free drug (0 mg/ml collagen) infused by the same route. At the highest collagen concentration tested, the drug accumulation in the infused kidney was over 100 times that in the noninfused kidney.

Although the invention has been described with reference to particular embodiments and uses, it will be appreciated that various changes and modifications can be made without departing from the invention.

IT IS CLAIMED:

1. A method of achieving persistent occlusion of target tissue vessels, for a period of at least about two weeks, comprising

providing a vaso-occlusive material composed of a suspension of atelopeptide collagen fibers which are cross-linked under conditions which raise the melting temperature of the suspension at least about 10°C, and

introducing an occlusive amount of the material into the target tissue vessels.

2. The method of claim 1, wherein the fibers are cross-linked under conditions which raise the melting temperature by at least about 15°C.

3. The method of claim 1, wherein the fibers are cross-linked by glutaraldehyde at a concentration thereof between 0.005% and 0.01%.

4. The method of claim 1, wherein the collagen material provided includes a radio-opaque agent.

5. The method claim 1, for achieving vaso-occlusion for a selected period between about 2 weeks and 6 months, wherein the collagen fibers are cross-linked under conditions which lead to a selected degree of cross-linking, as measured by the percent of total lysine residues which are cross-linked, between about 15 and 85 percent.

6. The method of claim 1, for occluding a vessel whose lumen sizes is substantially larger than the sizes of the cross-linked fibers, wherein the

material provided has a pastelike consistency, and said introducing includes positioning a catheter device in the vessel to be occluded, and forcing the material into the vessel.

7. The method of claim 1, wherein said providing includes preparing a suspension of reconstituted atelopeptide collagen fibers, mechanically shearing the fibers to produce fiber sizes predominantly less than about 100 microns, and cross-linking the sheared fibers.

8. The method of claim 1, for occluding predominantly 20-250 micron arterial branch vessels fed from a segment of a supply artery having a diameter of at least about 2 mm, wherein the material provided has a fiber protein concentration of less than about 10 mg/ml, and said introducing includes positioning a catheter device in the supply artery segment, delivering the collagen into the supply artery, and by said delivering, producing flow-directed occlusion of the branch vessels of the specified size range.

9. The method of claim 8, wherein the catheter device has a pair of relatively movable balloons, and said positioning includes moving the two balloons to positions just upstream and downsteam of the ends of such arterial segment.

10. The method of claim 8, for use in treating a solid tumor having 20-250 micron arterial branch vessels fed by such segment of a supply artery, which further includes heating the region of the solid tumor

to raise the temperature of such region to at least about 42° C.

11. The method of claim 10, wherein said heating is effective to raise the temperature of the tumor region to between about 44-46°C.

12. The method of claim 11, wherein said heating is applied for a period of at least about 30 minutes.

13. The method of claim 1, for administering a drug to a target tissue supplied by such vessels, wherein the drug is included in the collagen vaso-occlusive material.

14. The method of claim 13, for treating a solid tumor having 20-250 micron arterial branch vessels fed by a segment of a supply artery, wherein the collagen vaso-occlusive material contains an antitumor drug and a collagen concentration of less than about 15 mg/ml, and said introducing includes positioning a catheter device in the supply artery segment, delivering the collagen material into the supply artery, and by said delivering, producing flow-directed occlusion of the branch vessels of the specified size range.

15. The method of claim 1, which further includes, following said introducing, infusing a selected drug into the vessel proximal to the vaso-occlusive material introduced into the vessel.

16. A nonimmunogenic vaso-occlusive composition having a biological persistence of greater

than about two weeks, comprising a vaso-occlusive material composed of a suspension of atelopeptide collagen fibers which are cross-linked under conditions which raise the melting temperature of the suspension at least about 10°C, and a radio-opaque agent, at a concentration sufficient to allow fluoroscopic monitoring of the distribution of the composition, when infused into a vascular site.

17. The composition of claim 16, for use in occluding predominantly 20-250 micron arterial vessels, wherein the material has a concentration of less than about 15 mg collagen/ml, and collagen fiber sizes predominantly less than about 100 microns.

18. The composition of claim 16, for use in delivering a drug at a vaso-occluded target site, wherein the composition includes such drug.

19. A method of treating a solid tumor comprising:

a. localizing the tumor and an arterial vessel that supplies it,

b. providing a collagen vaso-occlusive material adapted to produce occlusion of blood vessels having lumen diameters between about 10 and 250 microns,

c. injecting such material into the identified arterial vessel,

d. by said injecting, producing vaso-occlusion of the secondary and tertiary vessels supplying the tumor, distal to the collateral blood vessels which may also supply the tumor, and

e. heating the tumor under conditions which produce tissue necrosis selectively in the occluded tumor tissue.

20. A method of administering a drug to a target tissue supplied by a vessel, said method comprising

providing a mixture of collagen vaso-occlusive material and the drug,

injecting the mixture into the tissue-supply vessel, to produce localized vaso-occlusion of the target tissue, and

by said injecting, localizing the drug at the vessel site of vaso-occlusion, for slow release from the site into the surrounding target tissue.

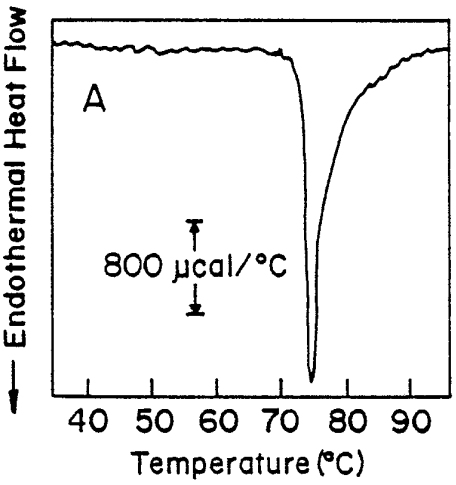


FIG. 1A

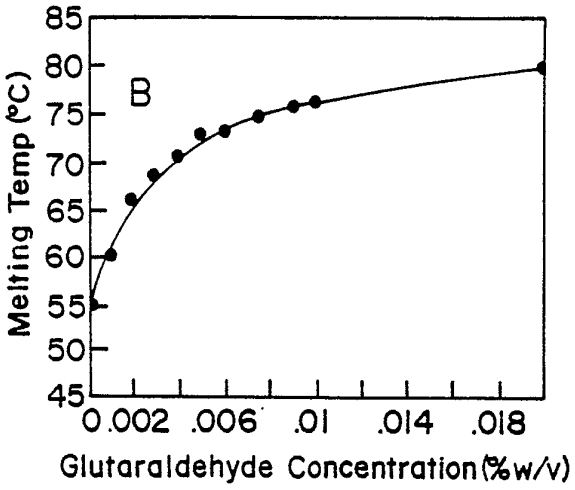


FIG. 1B

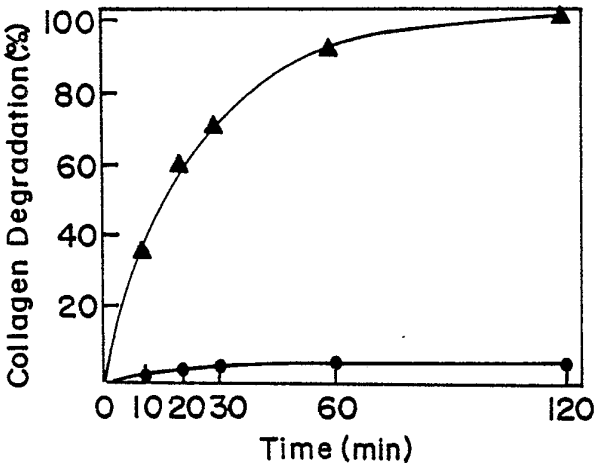


FIG. 3

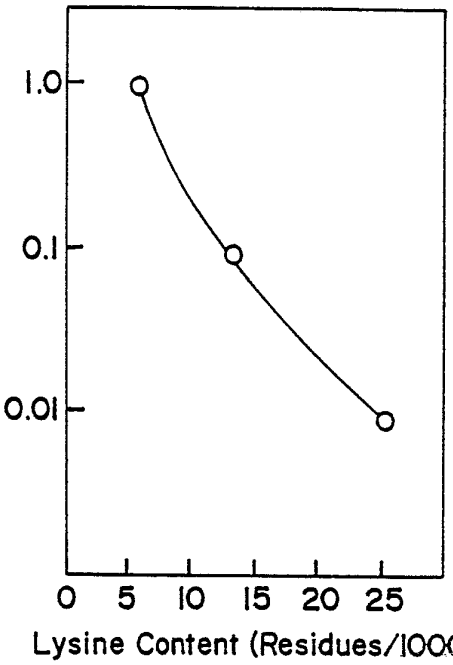
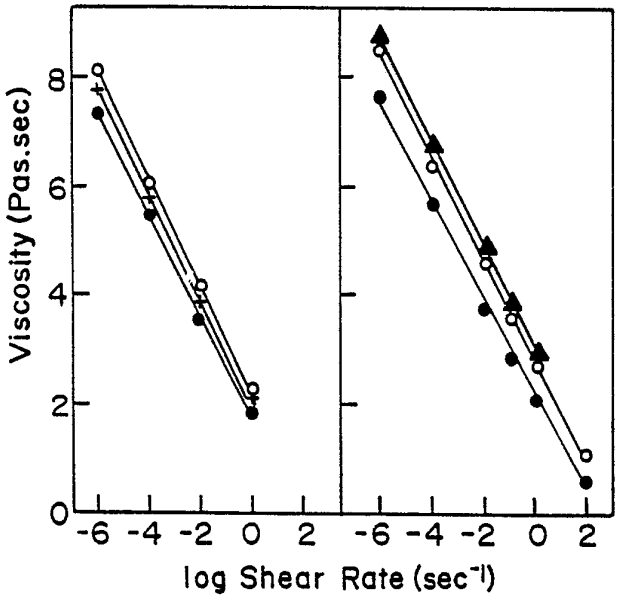


FIG. 2

FIG. 4A FIG. 4B



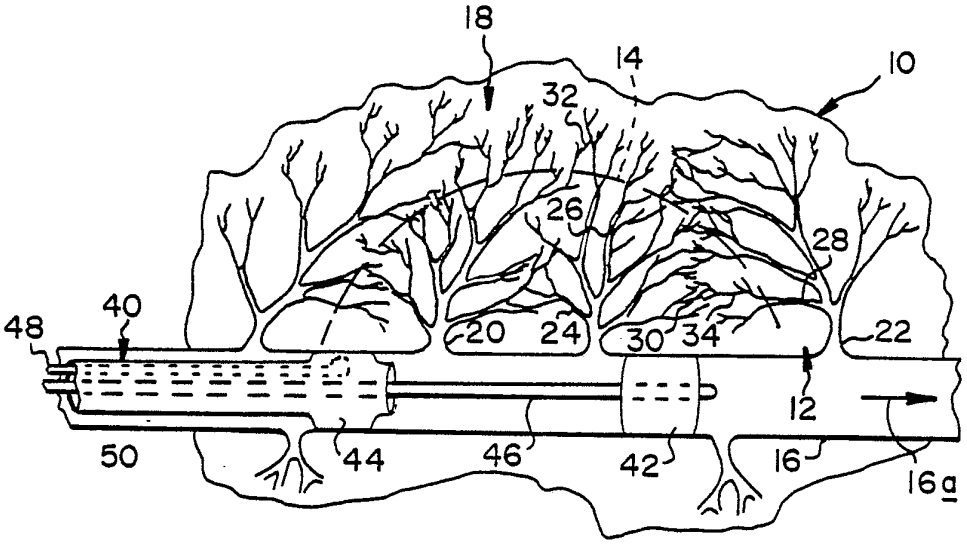


FIG. 5

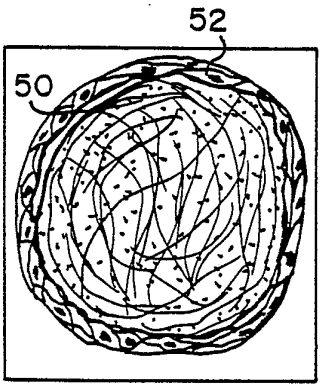


FIG. 6A

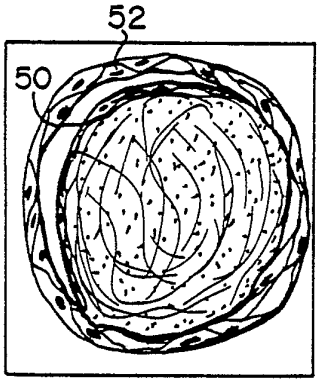


FIG. 6B

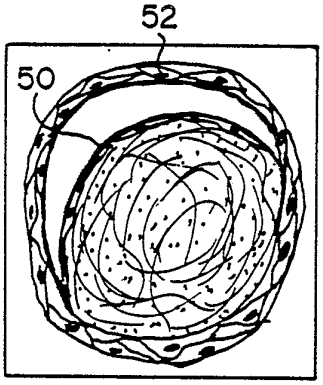


FIG. 6C

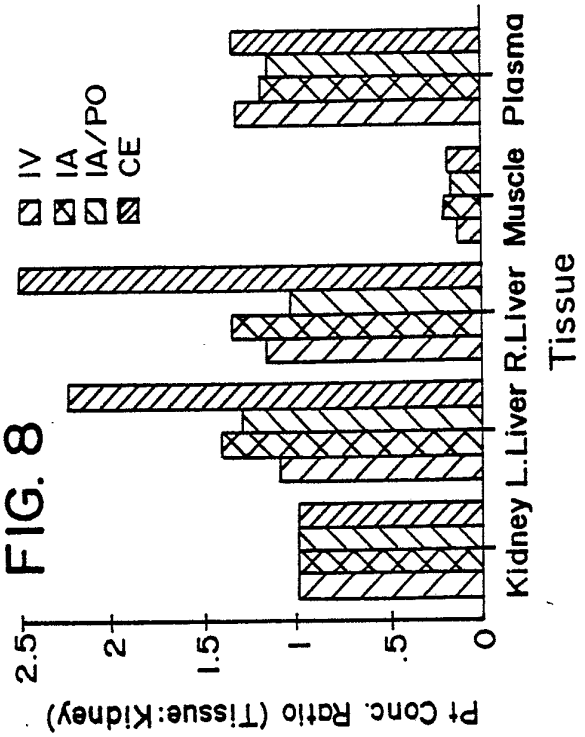


FIG. 8

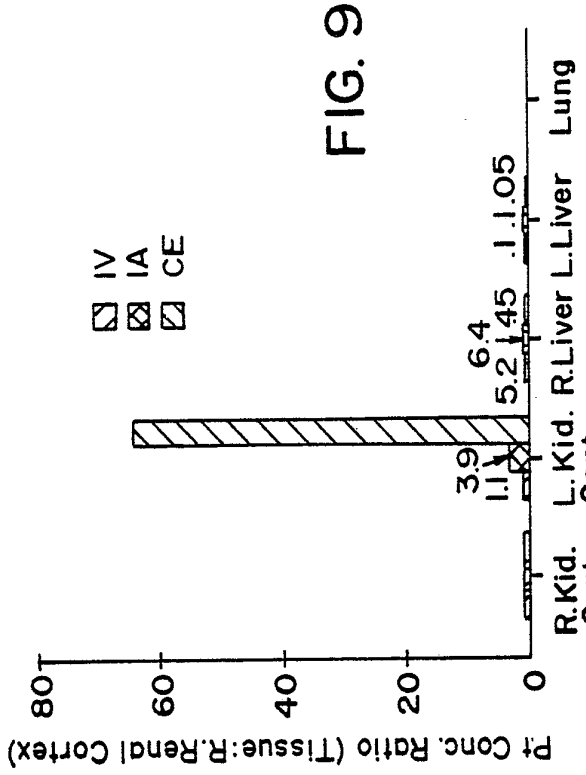


FIG. 9

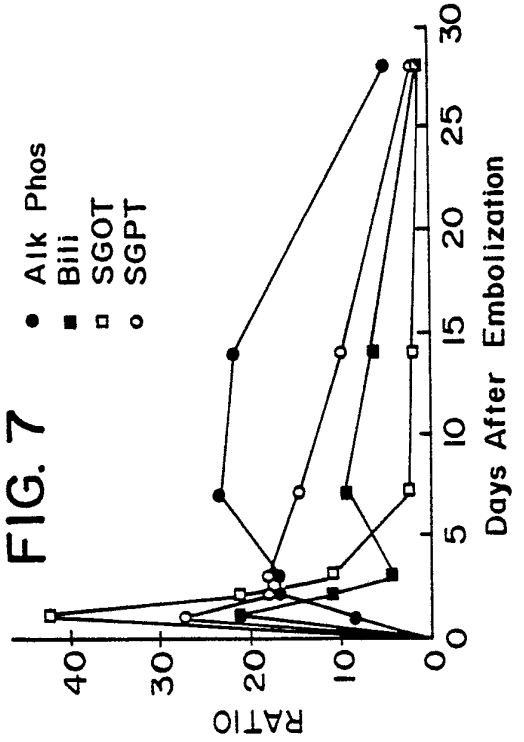


FIG. 7

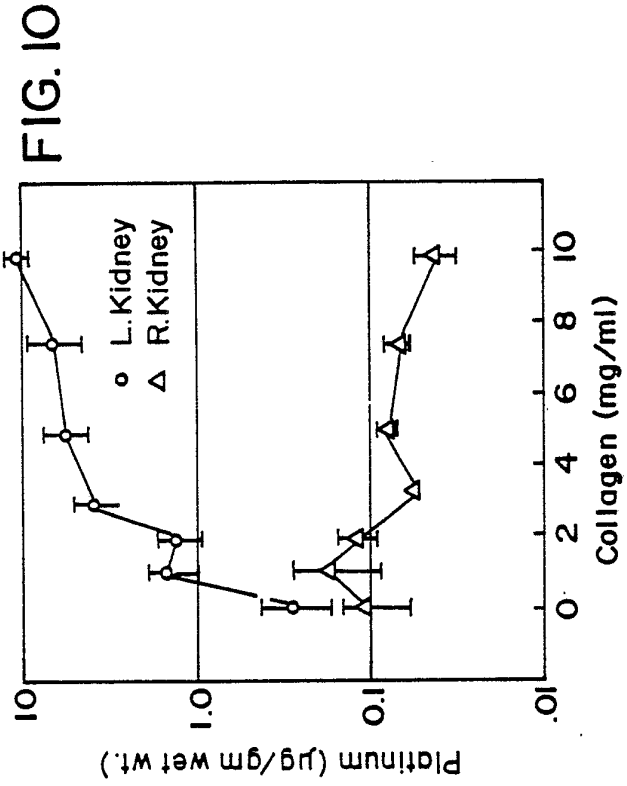


FIG. 10

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/01408

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4) A61N 1/30, 5/12

U.S. Cl. 604/20, 52; 128/1.1; 530/356

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System	Classification Symbols
U.S.	604/20,52,53,97,98,101,368; 128/1.1, 334R, Dig. 8; 424/14; 530/356

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 5

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
A,P	US, A, 4,558,690 (JOYCE) 17 December 1985. See entire document.	1-15,19-20
A,P	US, A, 4,569,836 (GORDON) 11 February 1986. See entire document.	1-15,19-20
A	H. LeVEEN, "Radiofrequency Thermotherapy, Local Chemotherapy, and Arterial Occlusion in the Treatment of Nonresectable Cancer," American Surgeon, Vol. 50, February 1984. See page 62.	1-15, 19-20
A	J. R. Stewart "Change in the IN VIVO Hyperthermic Response Resulting From the Metabolic Effects of Temporary Vascular Occlusion, "International Journal of Radiation Oncology Biology & Physics, February 1983, Vol. 9, No. 2. See page 197.	1-15,19-20
A	E.K. Rofstad, "Response of Human Malignant Melanoma Xenografts to Hyperthermia: Effect of Vascular Occlusion,""International Journal of Radiation Oncology Biology & Physics, December 1981, Vol. 7, No. 12. cont'd next pg.	

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
08 September 1986	18 SEP 1986
International Searching Authority 1	**Signature of Authorized Officer 10**
ISA/US	John Ferros

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

	See pages 1685-1686.	1-15,19-20
A	US, A, 4,238,480 (SAWYER) 09 December 1980. See entire document.	16-18
A	US, A, 4,374,830 (SCHNEIDER) 22 February 1983. See entire document.	16-18

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This international Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.