Title: ANTI-VEGF ANTIBODY/FRAGMENT CONJUGATED GOLD NANOPARTICLES, AND FABRICATION AND THERAPEUTIC METHODS

Abstract: Preferred embodiments of the invention include gold nanoparticles, preferably less than about 20nm, that are conjugated with biologically active anti-VEGF antibody or a fragment derived therefrom. The particles can be formed by a "one-pot" synthesis method of the invention, in which the biologically active anti-VEGF antibody or a fragment derived therefrom acts to stabilize formed gold nanoparticles while simultaneously retaining biological activity. The method uses a reducing agent of non-toxic trimeric alanine compound that is benign toward the biological activity of the anti-VEGF antibody or a fragment derived therefrom. Therapeutic methods of the invention include injection of a solution of conjugated gold nanoparticles of the invention at vascularisation sites such as cancer sites and intraocular injection.
ANTI-VEGF ANTIBODY/FRAGMENT CONJUGATED GOLD NANOPARTICLES, AND FABRICATION AND THERAPEUTIC METHODS

STATEMENT OF GOVERNMENT INTEREST

This invention was made with government support under Grant No. CA 119412 awarded by the National Institute for Health. The government has certain rights in the invention.

PRIORITY CLAIM AND REFERENCE TO RELATED APPLICATION

The application claims priority under 35 U.S.C. § 119 from prior provisional application serial number 61/455,322, which was filed October 19, 2010.

FIELD

A field of the invention is anti-VEGF (vascular endothelial growth factor) treatments. Example applications of the invention include a therapeutic method for slow responsive release of anti-VEGF antibodies and fragments thereof for treatment of diseases comprising neovascularisation, e.g. cancers and macular degeneration.

BACKGROUND

It is known that solid tumor growth in cancers is accompanied by increased vascularity. Compelling evidence has been established by various
researchers for the existence of a tumor-derived blood vessel growth stimulating factor that serves to induce a neovascular supply to the growing tumor. Therefore, anti-angiogenesis agents have gained prominence as effective therapeutic agents to treat cancer. The important role of vascular endothelial growth factor (VEGF) in the regulation of physiological and pathological angiogenesis has been well established. Research is focused on delivery of anti-VEGF agents to cancer sites \textit{in vivo} as cancer therapeutic methods.

Bevacizumab is an example humanized monoclonal antibody that inhibits vascular endothelial growth factor A (VEGF-A), which is a chemical signal that stimulates angiogenesis. Bevacizumab has been approved by the United States Food and Drug Administration (US FDA) for various metastatic cancers. An early approved use was for combined use with standard chemotherapy for metastatic colon cancer and non-small cell lung cancers. A once approved but currently withdrawn use by the US FDA was for breast cancer, but other countries still list bevacizumab for breast cancer. It is also prescribed "off-label" in the US for breast cancer. For the treatment of other cancers, effective delivery of bevacizumab or another anti-VEGF agent should target the location of the cancer.

Bevacizumab is commercially available as Avastin™ and has also been recognized as an effective treatment for eye diseases, such as age-related macular degeneration. Clinical studies and off-label prescriptions have been conducted to evaluate bevacizumab for treatment of macular degeneration, an eye disease also characterized by proliferation of blood vessels in the retina. One difficulty that remains concerns delivery. Free bevacizumab in the eye has a short duration of efficacy, which requires that a treatment procedure include multiple injections over a period of time. The practice of multiple injections creates additional risks that can outweigh the potential benefit of bevacizumab delivery to the eye.
Specifically, bevacizumab has been reported to have an intraocular half life of 4.3 days following intravitreal injection. See, Bakri S.J., et al., "Pharmacokinetics of Intravitreal Bevacizumab (Avastin)," Ophthalmology 2007; 114:855-9. This half life suggests a treatment regime that includes monthly repeat injections to maintain therapeutic efficacy. Subramanian M.L, et al. "Bevacizumab vs Ranibizumab for Age-Related Macular Degeneration: 1-year Outcomes of a Prospective, Double-Masked Randomised Clinical Trial," Eye (Lond) 2010; 24:1708-15. The risks presented by such frequent bevacizumab intraocular injections are shared with any other intraocular injections and include: increased risk of infection, bleeding, retinal detachment, cataract formation and subsequent visual loss. Despite this risks and the known complications with frequent injections, the present inventors are not aware of any commercially available sustained release forms of bevacizumab.

Ranibizumab (trade name Lucentis) is a monoclonal antibody fragment (Fab) derived from the same antibody as bevacizumab. Ranibizumab is offered commercially tinder the tradename of Lucentis ™. It also binds to VEGF-A. Ranibizumab has been approved to treat the "wet" type of age-related macular degeneration, but the intraocular injection regime is like that of bevacizumab and presents the same risks.


When gold nanoparticles are used in testing and therapeutic methods in the art, the nanoparticles are formed and then any ligands are chemically attached. Molecules being attached must withstand the procedure of attachment. Unfortunately, the chemical attachment of monoclonal antibodies can destroy the biological activity of the antibody. Other delivery vehicles have therefore been the focus of research and investigation.

For example, efforts have been made to investigate a delivery vehicle for bevacizumab into the eye. One effort involved encapsulation of bevacizumab in Poly(lactide-co-glycolide) (PLGA) nanoparticles. See, Hao et al., "Preparation and Characterization of Bevacizumab (Avastin) Nanoparticles for the Treatment of Age Related Macular Degeneration," Proceedings of the 2009 AAPS Annual Meeting and Exposition; Nov. 2009. A concern with any association of bevacizumab, or monoclonal antibodies in general, is that the biological activity can be affected or hampered in many processes. The Hao et al. paper reported that the PLGA encapsulation formed by a double emission method resulted in 230nm particles that retained biological activity and maintained for up to 4 weeks. This suggests an injection regime that is not radically different than injection of free bevacizumab. Xu et al., U.S. patent application 201110104069 also considers PLGA particles to form a biodegradable shell with an ocular targeting agent coupled to the exterior surface of the shell, a filler agent encapsulated within the shell; and a therapeutic agent encapsulated within the shell. The therapeutic agent can be an anti-VEGF therapeutic.
SUMMARY OF THE INVENTION

Preferred embodiments of the invention include gold nanoparticles, preferably less than about 20nm, that are conjugated with biologically active anti-VEGF antibody or a fragment derived therefrom. The particles can be formed by a "one-pot" synthesis method of the invention, in which the biologically active anti-VEGF antibody or a fragment derived therefrom acts to stabilize formed gold nanoparticles while simultaneously retaining biological activity. The method uses a reducing agent of nontoxic trimeric alanine compound that is benign toward the biological activity of the anti-VEGF antibody or a fragment derived therefrom. Therapeutic methods of the invention include injection of a solution of conjugated gold nanoparticles of the invention at vascularisation sites such as cancer sites and intraocular injection.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Preferred embodiments of the invention include gold nanoparticles, preferably less than about 20nm, conjugated with a biologically active anti-VEGF antibody or fragment derived therefrom. The particles can be formed by a "one-pot" synthesis method of the invention, in which the biologically active anti-VEGF antibody or fragment derived therefrom acts to stabilize formed gold nanoparticles while simultaneously retaining biological activity. The synthesis method in which the biologically active anti-VEGF antibody or fragment derived therefrom acts as a stabilizing agent is benign to the biological activity of the biologically active anti-VEGF antibody or fragment derived therefrom, which is therefore retained after conjugation.

The synthesis methods of the invention have been demonstrated to not affect biological activity of the anti-VEGF humanized monoclonal antibody bevacizumab when it is used as a stabilizing agent in the synthesis method. Ranibizumab, the monoclonal antibody fragment (Fab) derived from the same antibody as bevacizumab, has also been successfully conjugated when to gold
nanoparticles via the method of the invention when used as a stabilizing agent in the same manner. Animal testing had not yet been completed on ranibizumab at the time of this application’s filing, however, the inventors believe that biological activity will be retained because the preferred synthesis methods utilize a reducing agent of nontoxic trimeric alanine compound. This reducing agent has been demonstrated to not affect the biological activity of bevacizumab and its benign nature is believed, based upon the results, to be benign toward the biological activity of anti-VEGF antibodies or fragments thereof, toward anti-VEGF monoclonal antibodies or fragments thereof, and toward the specific anti-VEGF humanized monoclonal antibody bevacizumab and its fragment ranibizumab.

The testing of gold nanoparticles of the invention conjugated with biologically active bevacizumab has shown that the formation process does not affect biological activity of the bevacizumab. Tests have also shown that the biological activity and release can be retained for exceptionally long periods that would permit less frequent injections in a therapeutic method of the invention. The release, as shown by clinical studies, is triggered by biological events (e.g., intraocular neovascularization) in the eye over a period of months, instead of weeks. The longevity and long dwell time in the eye have been demonstrated with animal studies. The preferential binding to new blood vessels provided by the gold nanoparticles also provides benefits not available with other delivery vehicles. As cancers also involve neovascularisation, the therapeutic methods and preferential of the invention include the injection at cancer sites where the anti-VEGF antibody or fragment derived therefrom conjugated gold nanoparticles will preferentially bind at the cancer site.

Preferred embodiments of the invention will now be discussed with respect to experiments and animal studies that have been conducted for the formation, longevity, and animal model testing of gold nanoparticles conjugated with biologically active bevacizumab. Artisans will recognized broader aspects of the invention from the discussion of the experiments and studies, including the
benign nature of the nontoxic trimeric alanine compound toward biological activity of anti-VEGF antibodies and fragments thereof.

The experiments and studies have shown that gold nanoparticles preferentially bind to "leaky" blood vessels, such as those observed with intraocular neovascularization. With the example methods of the invention, bevacizumab is conjugated to gold nanoparticles with its biological activity being retained, allowing free bevacizumab to disassociate from the gold nanoparticles when increased VEGF concentrations are present in the ocular environment. Without being bound by any theory and without any such theory being necessary to support the invention, it is believed that the methods of the invention produce a non-covalent bond and that the bond might contribute to responsive dissociation. No cellular toxicity has been shown to be present with the experimental nanoparticles of the invention in animal model testing. Gold nanoparticles themselves may also contribute some long-term anti-VEGF properties, augmenting the angiogenesis inhibitory effect of the bevacizumab itself.

For the fabrication of bevacizumab nanoparticles in experiments, the precursors were obtained from commercial vendors: NaAuCl$_4$ (Alfa-Aesar); Avastin™ (Genentech); THPAL (Nanoparticles Biochem Inc) grids and other chemicals through Fisher Scientific. Preferred embodiment gold nanoparticles with non-covalently bonded bevacizumab were formed by the reduction of gold precursors (a source of Au$^{3+}$ ions) by a reducing agent. Particularly, sodium tetrachloro aurate (NaAuCl$_4$) with a nontoxic trimeric alanine compound, trimetric alanine peptide, ($P[CH_2NHCH(CH)_3COOH]_3$;THPAL(Trimeric Alanine Phosphine conjugate)) reducing agent in the presence of bevacizumab, which acted as a stabilizing solution. Specifically, to a glass vial was added 4 ml of sterile de-ionized water followed by the addition of 80 μL of Avastin solution (25 mg/ mL). To the stirring solution was added 160 μL of 0.0188 M NaAuCl$_4$ solution (in de-ionized, degassed water sterilized through membrane filtration) followed by the addition of 128 μL of 0.0188 M THPAL solution sterilized
through membrane filtration. The color of the solution gradually changed from colorless to red color indicating the generation of gold nanoparticles. Continuous stirring for 2h brings the reaction to completion. The nano-construct was stored at 4 °C.

Once synthesized, the gold nanoparticles were characterized by UV-Vis spectroscopy, Transmission electron microscopy and Malvern zeta seizer. The UV-Vis spectrum of gold nanoparticles showed a strong absorption band at ca. 530 nm is observed that corresponds to surface plasmon resonance of the gold nanoparticles. The presence of a ca. 530 peak is an indicative of the bevacizumab gold nanoparticle synthesis. The nanoparticles were further characterized by TEM imaging, which showed that the average morphology of the nanoparticles to be quasi-spherical and that the particles appear to be nearly monodispersed in size distribution histogram. The core particle size as determined by TEM was 9.4 nm (SD 1.1 nm). Hydrodynamic size as measured by zeta seizer nano S90 is 19 ± 1 nm, indicating the protein covering the nanoparticle surface is 8-10 nm (including its hydrodynamic environment. Zeta potential of nanoparticles as measured ranges from +32.5±1 mV. The solution of bevacizumab gold nanoparticles has a dark reddish appearance that remains in suspension for more than 6 months, indicating a very long shelf life of nanoparticles. Synthesis and stability of all gold nanoparticles was monitored by UV-vis spectroscopy on a Varian Cary 50 UV-Vis- spectrophotometer operated at a resolution of 5 nm. Physicochemical properties, such as size, charge, and morphology of bevacizumab gold nanoparticles were determined by two independent techniques; transmission electron microscopy (TEM) and dynamic light scattering (DLS). Dynamic light scattering method was employed to calculate the hydrodynamic size of bevacizumab gold nanoparticles. Zeta Potential (ζ), provides information on the stability and shelf life of the nanoparticle dispersion. TEM was used to determine the core size of bevacizumab gold nanoparticles and DLS was used to evaluate the hydrodyanamic size of the bevacizumab gold nanoparticles. Samples were
prepared by drop-coating films of the bevacizuraab gold nanoparticles solution on 300 mesh carbon-coated copper TEM grids followed by measurements on a JEOL model 1400 instrument, JOEL Ltd., Tokyo Japan, operated at an accelerating voltage of 100 kV.

The in vitro stability of bevacizumab gold nanoparticles in aqueous solutions of NaCl, histidine, HSA, BSA, as well as in pH 5, 7 and 9 phosphate buffer solutions was confirmed by UV absorbance. The plasmon wavelength and width in all the above formulations shifts -10 nm. This indicates that the bevacizumab gold nanoparticles are intact and thus, demonstrates the adequate in vitro stability of bevacizumab gold nanoparticles in biological fluids at physiological pH. In the testing, 0.5 mL of Bevacizumab gold nanoparticle solution was added to 0.25 mL aliquots of 2.5 % NaCl, 0.2 m histidine, 0.5 % BSA or 0.5 % HSA respectively. We also investigated the stability of Bevacizumab gold nanoparticles in pI 5, 7 and pH 9 phosphate buffer solutions. The stability and the identity of Bevacizumab gold nanoparticles were measured by recording UV absorbance.

**MTT cytotoxicity assay**

MTT assay was performed as described by the manufacturer (ATCC, USA) to evaluate the cytotoxicity of the bevacizumab gold nanoparticle. Human fibroblasts primary cultures were obtained as a gift from Prof. Cris Lorson, Bond Life Science Centre, University of Missouri-Columbia. The cells were maintained in DMEM (Gibco BRL) supplemented with 10% donor bovine serum, 10 pgmL⁻¹ phenol red, 100 units mL⁻¹ penicillin, 100 pgmL⁻¹ streptomycin and 10 mm HEPES. Briefly, 2 × 10⁴ cells at the exponential growth phase were seeded in each well of a flat-bottomed 96-well polystyrene-coated plate and were incubated for 24 h in CO₂ incubator at 5% CO₂ and 37 °C. Series of dilutions like 25, 50, 125 and 250 µM of these nanoparticles (gold atoms) were made in the medium. Each concentration was added to the plate in pentaplet manner. After 24 h incubation, 10 µL per well MTT (stock solution 5mgmL⁻¹ PBS)(ATCC, USA) was
added for 24 h and Formosan crystals so formed were dissolved in 100 µL detergent. The plates were kept for 18 h in dark at 25 °C to dissolve all the crystals and the intensity of color development was measured by micro plate reader (Dynastic MR 5000, USA) operating at 570 nm. Wells with complete medium, nanoparticles, and MTT, but without cells were used as blanks. Untreated cells were considered 100 % viable.

**ELISA Studies**

The biological activity of bevacizumab gold nanoparticles was measured through highly specific sandwiched ELISA assay. This assay is critical in the sense that it provides the estimates of VEGF binding sites available on conjugated bevacizumab gold nanoparticles. Briefly, wells of maxisorp ELISA plate (Nunc) were coated with 50 µl/ well predetermined concentrations of free bevacizumab, bevacizumab gold nanoparticles, human IgG conjugated gold nanoparticles and PEG (polyethylene glycol to increase the aqueous solubility) conjugated gold nanoparticles as controls in 0.1 M sodium carbonate coating buffer (pH 9.5) at 4 °C for 16h. The plates were washed twice with wash buffer provided with BD-OptEIA reagent set followed by blocking in Assay diluent (BD) for 2 h at room temperature. After incubation the well were washed 5 times with wash buffer and biological activity of bevacizumab in nanoparticulate suspension was targeted by reincubation of plate with biotinylated Fluorikine VEGF (R&D systems) for 30 min at room temperature. Following VEGF incubation the wells were further washed for 5 times with wash buffer and 100 µl/ well Streptavidin HRP (Sigma)(1:1000 dilution) added in dark for color development. The assay was terminated by addition of 1M phosphoric acid and plates were read at 450 nm in Powerwave plate reader (Bioteck Instruments, USA). A standard graph of bevacizumab concentration was plotted against OD and bound bevacizumab concentrations were measured.

**Animals Studies**
Brown Norway rats and New Zealand white rabbits of either sex, of 6-8 weeks age were used in accordance with the institutional ACLJC committee adhering to ARVO statement on treatment of laboratory animals. Anesthesia was induced with intraperitoneal injection of ketamine and xylazine.

**Injection of bevacizumab gold nanoparticles or free bevacizumab**

Intravitreal injection of 5 µL of Bevacizumab gold nanoparticle solution or 5 µL commercially available Avastin solution (25mg/ml) was performed using a 10 µL Hamilton syringe with a 32-gauge 12° beveled needle (Hamilton) in rat. The needle was placed 1.5-2 mm posterior to the corneal limbus and was inserted into the mid-vitreous cavity with careful injection of Bevacizumab gold nanoparticle solution. The needle was left in position for 60 seconds and then slowly withdrawn to minimize fluid loss from the eye. Control rats received 5 µL of sterile balanced salt solution and 2 rats received intravitreal injection in both eyes.

**Retinal photography**

Rat fundus photography was performed immediately and seven days after intravitreal Bevacizumab gold nanoparticle injection using a Carl Zeiss 450 digital camera modified with a Volk 2.2 condensing lens (Volk Ophthalmic,). Angiography was performed 10 minutes after intraperitoneal injection of 0.1ml of 2.5% fluorescein sodium solution.

**Histology and Immunohistochemistry**

Immediately after sacrifice, rat eyes were carefully enucleated and placed in modified Davidson's fixative for at least 24 hours. Fixed eyes were processed for histological evaluation using H & E staining. To determine the presence of bevacizumab in rat eyes following intravitreal injection with either bevacizumab gold nanoparticle or free bevacizumab, two rats from each injection group were sacrificed at one hour, one day and seven days. The four eyes from each time point were fixed in modified Davidson fixative for 24 h and processed for Immunohistochemistry. Briefly the tissues were processed and 5 micron
sections were cut. The slides were placed in a 60 °C oven for 30 min. Slides were
deparafinized by placing them in Xylene for 10 min by two changes of xylene, 2
changes of absolute alcohol and 2 changes of 95% alcohol and then rinsed in
distilled water. The slides were kept in wash buffer (Dako, Carpinteria, CA cat# S3006) for 5 min followed by 20 min incubation with FITC conjugated affinity
purified goat anti human IgG, F(ab’) (Jackson Immuno Research Labs, Inc, PA, Cat# 109-095-097) in dark at 25 °C. Since, bevacizumab is a humanized
monoclonal antibody, antibody specific for human IgG allowed the presence of
bevacizumab to be determined in rat eyes.

Distribution of bevacizumab gold nanoparticle in Rat Eye

Eyes injected with 5 µL of Bevacizumab gold nanoparticle solution intravitreallly were enucleated at one week in modified Davidson fixative. The
retinas were detached from the sclera and both sides of the retina were processed
for scanning electron microscopy (SEM). The samples after 24 h of fixation were
washed with sodium cacodylate buffer followed by secondary fixation in osmium
tetraoxide for 1 h on rocking platform at 25 °C. The samples were washed thrice
with ultrapure distilled water and dehydrated in graded acetone series. After
acetone dehydration samples were subjected to critical point drying (Tousimis
Auto-Samidri 815) and mounted on SEM stub. Mounted samples were sputter
coated with carbon and observed under Hitachi H4700 Hitachi S-4700 Scanning
Electron Microscope. Secondary electron and backscatter images of both sides of
the retina were obtained to identify the presence of bevacizumab gold nanoparticle.

Distribution of bevacizumab gold nanoparticle in Rabbit Eye

New Zealand white rabbits were injected with 50 µL of
Bevacizumab gold nanoparticle solution intravitreallly. Four eyes were injected
immediately after sacrifice and then enucleated to avoid any possible systemic
absorption or metabolism of the Bevacizumab gold nanoparticle. Four eyes were
enucleated for each time point at seven days and 21 days post injection and frozen
at -70 degrees. The frozen eyes were dissected, separating the tissue into three groups; 1) retina, choroid and sclera, 2) vitreous, and 3) lens, iris, aqueous and cornea. Neutron activation analysis (NAA) was used to determine whether bevacizumab gold nanoparticle remained localized to the eye after intravitreal injection or was absorbed systemically. NAA was performed to determine the gold content of each group in each eye. Retina, sclera and choroid of the dissected eye were analyzed as a single fraction. It was assumed that the majority of the gold in this fraction was within the retina, with minimal penetration to the sclera. Accordingly, an average rabbit retinal mass of 150 mg for analysis was decided from the available literature.

In vitro toxicity assessment

Following physico-chemical characterization dose dependent in vitro toxicity studies were performed on primary cultures of human fibroblast cells. Fibroblast cells treated with 0, 25, 50, 125, 150, 165 and 247 \( \mu \text{gole} \) of bevacizumab gold nanoparticles for 24 h were subjected to MTT assay for cell-viability determination. After 24 h of bevacizumab gold nanoparticle treatment, cells showed more than 90\% viability up to 150 \( \mu \text{gole} \) of bevacizumab gold nanoparticles, and more than 80\% viability for up to 250 \( \mu \text{gole} \).

Intraocular localization

Intravitreal bevacizumab gold nanoparticle injection results in a dark reddish vitreous opacity. Immediately following bevacizumab gold nanoparticle injection into rabbit and rat eyes, little retinal detail can be seen, although more detail was seen with fluorescein angiography. However, at one week, the diffuse vitreous haze cleared with only fine reddish clumping of bevacizumab gold nanoparticle remains to be seen with retinal examination. These clumps are manifested as focal blockage on fluorescein angiography. These clumps represent aggregates of bevacizumab gold nanoparticles since individual nanoparticles are not discernable without electron microscopy. Testing showed that the vast
majority of the bevacizumab gold nanoparticles have migrated out of the vitreous by the first week, resulting in remarkably clear ocular media.

No retinal, ciliary body, iris or corneal abnormalities were noted at either one hour or up to 3 week after bevacizumab gold nanoparticle injection into the vitreous. In order to understand the distribution and intraocular localization profile of Bevacizumab gold nanoparticle in rabbit eye the eye was dissected and different tissues were carefully separated. The amount of gold in different eye tissues was measured through neutron activation analysis. The average wet weight of rabbit retina has been reported to be 150 mg. To determine the amount of gold per tissue weight, it was assumed that the gold that deposited in the retina did not move through the retina in appreciable amounts to penetrate the choroid and sclera. Thus the gold measured in the retina/choroid/sclera combined tissue was calculated per tissue weight as if only in the retina. SEM images with associated backscattering confirmed the presence of bevacizumab gold nanoparticle within the retina. The presence of the bevacizumab antibody coating was seen on SEM images surrounding the bevacizumab gold nanoparticles seen on backscatter images.

**Long term stability of biologically active bevacizumab following conjugation (ELISA)**

Bevacizumab gold nanoparticle was tested for its immunoreactivity toward human VEGF and long term biological activity of bevacizumab gold nanoparticles was measured through ELISA assay. A highly specific in vitro ELISA assay was used to measure biological activity of bevacizumab in bevacizumab gold nanoparticle using biotinylated VEGF as a detection.

Freshly prepared bevacizumab was compared with two different 6 months old batches of Bevacizumab gold nanoparticle stored at 4°C. Human IgG conjugated gold nanoparticles, and gum arabic glycoprotein conjugated nanoparticles were used as negative control. These results demonstrated long term VEGF immunoreactivity of bevacizumab gold nanoparticles. The results did not
make clear how much amount of bevacizumab is conjugated per unit of bevacizumab gold nanoparticle, but it the amount of conjugated nanoparticles exhibited a long term stability and preservation of biological activity of bevacizumab towards human VEGF.

**Intraocular bioavailability of Bevacizumab**

When free bevacizumab was injected intravitreally, fluorescent staining for human IgG demonstrated bevacizumab to be diffusely present in the rat vitreous at one hour. However, at one week no bevacizumab remained, consistent with the short half life observed with this antibody.

Similar anti human IgG staining in eyes injected with bevacizumab gold nanoparticle demonstrated focal areas of fluorescent staining both at one hour and at one week, indicating continued presence of intraocular bevacizumab.

**Therapeutic Release**

The tests demonstrated the suitability of the present bevacizumab gold nanoparticles for therapeutic treatment of intraocular neovascular disorders. The synthesis process was a one-pot synthesis that is scalable, and does not require or produce any toxic by-products. Advantageously, the bevacizumab gold nanoparticles clear from the vitreous by dispersing into the retina. The studies showed that after one week, the majority of bevacizumab gold particles remained within the retina. This provides a reservoir of bevacizumab gold particles that can release bevacizumab that can function as a sustained release in response to retinal neovascularisation. Though little bevacizumab staining was noted after two weeks, the bevacizumab gold particles clump in inflammatory cells. It is likely that bevacizumab, which is a humanized IgG antibody, is recognized as foreign in a rat model and a resultant immunological reaction is being mounted against this foreign protein, which would explain the degradation of bevacizumab by two weeks.

The ideal amount of solution including bevacizumab nanoparticles for is determined largely by the practical consideration of the need to inject an
amount that is physically tolerable for a human eye (0.1 ml or less) with a concentration that is already commercially available as free bevacizumab (25 mg/ml). A therapeutic method of the invention involves initially injecting a combination of free bevacizumab and bevacizumab gold nanoparticles, so that the free bevacizumab is present to inhibit retinal neovascularization and the bevacizumab gold nanoparticles to maintain this inhibition for an extended period of time.

Bevacizumab is non-covalently bound to the gold nanoparticles in the invention. This allows for dissociation in the presence of high levels of VEGF in the local intraocular environment; this dissociation being driven by the concentration gradient of increased levels of VEGF. This dissociation of bevacizumab can likely decrease as local VEGF levels decrease, allowing a store of bevacizumab to be maintained until needed by increased activity of retinal neovascularization. The ability of bevacizumab gold nanoparticles to inhibit retinal neovascularization, both initially and after recurrent activity can provide an important therapeutic benefit. The ability of gold nanoparticles to bind preferentially to neovascularization makes it a useful platform for targeted delivery in many retinal diseases.

While specific embodiments of the present invention have been shown and described, it should be understood that other modifications, substitutions and alternatives are apparent to one of ordinary skill in the art. Such modifications, substitutions and alternatives can be made without departing from the spirit and scope of the invention, which should be determined from the appended claims.

Various features of the invention are set forth in the appended claims.
CLAIMS

1. A gold nanoparticle having a biologically active anti-VEGF antibody or fragment derived therefrom conjugated to its surface.

2. The gold nanoparticle of claim 1, wherein said anti-VEGF antibody or fragment derived therefrom comprises an anti-VEGF monoclonal antibody or fragment derived therefrom.

3. The gold nanoparticle of claim 1, wherein said anti-VEGF antibody or fragment derived therefrom comprises an anti-VEGF humanized monoclonal antibody or fragment derived therefrom.

4. The gold nanoparticle of claim 3, wherein said anti-VEGF humanized monoclonal antibody or fragment derived therefrom comprises bevacizumab.

5. The gold nanoparticle of claim 3, wherein said anti-VEGF humanized monoclonal antibody or fragment derived therefrom comprises ranibizumab.

6. An aqueous solution for injection at a neovascularization site, the solution comprising a plurality of gold nanoparticles according to claim 1 and a biologically compatible buffer.

7. An aqueous solution for intraocular injection, the solution comprising a plurality of gold nanoparticles according to claim 1 and a biologically compatible buffer.
8. The aqueous solution of claim 7, having gold nanoparticles of about 250 micromoles or less.


10. The method of claim 9, wherein the solution further comprises free anti-VEGF antibodies or fragments derived therefrom.

11. The nanoparticle of claim 1, wherein the gold nanoparticle is about 9-10nm and the biologically active anti-VEGF antibody or fragment derived therefrom forms an about 8-10nm coating.

12. A method for fabrication of gold nanoparticles conjugated with biologically active anti-VEGF antibody or a fragment derived therefrom, the method comprising:

   providing gold precursors including a gold containing molecule and a reducing agent of nontoxic trimeric alanine compound in solution in the presence of biologically active anti-VEGF antibody or fragment derived therefrom;

   mixing the solution for a time period sufficient to permit formation of the gold nanoparticles conjugated with biologically active anti-VEGF antibody or a fragment derived therefrom.

13. The method of claim 12, wherein the gold containing molecule comprises sodium tetrachloro aurate (NaAuCl₄) and a reducing agent is trimetric alanine peptide.

14. The method of claim 12, wherein said providing comprises combining the gold containing molecule with the anti-VEGF antibody or fragment
derived therefrom in de-ionized, degassed water sterilized through membrane filtration, followed by the addition of trimeric alanine phosphine conjugate solution sterilized through membrane filtration.

15. The method of claim 12, wherein said anti-VEGF antibody or fragment derived therefrom comprises an anti-VEGF monoclonal antibody or fragment derived therefrom.

16. The method of claim 12, wherein said anti-VEGF antibody or fragment derived therefrom comprises an anti-VEGF humanized monoclonal antibody or fragment derived therefrom.

17. The method of claim 12, wherein said anti-VEGF humanized monoclonal antibody or fragment derived therefrom comprises bevacizumab.

18. The method of claim 17, wherein said anti-VEGF humanized monoclonal antibody or fragment derived therefrom comprises ranibizumab.