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Targeted imaging of VCAM-1 mRNA in a mouse model of laser-induced choroidal neovascularization (LCNV) using antisense hairpin-DNA functionalized gold-nanoparticles

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Abstract

Mouse laser-induced choroidal neovascularization (mouse LCNV) recapitulates the ‘wet’ form of human age-related macular degeneration (AMD). Vascular cell adhesion molecule-1 (VCAM-1) is a known inflammatory biomarker and it increases in the choroidal neovascular tissues characteristic of this experimental model. We have designed and constructed gold nanoparticles (AuNP) functionalized with hairpin-DNA that incorporates an anti-sense sequence complementary to VCAM-1 mRNA (AS-VCAM-1 hAuNP), and tested them as optical imaging probes. The 3’ end of the hairpin is coupled to a near-infrared fluorophore that is quenched by the AuNP surface via Förster resonance energy transfer (FRET). Hybridization of the anti-sense sequence to VCAM-1 mRNA, displaces the fluorophore away from the AuNP surface, inducing fluorescent activity. *In vitro* testing showed that hAuNP hybridize to an exogenous complementary oligonucleotide within a pH range of 4.5 to 7.4 and that they are stable at reduced pH. LCNV mice received tail-vein injections of AS-VCAM-1 hAuNP. Hyperspectral imaging revealed the delivery of AS-VCAM-1 hAuNP to excised choroidal tissues. Fluorescent images of CNV lesions were obtained, presumably in response to hybridization of AS-hAuNP to LCNV-induced VCAM-1 mRNA. This is the first demonstration of systemic delivery of hAuNP to ocular tissues to facilitate mRNA imaging of any target.

Keywords

Hairpin-DNA; gold nanoparticle; VCAM-1 mRNA; imaging; LCNV

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Author Contributions

M.I.U designed and supervised the project, performed the imaging studies, analyzed the data and wrote the manuscript; T.C.K. performed the animal husbandry and *ex vivo* assays; R.Y. performed dissection of the choroid-RPE tissues; G.W.M. helped revise the manuscript, D.W.W. and J.S.P. supervised the project; All authors reviewed the manuscript.

INTRODUCTION

Imaging mRNA in cells and tissues is of great interest, and it is commonly achieved by *in situ* hybridization (ISH). Originally, this technique relied on the hybridization of mRNAs to radiolabeled complementary oligonucleotides ¹. Fluorescence *in situ* hybridization (FISH) is a more recent adaptation, eliminating the need of radioisotopes, and it is capable of distinguishing mRNAs that differ by only a single base ². Other hybridization methods include the application of molecular beacons ³ and forced intercalation probes (FIT) ⁴. Molecular beacons hybridize with specific mRNA biomarkers, allowing their detection and imaging in cells and tissues. A molecular beacon consists of a hairpin-shaped oligonucleotide coupled to a fluorophore and a quencher on opposite ends. In the hairpin conformation, the quencher and fluorophore are juxtaposed, causing fluorescence emission to be blocked by distance-dependent Förster resonance energy transfer (FRET). Upon hybridization to the target mRNA, the hairpin opens up, triggering fluorescent activity. Non-hybridization mRNA imaging methods exist, some of which depend on the covalent modification of mRNA, mRNA protein binding ⁵ and reporter protein expression by trans-splicing ⁶. Hybridization methods using antisense-DNA oligonucleotides (AS-DNA) are attractive because they regulate gene expression by reducing mRNA levels; however, they are limited due to the short half-lives of AS-DNAs that require covalent modification to enhance their stability ⁷. Furthermore, AS-DNA uptake often requires transfection reagents that are toxic in some cases. Hence, these drawbacks have heretofore precluded the use of AS-DNA for imaging mRNAs *in vivo*.

Gold nanoparticles (AuNP) are readily internalized by cells and tissues and have numerous potential medical applications. AuNP, specifically gold nanorods, can be used to visualize laser-induced choroidal neovascular lesions in mice using photothermal-optical coherence tomography (PT-OCT). However, without the correct functionalization, AuNP are not capable of detecting specific mRNA biomarkers ⁸. If they are conjugated to AS-DNA, the conjugates (AS-AuNP) are fully capable of hybridizing to an mRNA of interest as well as cellular internalization. Thus, visualization and localization of specific target mRNAs in cells and tissue becomes feasible. In effect, AuNP are exploited as non-viral vectors to deliver oligonucleotides that are otherwise unstable *in vivo*. In the current report we have used spherical 15 nm AuNP to prepare our AS-AuNP since these nanoparticles readily cross tissue barriers. Recently, others and we have structurally modified AS-DNAs to include self-complementary sequences causing them to adopt a hairpin-loop conformation, and a fluorophore coupled to the 3' end, to impart optical activity. We have coupled these antisense hairpin DNAs to 15 nm AuNP (AS-hAuNP) and used them as optical probes to image mRNA in living cells ⁹. The mechanism of mRNA detection is similar to that of molecular beacons described above. Presumably the AS-hairpin DNA stands erect, juxtapositioning the fluorophore to the hAuNP surface, causing it to be quenched by FRET. When AS-hAuNP hybridize to target mRNAs, the hairpin opens up, displacing the fluorophore to a distance beyond that required for quenching. Thus, fluorescence emission is induced reporting the hybridization event.

Vascular cell adhesion molecule 1 (VCAM-1) is over-expressed in a number of human ocular diseases^{10–12} and in experimental ocular disease models^{13–14}. VCAM-1 is induced in cultured cells and tissues by the pro-inflammatory cytokine tumour necrosis factor- α (TNF- α)¹⁵. Thus, VCAM-1 is a putative biomarker of ocular disease and its *in vivo* spatiotemporal imaging might be useful for disease staging. Several attempts have been made to visualize VCAM-1 protein levels using radiolabeled peptides, but these efforts were limited by low signal-to-background ratios^{16–17}. Recent advances in nanomaterial-based approaches targeting VCAM-1 protein have overcome several of these limitations, but still results have proven less than optimal^{18–20}. As an alternative, an imaging method to visualize VCAM-1 mRNA in tissues may overcome some of these limitations and, if successful, could provide useful information to understand disease onset, progression or response to therapy.

In vivo imaging of VCAM-1 mRNA expression could potentially be used as a predictor of an incipient neovascular response, allowing the clinician a therapeutic window to pre-emptively abort its onset and progression. However, real time mRNA imaging remains a major challenge²¹. We have previously reported the advantages of AS-hAuNP related to their application to imaging mRNAs in living retinal cells and cancer cells^{9, 22–23}. In the current study, we have constructed AS-hAuNP incorporating an anti-sense sequence complementary to VCAM-1 mRNA (AS-VCAM-1 hAuNP). We have used these imaging probes to visualize VCAM-1 mRNA after systemic administration followed by *ex vivo* detection in excised neovascular choroidal tissues. Herein we report the results.

MATERIALS AND METHODS

Materials. Gold nanoparticles with diameters ranging from 15–20 nm were obtained from Nanopartz Inc (Loveland, CO). Custom designed molecular beacons (MB) and custom oligonucleotides were purchased from Integrated DNA Technologies Inc. (Coralville, IA). Synthesis of AS-VCAM-1 hAuNP and nonsense NS-hAuNP are described elsewhere⁹. Briefly, DNA oligonucleotides were synthesized that incorporated the mouse anti-sense VCAM-1 sequence GCC TCC ACC AGA CTG TAC GAT CCT or a scrambled version of this sequence (nonsense sequence). The anti-sense VCAM-1 and the nonsense sequences are located within the loop of the hairpin structure (Figure 1A). The DNA-oligonucleotides were computationally designed to achieve energy-minimalized conformation when in the hairpin configuration. Each of the optimized DNA oligonucleotides, including the NS control, were coupled to an Alexafluor-647 near-infrared (NIR) dye (fluorophore) at the 3' end. The 5' ends were modified with a thiol (–SH) group that forms a strong Au-S bond that anchors the DNA hairpin to the surface of the gold nanoparticle. The diameters of the gold nanoparticles used to prepare the hAuNP ranged from 15–20 nm and the average diameter of the entire hAuNP was determined to be approximately 37 $\pm$ 4 nm by dynamic light scattering (DLS). The number of DNA oligonucleotides per gold nanoparticle was approximately 48⁹.

Animals:

C57BL/6J male mice 4–6 weeks of age were purchased from Charles River Laboratories. All animal procedures used in this study were approved by the Vanderbilt University

Institutional Animal Care and Use Committee and were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Mouse model of laser-induced choroidal neovascularization (LCNV):

To induce CNV in C57BL/6J mice, laser-induced ruptures of Bruch's membrane were performed with an argon laser photocoagulator (blue-green light) mounted on a slit-lamp (Space Coast Laser Inc.; Palm Bay, FL). Four lesions were created in both the left and right eyes of each mouse. Laser parameters used were 100- μ m spot size, 0.1-second duration, and 0.1 Watts²⁴⁻²⁷. On day-3 post-laser treatment, mice were divided into two groups and received tail-vein injections of AS-VCAM-1 hAuNP or NS-hAuNP at a concentration of 0.5 mg/kg in PBS. Twenty-four hours after the probe was incorporated, the mice were sacrificed and the eyes were fixed in neutral buffer formalin (NBF) for 30 min. The anterior segments and lenses were removed while submerging the eye in NBF solution. The choroid-Bruch's membrane-RPE complex was dissected as previously described²⁸⁻²⁹ and transferred into tris-buffered saline (TBS) before immunostaining. Tissues were blocked/permeabilized in 10% donkey serum with 1% Triton X-100 and 0.05% Tween 20 in TBS for 2 hours and were then counter-stained for IB4 conjugated to Alexafluor-488 (Life Technologies; Grand Island, NY). The tissues were then mounted with Prolong Gold mounting medium (Life Technologies; Grand Island, NY). Images were taken using an epifluorescence Nikon Eclipse Ti-E inverted microscope (Melville, NY).

Electroretinogram (ERG) measurements:

ERG measurements were performed according to previously published methods³⁰⁻³¹. Briefly, ERG analysis was performed on mice injected with AS-VCAM-1 hAuNP (0.5 mg/kg) at one week post-injection. Mice were dark adapted overnight, anesthetized with ketamine/xylazine, eyes were dilated with 1% tropicamide, and placed on a warm platform within the Ganzfeld dome of a Diagnosys LLC Espion Electrophysiology system (Lowell, MA). Mice were exposed to flashes of light ranging from -4 to 2 log cd.s/m² and the amplitudes of *a*-wave and *b*-wave were measured from baseline to peak. The amplitude of the *a*-wave and *b*-wave were plotted as a function of luminance.

Hyperspectral Imaging:

Adult C57BL/6 mice were injected with AS-VCAM-1 hAuNP at a dose of 0.5 mg/kg. At 1 week post-injection, mice were sacrificed and choroidal tissues were dissected as described above. Spectral scans and images were taken using the CytoViva Hyperspectral Imaging System (CytoViva Inc.; Auburn, AL) and archived with ENVI software.

RESULTS

The design and synthesis of AS-VCAM-1 hAuNP and NS-hAuNP were carried out according to the methods described previously and characterized using dynamic light scattering (DLS) and transmission electron microscopic (TEM) analysis⁹. Method for the synthesis is briefly described in the method section.

The pH-dependent stability of AS-VCAM-1 hAuNP and hybridization motif:

Mouse LCNV has an inflammatory component and inflammation may cause reduced tissue pH. We speculated that probe degradation might occur at a reduced pH in tissue inflammation. Therefore, we tested and compared the stabilities of the AS-VCAM-1 hAuNP in pure water or isotonic media with pH levels ranging from 1.4 to 7.4 (Figure 1B). The base-line fluorescence was minimal at pH=1.6 and there were no changes in fluorescent activity over 8 hours, at any of the pHs tested (Figure 1B). After 8 hours incubation, synthetic complementary oligonucleotide was added and the relative hybridization rates were assessed. They were highest at pHs of 4.5, 5.8, and 6.5, and declined at pH=1.6. Surprisingly, the hybridization was very slow in pure water, perhaps owing to the absence of ions that facilitate melting of the DNA hairpin.

AS-VCAM-1 hAuNP dependent ex vivo imaging of VCAM-1 mRNA:

We performed fluorescence imaging of CNV lesions from LCNV mice receiving systemic injections of AS-VCAM-1 hAuNP through tail-vein. The CNV lesions were counter-stained with isolectin B4 (Figure 2 A). We observed AS-VCAM-1 hAuNP dependent fluorescence that co-localized with isolectin B4 staining indicating focally expressed VCAM-1 mRNA in CNV lesions (Figure 2 B) ³². Only minimal fluorescence was observed in CNV lesions when mice were treated with nonsense probe (NS-hAuNP). A 2.6-fold fluorescence enhancement was observed in AS-VCAM-1 hAuNP treated CNV lesions compared to the NS-hAuNP treated CNV lesions.

We also confirmed that the fluorescence signals were associated with intact AS-VCAM-1 hAuNP sequestered in CNV lesions, by using hyperspectral imaging microscopy with an adaptive detection (HSM-AD) technique (Figure 3 A–B). AS-VCAM-1 hAuNP (0.5 mg/kg) were injected into LCNV mice at three-days post-laser injury. The mice were euthanized at one-week post-injection of the probe. Punctate staining of CNV lesions clearly showed AS-VCAM-1 hAuNP accumulation. Though the injected AS-VCAM-1 hAuNP exhibited spectral shifting to approximately 634 nm from the native 520 nm surface plasmon resonance of AuNP, this may be due to clustered accumulation of AS-VCAM-1 hAuNP in the CNV lesion (Figure 3C).

We confirmed that VCAM-1 expression is associated with CNV lesions (Figure 4). Immunofluorescence detection using an antibody against VCAM-1 followed by secondary antibody conjugated to Alexafluor 488 specific to the VCAM-1 antigen clearly visualized the expression of VCAM-1 in mouse CNV lesions.

Toxicity of AS-VCAM-1 hAuNP:

In vivo toxicity was assessed by electroretinography (ERG) measurements in retinas from adult mice injected with AS-VCAM-1 hAuNP or vehicle control. AS-VCAM-1 hAuNP (0.5 mg/kg) was injected systemically, and ERG measurements were recorded in dark-adapted mice seven days post-administration. No statistically significant changes in mean *a*-wave and *b*-wave amplitudes at various flash intensities were observed as compared to vehicle-treated mice (Figure 5), suggesting that AS-VCAM-1 hAuNP probes are not toxic to retinal tissue.

DISCUSSION

Mouse LCNV has an inherent inflammatory component and fluctuations trending toward reduced pH have been associated with tissue inflammation^{33–34}. Accordingly, we performed tests to rule out not specific signal at reduced pH. We were concerned that Au-S bond dissociation and/or the melting of the duplex neck of the hairpin AS-DNA may occur at reduced pH, displacing the fluorophore from the AuNP surface and inducing optical activity independent of any hybridization event. We added AS-VCAM-1 hAuNP to media over a pH range of (7.4–1.6), in the absence of a complementary target oligonucleotide, while monitoring the fluorescent activity. We found that AS-VCAM-1 hAuNP were stable yielding no increased fluorescence over this pH range. After these incubations, we added a complementary oligonucleotide. Hybridization rates were maximum at pHs 7.4 to 4.5, representing a range most likely observed *in vivo*. Therefore, we conclude that non-specific signal due to any pH decreases in tissue inflammation is not likely to occur. Recent studies indicate that AS-hairpin DNAs conjugated to 15 nm AuNP are protected against DNase activity and that AuNP in this size range are optimal to facilitate hairpin DNA uptake³⁵. We have shown that citrate-capped 15 nm gold nanoparticles are susceptible to aggregation in isotonic medium⁹. However, 15 nm AuNP modified with hairpin-DNA similar to the AS-VCAM-1 hAuNP used in this study, were stable in serum-containing medium with high ionic strength⁹. Presumably these observations are related to a steric hindrance attributed to the DNA hairpins covering the AuNP surface that block cohesive forces of the AuNP cores. Reduced aggregation may confer enhanced pharmacokinetic properties to AS-hAuNP. Although we have not rigorously investigated the intracellular trafficking of AS-hAuNP, transmission electron microscopic (TEM) images we have obtained from retinal cells, suggest that AS-hAuNP are endocytosed, escape to the cytosol and become fluorescent due to the binding of their target mRNA.

We proceeded to administer AS-VCAM-1 hAuNP to LCNV mice via systemic injection through tail vein. This route was used to increase the bioavailability of the probe *in vivo*. Optical imaging of the excised choroidal tissues showed intense fluorescence activity, corresponding to a 2.6 fold enhancement over the control group, overlapping with counterstaining of neovessels with the vascular marker IB4. The fluorescent signal most likely depended on VCAM-1 mRNA hybridization because LCNV mice injected with NS-hAuNP demonstrated only minimal fluorescence (Figure 2). Hyperspectral imaging of the excised choroidal tissues confirmed the presence of hAuNP (Figure 3). We are currently working to improve the signal-to-background ratios by developing improved methods for delivery of hAuNP to pathologic ocular tissues. We did not do the requisite pharmacokinetic experiments to conclude that the AS-hAuNP are stable at the times we performed *ex-vivo* imaging. It is conceivable that the AS-DNA hairpins are released from the gold surface and form aggregates with quenched fluorescence, explaining the modest signal we observed. Furthermore, we do not discount the possibility that at least some of the fluorescence intensities from the AS- and NS-hAuNP may have an extracellular origin due to probe degradation. On the other hand, if these two processes were dominant, we would not expect to observe a difference between the AS- and NS-hAuNP. In addition, AuNP can be conjugated to antibodies and/or peptide fragments (pro-AuNP) and used to image cellular

proteins in their activated and/or unactivated states. This remains an intriguing line of investigation for future consideration.

CONCLUSIONS

The hAuNP-mediated imaging of VCAM-1 mRNA in mouse LCNV reported herein is the first description of the direct imaging of an endogenous mRNA in a tissue via systemic injection of an AS-hAuNP through tail vein. This technique has several advantages over traditional imaging methods, including a facile delivery method for oligonucleotides that does not require potentially toxic transfection reagents.

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ABBREVIATIONS

LCNV	laser-induced choroidal neovascularization
VCAM-1	vascular cell adhesion molecule 1
FRET	Förster resonance energy transfer
TNF-α	tumour necrosis factor- α

REFERENCES

1. Weil TT; Parton RM; Davis I, Making the message clear: visualizing mRNA localization. Trends Cell Biol 2010, 20 (7), 380–390. [PubMed: 20444605]
2. Larsson C; Grundberg I; Soderberg O; Nilsson M, In situ detection and genotyping of individual mRNA molecules. Nat Methods 2010, 7 (5), 395–7. [PubMed: 20383134]
3. Tyagi S; Kramer FR, Molecular beacons: Probes that fluoresce upon hybridization. Nat Biotechnol 1996, 14 (3), 303–308. [PubMed: 9630890]
4. Kohler O; Venkatrao D; Jarikote DV; Seitz O, Forced intercalation probes (FIT probes): Thiazole orange as a fluorescent base in peptide nucleic acids for homogeneous single-nucleotide-polymorphism detection. Chembiochem 2005, 6 (1), 69–77. [PubMed: 15584015]
5. Jao CY; Salic A, Exploring RNA transcription and turnover in vivo by using click chemistry. Proc Natl Acad Sci U S A 2008, 105 (41), 15779–84. [PubMed: 18840688]
6. Bhaumik S; Walls Z; Puttaraju M; Mitchell LG; Gambhir SS, Molecular imaging of gene expression in living subjects by spliceosome-mediated RNA trans-splicing. P Natl Acad Sci USA 2004, 101 (23), 8693–8698.
7. Stein CA; Hansen JB; Lai J; Wu SJ; Voskresenskiy A; Hog A; Worm J; Hedtjarn M; Souleimanian N; Miller P; Soifer HS; Castanotto D; Benimetskaya L; Orum H; Koch T, Efficient gene silencing by delivery of locked nucleic acid antisense oligonucleotides, unassisted by transfection reagents. Nucleic Acids Res 2010, 38 (1).

8. Lapierre-Landry M; Gordon AY; Penn JS; Skala MC, In vivo photothermal optical coherence tomography of endogenous and exogenous contrast agents in the eye. *Sci Rep* 2017, 7 (1), 9228. [PubMed: 28835698]
9. Uddin MDI; Jayagopal A; Wong A; McCollum GW; Wright DW; Penn JS, Real-time imaging of VCAM-1 mRNA in TNF-alpha activated retinal microvascular endothelial cells using antisense hairpin-DNA functionalized gold nanoparticles. *Nanomed-Nanotechnol* 2018, 14 (1), 63–71.
10. Hernandez C; Burgos R; Canton A; Garcia-Arumi J; Segura RM; Simo R, Vitreous levels of vascular cell adhesion molecule and vascular endothelial growth factor in patients with proliferative diabetic retinopathy: a case-control study. *Diabetes Care* 2001, 24 (3), 516–21. [PubMed: 11289478]
11. Seddon JM; George S; Rosner B; Rifai N, Progression of age-related macular degeneration: prospective assessment of C-reactive protein, interleukin 6, and other cardiovascular biomarkers. *Arch Ophthalmol* 2005, 123 (6), 774–82. [PubMed: 15955978]
12. Toker E; Kazokoglu H; Sahin S, Cell adhesion molecules in subretinal fluid: Soluble forms of VCAM-1 (vascular cell adhesion molecule-1) and L-selectin. *Int Ophthalmol* 1998, 22 (2), 71–76. [PubMed: 10472764]
13. Shen WY; Yu MJT; Barry CJ; Constable IJ; Rakoczy PE, Expression of cell adhesion molecules and vascular endothelial growth factor in experimental choroidal neovascularisation in the rat. *Brit J Ophthalmol* 1998, 82 (9), 1063–1071. [PubMed: 9893599]
14. Caicedo A; Espinosa-Heidmann DG; Pina Y; Hernandez EP; Cousins SW, Blood-derived macrophages infiltrate the retina and activate Muller glial cells under experimental choroidal neovascularization. *Exp Eye Res* 2005, 81 (1), 38–47. [PubMed: 15978253]
15. Sawa Y; Sugimoto Y; Ueki T; Ishikawa H; Sato A; Nagato T; Yoshida S, Effects of TNF-alpha on leukocyte adhesion molecule expressions in cultured human lymphatic endothelium. *J Histochem Cytochem* 2007, 55 (7), 721–33. [PubMed: 17371935]
16. Nahrendorf M; Keliher E; Panizzi P; Zhang H; Hembrador S; Figueiredo JL; Aikawa E; Kelly K; Libby P; Weissleder R, 18F-4V for PET-CT imaging of VCAM-1 expression in atherosclerosis. *JACC Cardiovasc Imaging* 2009, 2 (10), 1213–22. [PubMed: 19833312]
17. McHale JF; Harari OA; Marshall D; Haskard DO, Vascular endothelial cell expression of ICAM-1 and VCAM-1 at the onset of eliciting contact hypersensitivity in mice: Evidence for a dominant role of TNF-alpha. *J Immunol* 1999, 162 (3), 1648–1655. [PubMed: 9973425]
18. Wang YG; Zhou KJ; Huang G; Hensley C; Huang XN; Ma XP; Zhao T; Sumer BD; DeBerardinis RJ; Gao JM, A nanoparticle-based strategy for the imaging of a broad range of tumours by nonlinear amplification of microenvironment signals. *Nat Mater* 2014, 13 (2), 204–212. [PubMed: 24317187]
19. Tsourkas A; Shinde-Patil VR; Kelly KA; Patel P; Wolley A; Allport JR; Weissleder R, In vivo imaging of activated endothelium using an anti-VCAM-1 magnetooptical probe. *Bioconjugate Chem* 2005, 16 (3), 576–581.
20. Yang C; Uertz J; Yohan D; Chithrani BD, Peptide modified gold nanoparticles for improved cellular uptake, nuclear transport, and intracellular retention. *Nanoscale* 2014, 6 (20), 12026–12033. [PubMed: 25182693]
21. Buxbaum AR; Haimovich G; Singer RH, In the right place at the right time: visualizing and understanding mRNA localization. *Nat Rev Mol Cell Bio* 2015, 16 (2), 95–109. [PubMed: 25549890]
22. Harry SR; Hicks DJ; Amiri KI; Wright DW, Hairpin DNA coated gold nanoparticles as intracellular mRNA probes for the detection of tyrosinase gene expression in melanoma cells. *Chem Commun* 2010, 46 (30), 5557–5559.
23. Jayagopal A; Halfpenny KC; Perez JW; Wright DW, Hairpin DNA-Functionalized Gold Colloids for the Imaging of mRNA in Live Cells. *J Am Chem Soc* 2010, 132 (28), 9789–9796. [PubMed: 20586450]
24. Shi X; Semkova I; Muther PS; Dell S; Kociok N; Jousen AM, Inhibition of TNF-alpha reduces laser-induced choroidal neovascularization. *Exp Eye Res* 2006, 83 (6), 1325–34. [PubMed: 16959248]

25. Zhang P; Zhang X; Fan JL; Hao XF; Wang YS; Hui YN; Hu D; Zhou J, Rac1 activates HIF-1 in laser induced choroidal neovascularization. *International journal of ophthalmology* 2011, 4 (1), 14–8. [PubMed: 22553600]
26. Lu H; Lu Q; Gaddipati S; Kasetti RB; Wang W; Pasparakis M; Kaplan HJ; Li Q, IKK2 inhibition attenuates laser-induced choroidal neovascularization. *PloS one* 2014, 9 (1), e87530.
27. Yang XM; Wang YS; Zhang J; Li Y; Xu JF; Zhu J; Zhao W; Chu DK; Wiedemann P, Role of PI3K/Akt and MEK/ERK in mediating hypoxia-induced expression of HIF-1 α and VEGF in laser-induced rat choroidal neovascularization. *Invest Ophthalmol Vis Sci* 2009, 50 (4), 1873–9. [PubMed: 19098317]
28. Lambert V; Lecomte J; Hansen S; Blacher S; Gonzalez ML; Struman I; Sounni NE; Rozet E; de Tullio P; Foidart JM; Rakic JM; Noel A, Laser-induced choroidal neovascularization model to study age-related macular degeneration in mice. *Nature protocols* 2013, 8 (11), 2197–211. [PubMed: 24136346]
29. Evans SM; Kim K; Moore CE; Uddin MI; Capozzi ME; Craft JR; Sulikowski GA; Jayagopal A, Molecular probes for imaging of hypoxia in the retina. *Bioconjug Chem* 2014, 25 (11), 2030–7. [PubMed: 25250692]
30. Domenici L; Berardi N; Carmignoto G; Vantini G; Maffei L, Nerve growth factor prevents the amblyopic effects of monocular deprivation. *Proc Natl Acad Sci U S A* 1991, 88 (19), 8811–5. [PubMed: 1924342]
31. Rex TS; Allocca M; Domenici L; Surace EM; Maguire AM; Lyubarsky A; Cellerino A; Bennett J; Auricchio A, Systemic but not intraocular Epo gene transfer protects the retina from light-and genetic-induced degeneration. *Molecular therapy : the journal of the American Society of Gene Therapy* 2004, 10 (5), 855–61. [PubMed: 15509503]
32. Bretz CA; Divoky V; Prchal J; Kunz E; Simmons AB; Wang H; Hartnett ME, Erythropoietin Signaling Increases Choroidal Macrophages and Cytokine Expression, and Exacerbates Choroidal Neovascularization. *Sci Rep* 2018, 8 (1), 2161. [PubMed: 29391474]
33. Rezaei KA; Toma HS; Cai J; Penn JS; Sternberg P; Kim SJ, Reduced choroidal neovascular membrane formation in cyclooxygenase-2 null mice. *Invest Ophthalmol Vis Sci* 2011, 52 (2), 701–7. [PubMed: 20881304]
34. Steen KH; Steen AE; Reeh PW, A dominant role of acid pH in inflammatory excitation and sensitization of nociceptors in rat skin, in vitro. *J Neurosci* 1995, 15 (5 Pt 2), 3982–9. [PubMed: 7751959]
35. Wong AC; Wright DW, Size-Dependent Cellular Uptake of DNA Functionalized Gold Nanoparticles. *Small* 2016, 12 (40), 5592–5600. [PubMed: 27562251]

Figure 1:

Structural design, hybridization mechanism and pH sensitivity of AS-VCAM-1 hAuNP for targeted imaging of VCAM-1 mRNA. (A) Gold nanoparticles were functionalized with DNA hairpin-loops incorporating an anti-sense sequence specific for mouse VCAM-1 mRNA (AS-VCAM-1 hAuNP) or a scrambled version of this sequence (NS-hAuNP). (B) AS-VCAM-1 hAuNP were stable in a wide range of acidic and neutral media, such as PBS and phosphate buffer containing citric acid. Hybridization was only observed at pH ranges from 4.5 to 7.4 when a complementary single strand DNA oligonucleotide (VCAM-1 oligo) was present indicating the specificity and stability of the AS-VCAM-1 hAuNP. The hybridization was slower in water and under strongly acidic conditions (pH 1.6).

Figure 2:

Ex vivo fluorescence imaging of VCAM-1 mRNA using AS-VCAM-1 hAuNP in choroidal flat-mounts from LCNV animals. Nonspecific NS-hAuNP were used as a control. C57BL/6 mice were laser-injured and AS-VCAM-1 hAuNP or NS-hAuNP were systemically injected into LCNV mice at day-3 post-laser injury. 24 hours post-injection of the probe, the mice were euthanized and choroidal tissues were dissected and flat-mounted for *ex vivo* imaging using confocal microscopy. (A, C) LCNV lesions were counter-stained with isolectin B4 (IB4). (B) Strong fluorescence emission correlating with activated AS-VCAM-1 hAuNP indicated the localization of VCAM-1 mRNA within the LCNV lesion. (D) Only minimal fluorescence was detected in the LCNV lesions from NS-hAuNP-treated animals. (E) Fluorescence intensity measurements expressed in relative fluorescence units (RFU) per lesion in two different treatment groups were measured using ImageJ software. A nearly 2.6-fold fluorescence enhancement was observed in AS-VCAM-1 hAuNP-treated LCNV lesions compared to those in the NS-hAuNP control group.

Figure 3:

Outline of the AS-VCAM-1 hAuNP localization in LCNV lesion using hyperspectral microscopy with adaptive detection (HSM-AD). (A) AS-VCAM-1 hAuNP were systemically injected into LCNV mice at day-3 post-laser injury. 24 hours post-injection of the probe, the mice were euthanized and choroidal tissues were dissected and flat-mounted for characterization using HSM-AD. (B) Clusters of AS-VCAM-1 hAuNP in LCNV lesions were clearly detected using HSM-AD. (C) The spectral profile was visualized as bright punctate staining of LCNV lesions depicting AS-VCAM-1 hAuNP accumulation in (B). The injected AS-VCAM-1 hAuNP exhibiting 520 nm plasmon resonance showed spectral shifting of plasmon resonance to around 634 nm, perhaps due to clustering of AS-VCAM-1 hAuNP in the LCNV lesion.

Figure 4:

Assessment of VCAM-1, an inflammatory biomarker expressed in LCNV lesions of adult C57BL/6 mice. (A) Fluorescence dye conjugated isolectin-B4 (IB4) was used as counter stain to visualize choroidal neovessels in LCNV lesions. (B) VCAM-1 expression was observed in LCNV lesions using an antibody specific for VCAM-1. Adult mice were sacrificed at day-3 post-laser injury and choroidal tissues were dissected and flatmounted for fluorescence immunohistochemical (IHC) staining using an antibody specific for VCAM-1. (D) A non-laser control choroidal tissues showed no positive staining for VCAM-1, suggesting that VCAM-1 is associated with injured LCNV lesions.

Figure 5: Assessment of *in vivo* retinal toxicity using response to electroretinography (ERG) analysis. ERGs were recorded one-week post-systemic injection of AS-VCAM-1 hAuNP in adult C57BL/6 mice through tail vein. No significant differences were observed in a- and b-waves of healthy control and AS-VCAM-1 hAuNP treated animals, suggesting that AS-VCAM-1 hAuNP were not acutely toxic to the retinal tissues.