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Application of INAA to the build-up and clearance of gold nanoshells in clinical studies in mice

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Clinical studies have been carried out for detailed measurements of the build-up and clearance of engineered gold nanoshell in the tissues of dosed mice. These optically tunable nanoshells are under consideration for a new therapy for tumors. The proposed therapy would involve the injection of the nanoshells and their preferential accumulation in tumor sites. This will be followed by irradiation with a monochromatic near infrared laser, which will induce cellular hyperthermia, thereby eradicating the tumor. Neutron activation analysis has been used for the detection and quantitation of gold, and therefore, the nanoshells, in dosing materials, blood, bones and other tissues as well as tumors at varioius sacrifice times following dosing. Feasibility studies have shown instrumental neutron activation analysis to be uniquely suited for detection of the gold nanoshells over a wide dynamic range. This allows for the study of high concentrations of gold in tissues which scavenge the shells from the blood (liver, spleen, kidney) as well as for much lower concentrations in those which do not (muscle, brain). In particular, the tissues from animals sacrificed after the longest post dose delay (28 days) and the control animals required experimental optimization to ensure the lowest possible determination limits. The mass of gold in the tissue samples ranged from our determination limit (about 70 pg) to a few micrograms.

Introduction

Optically tunable nanoshells, constructed from an insulating core and outer metal shell, are a new class of nanoparticles which is under consideration for possible new uses in medicine because of the particles' compatability with biological systems and unique ability to be tuned for near-infrared absorption.[1–3] The development of such diagnostic and therapeutic methods using metal laden nanoparticles will require corresponding analytical techniques capable of measuring the presence of the particles in the target tissues at concentrations likely to be encountered in clinical studies. In the present study, neutron activation analysis (NAA) methodology for the detection, buildup and clearance of gold nanoshells in mice has been evaluated as a component of the development of a novel method of cancer tumor treatment. The proposed treatment includes the introduction of some 20 billion gold nanoshells into the blood stream. Preferential migration of these shells to tumors with underdeveloped vascular systems is influenced by the particular size of the particles, resulting in a buildup of nanoshells in and around the unwanted lesions.[4] The frequency of light absorbed by the nanoshells is dependent on the relative dimensions of the insulating substrate and the metal shell. By designing and constructing the particles to appropriate dimensions, one can tune light absorption to a frequency at which tissue is least likely to absorb.[5] It is thus possible to construct gold nanaoshells which preferentially absorp in the near infrared region of the spectrum (700–1100 nm), a region where optical transmission through tissue is optimal.[6] This introduces the possibility of non-invasive excitation of nanoshells within tissue.

Gold nanoshells

The nanoshells used in this work are silica-gold core-shell nanoparticles, produced using techniques first employed by OLDENBERG et al.,[3] which are nominally 110 nm cores with 10 nm thick shells. The shells were stabilized by coating with polyethylene glycol (PEG). The PEGylation provides the steric repulsion to reduce aggregation of nanoparticles in blood as well as a deterrence to protein absorption. These nanoshells were designed for peak absorption resonances between 800 and 830 nm.

Clinical studies of therapeutic tumor ablation

Clinical animal trials are underway in which nanoshell-treated tissue have been confirmed to be destroyed photo-thermally while untreated specimen are unaffected by the laser alone. Early results indicate that 55% of the mice receiving nanoshell therapy/laser treatment survived to the end of the study (35 days) with complete tumor regression and no regrowth after 90 days. Conversely, no mice in the control groups survived beyond 35 days.

Biodistribution and clearance of gold nanoshells

It is imperative when considering the injection of gold nanoshells for therapeutic purposes to understand where, in addition to tumor tissue, the nanoshells might

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accumulate, in what quantities and over what timecourse. For quantification in tissue, analytical methodology of sufficient sensitivity must be employed to measure concentrations in the predicted ppb (ng/g) range. In this study, neutron activation analysis was chosen for its sensitivity and its robust nature, which allowed determination of gold (and thus nanoshells) in the complex tissue matrices with minimal matrix effects and freedom from sample preparation steps such as digestion.

Experimental

Long-term biodistribution studies in healthy mice

Healthy female albino mice of >6 weeks age and about 15 g body mass were anesthetized via isoflurane, then injected with 100 µl of PEGylated nanoshells suspended in 0.9% NaCl via tail vein. The estimated concentration of nanoshells in the injected solution was about $2.4 \cdot 10^{11}$/ml. The nanoshells were allowed to circulate for variable times ranging from 4 hours to 28 days, at which point the mice were exsanguinated and euthanized. Blood, liver, lung, spleen, muscle, kidney, bone and brain tissues were excised from each of five replicate mice at each of six sacrifice times. The samples were then lyophilized and weighed into precleaned and tared polyethylene irradiation vials for NAA gold measurements. For each batch of samples prepared for NAA, a separate sample vial containing 100 µl of the nanoshell dosing solution was prepared to quantify total amount of gold injected into the mice.

Short-term accumulation studies in tumored mice

In order to determine optimal times for maximum accumulation of nanoshells within tumors a separate study was performed. In this case, the mice had subcutaneous tumors of about 0.5 cm diameter. Three such mice were treated and sacrificed as described above at each of four circulation times (1, 4, 24 and 48 hours) and blood, tumor, spleen, muscle and lung tissues were prepared for NAA as described above.

Neutron activation analysis

Primary standard solutions containing gold were prepared and dispersed onto cellulose in polyethylene containers identical to those used for sample preparation. The mass of cellulose used in each case was selected to mimic the sample geometry of the various tissue types collected from the mice under study. One hundred nanograms of gold was dispersed into each standard vial in sufficient volume to wet the entire mass of cellulose and evaporated under a heat lamp. Orchard Leaves, NIST SRM 1571, utilized as a quality control (QC) material for this study, was prepared by weighing some 100 mg into the irradiation containers. Three standards, one quality control material, and one blank were irradiated together with sets of eight unknown samples in the Texas A&M University's Nuclear Science Center 1 MW Triga research reactor for 4 to 14 hours depending on the level of gold expected in the particular set of samples studied. The samples selected for inclusion into sets were of similar physical size to minimize solid angle uncertainties in irradiation and counting positions. Up to six such irradiation sets (standards, samples, QC and blank) were encapsulated in separate "cans" then irradiated together in a rotating position adjacent to the reactor core. The irradiation position used in this study has an average neutron flux of about $1 \cdot 10^{13}$ n·cm^{-2}·s^{-1}. Gamma-ray spectroscopy was carried out on all irradiated materials after a delay of 4 to 8 days to allow for decay of matrix activity, primarily ^{24}Na. High purity germanium detectors with nominal resolutions (FWHM) of 1.74 keV or better and efficiencies of 25 to 47% by industry standard relative measurement, were used to quantify the 412 keV gamma-line from ^{198}Au. The Canberra Industries' OpenVMS alpha processor-based Genie-ESP software was used for acquisition and computation of gold concentrations.

Results and discussion

Calculations revealed the determination limit for gold in mice muscle tissue to be about 70 pg. The average concentration of dose solutions was determined to be 154±19 µg per 100 µl portion which contained an estimated $2.4 \cdot 10^{10}$ nanoshells, or $6.45 \cdot 10^{-9}$ µg/nanoshell. This is in reasonable agreement with theoretical calculations which place the nanoshell gold content at $8.76 \cdot 10^{-9}$ µg/nanoshell for a 110 nm diameter core with 10 nm thick shell ($\rho_{gold} = 19.32$ g/cm^3). Using the experimental value, the determination limit of about 70 pg represents some 10,800 nanoshells, about one half of one millionth of the injected quantities.

Since whole organs were usually used in the analyses to avoid concerns of non-representative sampling of potentially inhomogeneous tissues, analytical portion sizes varied by nearly two orders of magnitude. Tumor masses were typically a few milligrams while livers were about 250 mg. This caused a disparity in the physical sample–reactor and sample–detector geometries (solid angles) between various samples. Therefore, we segregated and analyzed samples according to mass and prepared standards with comparable physical dimensions. In addition, the gold concentration levels in the study varied greatly, even in tissues from the same animals. Spleen, liver and kidney samples were generally much more active following irradiation, while blood, brain, bone, and tumor samples were often quite

low. This was especially true for animals with the longer post-dose sacrifice times. In addition, representative dose solutions used to normalize our animal studies were included in each analytical run and these tended to generate significantly higher count rates. As a result, standard gold concentrations were adjusted for sufficient induced activity to allow multiple countings. Low activity samples were generally counted about a week following irradiation, while high activity samples were delayed, sometimes as much as an additional week.

Biodistribution in healthy mice

To confirm the presence of low gold backgrounds within healthy mice, NAA was first conducted on samples from mice receiving no nanoshell injections. Gold levels in all tissue types were consistently lower than any levels expected post-treatment with nanoshells. Table 1 presents data obtained from these control animals as well as that showing the build-up and clearance of nanoshell gold concentrations from 4 hours to 28 days post-injection in seven tissue types. One can note that the nanoshells are quickly scavenged from the blood with most accumulating in the organs associated with macrophages from the reticuloendothelial system (RES). The elevated levels of nanoshells within the lungs and brain are thought to be associated with residual blood left behind even after exsanguination of the mice in these highly perfused tissues. It is evident that after 28 days, elevated levels of gold are still present within the liver and spleen as shown in Fig. 1. Likewise between 1–10 ppm levels are found in the bone, muscle, kidney and lung, levels which are still three orders of magnitude higher than pretreatment levels found within these tissues. Ideally, the tissues above would display clearance profiles similar to those of blood and brain by reducing to levels similar to those found pretreatment. Eventual clearance must be further investigated by extending the studies to a longer timecourse. However, no physiological complications have been seen in study animals due to the presence of these elevated levels.

Accumulation in tumored mice

The biodistribution study indicated that the nanoshells are cleared from the blood stream within hours allowing for little additional tumor accumulation after one day. Hence, an accumulation investigation period of 1–48 hours was chosen to capture the important phase of nanoshell accumulation within tumors. Figure 2, a plot of gold concentration in blood as a function of time, reveals that nanoshells are clearing from the blood in a predicted exponential fashion. The corresponding levels of nanoshells in muscle, lung and tumor are shown in Fig. 3. Also, as seen in the biodistribution study, a proportional increase in spleen and liver accumulation is associated with decrease in blood levels. Tumor levels reveal continual nanoshell accumulation until 24 hours and the beginning of clearance thereafter. Hence, this study suggests that optimal laser treatment should occur at 24 hours post-injection.

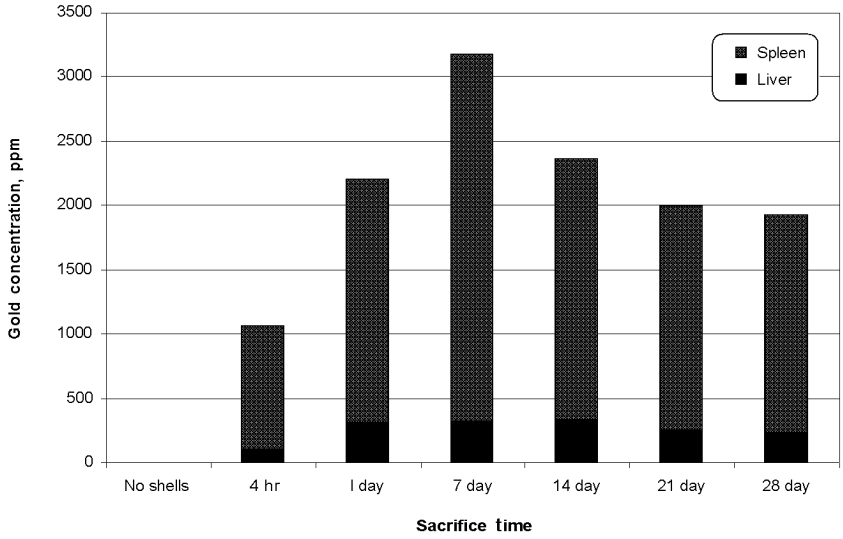

Fig. 1. Gold concentration of liver and spleen as a function of sacrifice time showing clearance between day 7 and day 28. The data represent means of 5 replicate gold determinations in dry tissue at each sacrifice time

Table 1. Accumulation of gold nanoshells in healthy mice

Sacrifice time	Gold concentration in dry tissue, Au μg/g							
	Blood	Liver	Kidney	Spleen	Lung	Muscle	Brain	Bone
Control	0.0009	0.0007	0.0011	0.0174	0.0021	0.0230	0.0011	0.0049
4 hr	313.7	103.8	52.22	952.2	88.58	3.796	7.187	9.531
1 day	29.17	311.8	27.61	1890	12.71	1.060	0.547	5.912
7 day	0.0187	313.4	21.49	2863	6.066	1.916	0.0684	7.319
14 day	0.0290	324.5	19.30	2039	3.738	0.779	0.0310	5.365
21 day	0.0430	252.0	23.53	1738	4.748	1.593	0.1243	8.333
28 day	0.0567	227.2	24.70	1703	3.781	1.023	0.0293	6.875

Data represent means of 5 replicate mice at each sacrifice time.

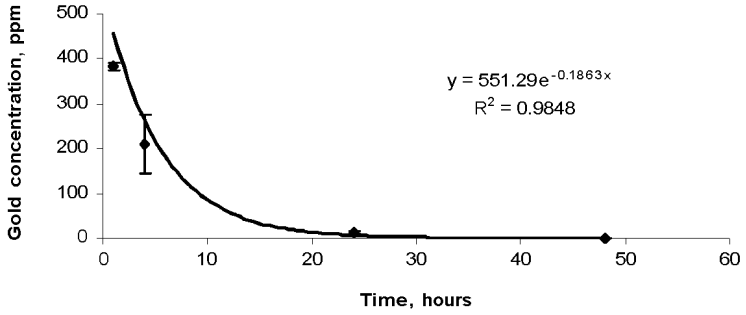

Fig. 2. Exponential decrease of gold concentration in blood reveals a circulation half-life of 3.7 hours. The data represent means of 5 replicate gold determinations at each sacrifice time

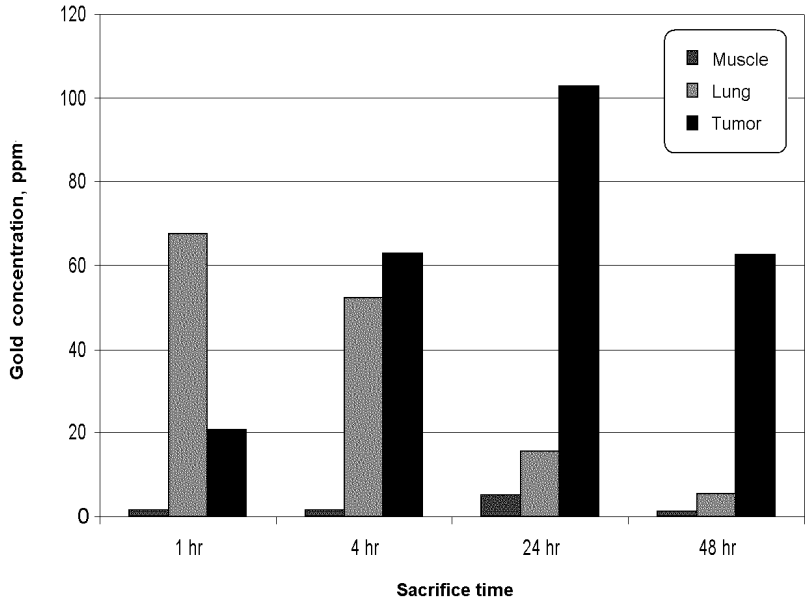

Fig. 3. Concentration of gold in muscle, lung and tumor as a function of sacrifice time. The data represent means of 5 replicate gold determinations in dry tissue at each sacrifice time

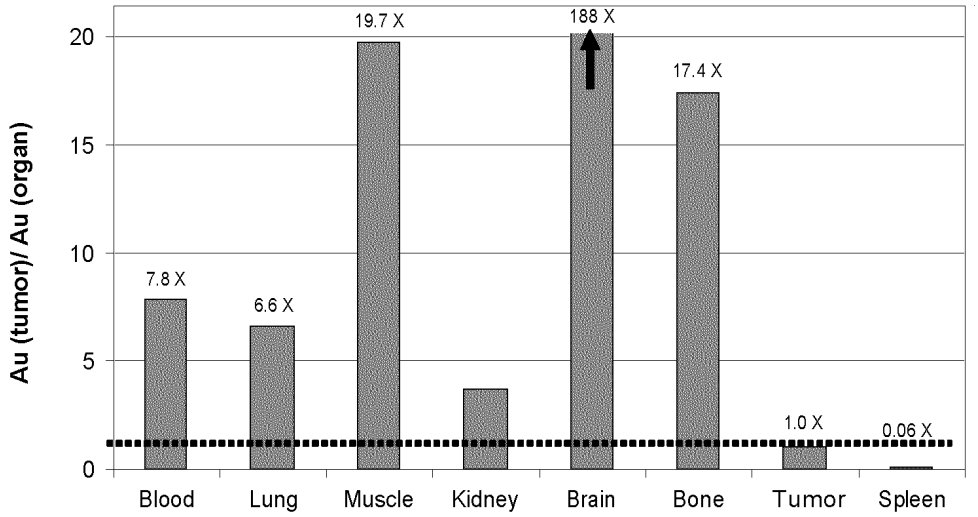

Fig. 4. Ratios of gold concentrations in each organ to that in tumor at 24 hours post-dose

Conclusions

Neutron activation analysis has been shown to have sufficient sensitivity and accuracy to provide the required analytical measurement of gold content in animal tissues to study the distribution, build-up and clearance of gold nanoshell particles from therapeutic injections. Determination limits for tissue samples were about 70 pg which corresponds to about 10,000 nanoshells.

Nanoshell concentrations have been shown to be quickly cleared from blood circulation and scavenged by the expected organs, primarily spleen and liver. Clearance from RES associated organs was slower than expected with little reduction in concentrations during the 28 day timecourse of the study. Only brain and blood gold concentrations approached control levels within the study period.

Maximum tumor accumulation was observed at 24 hours post-dose. Tumor accumulation at that time-point as measured by gold content exceeded that of blood, lung, muscle, kidney, brain and bone by factors of 7.8, 6.6, 19.7, 3.7, 188 and 17.4, respectively (Fig. 4). Total nanoshell tumor accumulation represented some 1% of the total dose administered. Methods for increasing circulation time in the bloodstream, including optimizing PEGylation chemistry are under consideration for future studies. An order of magnitude increase in total accumulation is thought possible.

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