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Nanoparticles functionalized with collagenase exhibit improved tumor accumulation in a murine xenograft model

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Abstract

Nanoparticles have garnered widespread interest for both the imaging and treatment of cancer due to their unique and tunable pharmacokinetics and their ability to carry a high payload of diverse compounds. However, despite these favorable attributes, the extent of tumor accumulation can be severely restricted due to the dense stroma surrounding the often-permeable blood vessel wall and high intratumoral pressure. In this study, we investigated whether modifying the surface of pegylated gold nanoparticles (AuNPs) with collagenase could improve the accumulation of nanoparticles within a murine tumor xenograft. It was determined that collagenase remains active after surface conjugation and the presence of collagenase has no measureable effect on cell proliferation in vitro. Following intravenous injection, the largest fractions of collagenase-labeled AuNPs were found in the liver and spleen. Histological analysis revealed no signs of toxicity in either of these organs. Blood chemistry revealed normal levels of liver enzymes, but a slightly elevated level of total bilirubin. Within the tumor, AuNPs labeled with collagenase exhibited a 35% increase in accumulation compared with unlabeled AuNPs. Therefore, these studies provide preliminary evidence that the functionalization of nanoparticles with collagenase represent an effective and safe approach to improve tumor accumulation.

Keywords

nanoparticles; collagenase; tumor; penetration; stroma

1. Introduction

The range of applications in which nanoparticles are used in the biomedical sciences continues to expand exponentially. One of the most prolific areas of nanoparticle research involves their use in the imaging and treatment of cancer. This is largely because

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nanoparticles offer many potential advantages over small molecule drugs/contrast agents, such as the ability to be retained largely within the vasculature preventing off-target toxicity, preferential delivery to tumors owing to the enhanced permeability and retention effect, the ability to carry high payloads of both hydrophilic and hydrophobic cargo, and the ability to protect cargo from degradation in serum.[1, 2] These favorable attributes have led to the approval of more than 20 nanoparticle therapeutics by the Federal Drug Administration (FDA) for clinical use.[3] However, despite the clear benefits of nanoparticles, their use as a drug delivery vehicle does not always lead to an improvement in clinical efficacy over free drug. For example, while the liposomal formulations of the anti-cancer drug doxorubicin, Doxil®, carries a reduced risk of cardiotoxicity, it does not afford a significant improvement in survival compared with doxorubicin when used as first-line therapy in breast cancer patients.[4]

It is widely speculated that the variable efficacy of nanoparticles that is observed in the clinic stems from their inability to penetrate deeply into the stroma of many tumors.[5] While leakiness of the tumor vasculature and poor lymphatic drainage can lead to some nanoparticle accumulation, this enhanced permeability and retention (EPR) effect is highly heterogeneous both within and between tumors. Moreover, some types of cancer, e.g. gastric and pancreatic, exhibit little to no EPR effect.[6] Even in cases where the vascular wall is highly permeable, the extracellular matrix (ECM) is composed of a dense collagen network embedded in a gel of glycosaminoglycans (GAGs), primarily hyaluronan, that can significantly impede the penetration of nanoparticles into tumors.[5] This ECM creates both a physical barrier and a hydrodynamic barrier in the form of intratumoral pressure that prevents most therapeutics and nanoparticles from reaching tumor foci.

A number of studies have explored the use of hyaluronidase and collagenase to degrade the extracellular matrix within the tumor microenvironment, as a means to improve the delivery of both small and large therapeutics.[7, 8] While the intravenous administration of both enzymes has been shown to improve drug delivery, multiple reports now suggest that degradation of the collagen networks has a greater effect on the delivery of macromolecules.[9, 10] Consequently, several groups have focused on whether collagenase can be used to improve the delivery of nanoparticles to tumors. In particular, it has been shown that functionalization of nanoparticles with collagenase can increase nanoparticle accumulation within tumor spheroids 4-fold, compared with control nanoparticles.[11] Moreover, it has been shown that the intravenous injection of tumor bearing mice with liposomes, 1-hr after the intravenous administration of collagenase, leads to a ~1.5-fold increase in tumor uptake.[12] Therefore, both of these studies highlight the potential use of collagenase as a means to improve nanoparticle delivery. In this study, we investigate whether nanoparticles covalently linked to collagenase exhibit an increase in tumor accumulation. In addition, we examine the biodistribution of the collagenase-labeled nanoparticles, perform histology on the organs exhibiting the highest nanoparticle uptake, and measure liver enzyme levels in the blood to assess the potential toxic side effects of this approach.

2. Results and Discussion

Hydrophilic gold Nanoparticles (AuNPs) were prepared using the Turkevich method.[13] Transmission electron microscopy indicated that the AuNPs were spherical in shape and highly uniform in size with a core diameter of 13.4 ± 1.3 nm (Figure 1A). These nanoparticles were used to prepare both control AuNPs and collagenase-labeled AuNPs. The control AuNPs were prepared by coating the surface with methoxy-polyethylene glycol (PEG, MW~3700). The AuNPs that were subsequently labeled with collagenase had their surface coated with a mixture of amine-terminated-PEG and methoxy-PEG at a molar ratio of 1:2. The surface-bound amine-terminated-PEGs were subsequently reacted with dibenzocyclooctyne-N-hydroxysuccinimidyl ester (DBCO-NHS) (Figure 1B). DBCO is a constrained alkyne capable of copper-free click reactions with azides.[14]

The free lysines on Collagenase Clostridium Histolyticum were reacted with azido-PEG^{12}-NHS (azide-NHS), enabling the enzyme to be attached to the DBCO-modified AuNP surface via click chemistry. The molar ratio of azide-NHS-to-collagenase was varied from 5:1 to 50:1 to identify a reaction condition that resulted in efficient attachment to DBCO-functionalized AuNPs without compromising enzymatic activity. Interestingly, the level of collagenase activity, prior to AuNP conjugation, was unchanged compared to unlabeled collagenase (i.e. 0:1 azide-NHS:collagenase) even at the highest labeling ratio tested (Figure 1C). Moreover, the same level of collagenase activity was observed following reaction with DBCO-functionalized AuNPs, for all labeling ratios tested (Figure 1D). AuNPs that were functionalized with collagenase had mean hydrodynamic diameters between ~25 to 27 nm. In comparison, the control AuNPs had a mean hydrodynamic diameter of ~23 nm. For all subsequent experiments, collagenase was reacted with a 20-fold excess of azide-NHS prior to labeling AuNPs.

To assess the cytotoxicity of the collagenase-coated AuNPs, an MTT cell proliferation assay (MTT=3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazoilum bromide) was performed (Figure 2). Specifically, increasing concentrations of collagenase-labeled AuNPs were incubated with human alveolar epithelial adenocarcinoma cells (A549), which has been shown to exhibit extensive connective tissue growth in tumor xenograft models.[15] Cell proliferation was compared to a positive control cell sample that was not incubated with any AuNPs and a negative control cell sample that was incubated with 20% DMSO. It was determined that the collagenase-labeled AuNPs had little to no effect on the viability of A549 cells up to a gold concentration of 500 μM. This is consistent with previous reports that indicate that collagenase is not toxic to cells.[16] In fact, collagenase is commonly used for cell isolation and is widely believed to have no obvious detrimental effects on normal cell function. Moreover, broad specificity proteases such as trypsin are also routinely used for cell dissociation with no obvious toxicity.

The biodistribution of both control AuNPs and collagenase-labeled AuNPs was evaluated 24 h after intravenous administration in healthy, non-tumor bearing mice. Gold content within the heart, kidneys, lungs, spleen, and liver was assessed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Figure 3). Interestingly, the animals injected with the control AuNPs had equal or higher levels of gold content in all of the organs evaluated,

compared with animals injected with collagenase-labeled AuNPs, although a statistically significant difference ($p<0.05$) was only observed in the spleen. As is consistently found with other nanoformulations of similar size, the largest fractions of gold were observed in the liver and spleen for both the control and collagenase-labeled AuNPs. Little to no gold was detected in the other organs. This is largely due to the hydrodynamic diameter of the AuNPs, which generally restricts nanoparticles of this size to the blood pool. As such, it was expected that the collagenase-labeled AuNPs would have little to no access to most collagen-rich tissues (e.g. cartilage). Our greatest concern was that there would be uncontrolled proteolytic activity in the liver or spleen due to the large fenestrae in these organs, which could provide AuNPs with access to extracellular matrix. However, neither group of mice exhibited any indication of toxicity, with no signs of illness or change in activity. Histological analysis (hematoxylin and eosin, H&E, staining) of the liver and spleen also showed no evidence of abnormal pathology or adverse effects (Figure 4). To further assess toxicity, blood chemistry was evaluated (Figure 5). Specifically, the levels of alkaline phosphatase, alanine transaminase, aspartate aminotransferase, and total bilirubin were measured. While the levels of liver enzymes were all within the normal range for both the control AuNPs and collagenase-labeled AuNPs, total bilirubin was slightly elevated in mice that received the collagenase-labeled AuNPs. This was somewhat surprising considering all other measures of liver function and morphology were normal. A potential alternative cause for an increase in total bilirubin is hemolysis, but previous studies do not seem to indicate any relationship between collagenase activity and hemolysis.[17] Notably, it has been reported that blood chemistry can be highly variable in animal models and is dependent on the method and rate of blood collection, time of day in which blood was collected, and the level of animal activity.[18] Therefore, this possibility cannot be ruled out. Despite the elevated level of total bilirubin, collagenase is still expected to be safe, especially considering that collagenase is already being evaluated in a variety of clinical applications, including for treatment of Dupuytren contracture, herniated discs and keloids, and ulcers.[19, 20] It remains unclear whether the collagenase-labeled AuNPs will elicit an immunogenic response, since all studies described here were performed in immunodeficient nude mice. Moreover, most of the prior clinical studies that utilized collagenase involved a local administration of enzyme, which will differ in the activation of an immune response compared with intravenously administered material.

To determine whether collagenase-labeled AuNPs exhibit improved tumor accumulation compared with control AuNPs, A549 cells were implanted subcutaneously into the rear left flank of ~6-week old female nu/nu mice. Tumors were grown until the mean diameter was approximately 4–6 mm. Mice were then injected intravenously with either the collagenase-labeled AuNPs or control AuNPs. Twenty-four hours post-injection the tumors were harvested and analyzed for gold content by ICP-OES. It was found that the collagenase-labeled AuNPs exhibited a 35% increase in tumor accumulation compared to control AuNPs (Figure 6). This is similar to what was observed in tumor-bearing mice that had collagenase administered intravenously 1 hr prior to injection of liposomes.[12] However, several potential advantages for the covalent attachment of collagenase to nanoparticles could include the need for only a single therapeutic injection, the ability to inject much lower concentrations of collagenase, and assurance that collagenase exhibits a similar

biodistribution as the nanoparticles and thus has a definitive impact at the site of nanoparticle accumulation.

3. Conclusions

In conclusion, we have determined that click chemistry can be used to efficiently attach collagenase to AuNPs and that collagenase remains active after surface conjugation. Moreover, collagenase does not exhibit any signs of cytotoxicity in cell culture and histological analysis did not reveal any signs of toxicity in either the liver or spleen - the two organs that exhibited the highest level of AuNP uptake. Blood chemistry also revealed normal levels of liver enzymes, although a slightly elevated level of total bilirubin was observed. Nonetheless, the administration of collagenase-labeled AuNPs is perceived to be safe. Within the tumor, AuNPs labeled with collagenase exhibited a 35% increase in accumulation compared with unlabeled AuNPs. Therefore, these studies provide preliminary evidence that the functionalization of nanoparticles with collagenase represent an effective and safe approach to improve tumor accumulation.

4. Experimental Section

Gold Nanoparticle (AuNP) Synthesis

AuNPs were synthesized using the Turkevich method.[13] Briefly, aqueous sodium citrate (15 mL, 55 mM) was added to a boiling solution of 60 mg of HAuCl4 (Sigma-Aldrich) dissolved in Millipore water (200 mL). The AuNP solution was filtered using a 0.2 μm pore size nylon filter system (Millipore). Control AuNPs were added to methoxy-terminated PEG thiol (MW~3700, Laysan Bio Inc.) at a 1:4 mass ratio (PEG:HAuCl4), while AuNPs that were subsequently labeled with collagenase were added to amine-terminated polyethylene glycol (MW~5000, Laysan Bio, Inc.) and methoxy-terminated polyethylene glycol (MW~3700, Laysan Bio, Inc.) at a 1:4 mass ratio (PEG:HAuCl4) and stirred overnight. The ratio of amine-to-methoxy-terminated PEG was 1:2. The AuNP solution was purified from excess reactants by repeated filtration and resuspension in phosphate buffered saline (PBS) using 50 K MWCO Amicon centrifugal filter devices (Millipore).

DBCO Modification of AuNPs

Dibenzocyclooctyne-N-hydroxysuccinimidyl ester (DBCO-NHS) was suspended in DMSO and reacted with AuNPs (5 mg/mL) at a final concentration of 27 μM. The reaction was allowed to proceed overnight at 4°C. The DBCO-modified AuNPs were sedimented by centrifugation and washed 3 times with 0.1 m sodium phosphate buffer, pH 7.4, and then passed through a 0.22 micron syringe filter.

Azide Modification of Collagenase

Surface amines on Clostridium histolyticum collagenase (Sigma-Aldrich, St. Louis, MO) were reacted with azido-dPEG$_{12}$-NHS (Quanta BioDesign) in 0.1 m sodium phosphate buffer, pH 9 for 4 h at room temperature. The linker was added at various ratios of azide-to-collagenase (5, 10, 20, 25, and 50). Modified collagenase (azide-collagenase) was purified

with 0.1 m sodium phosphate buffer, pH 7.4 by PD-10 desalting columns (GE Healthcare, Piscataway, NJ).

Collagenase-AuNP Conjugation

Purified azide-collagenase was added at 10 times molar excess to AuNPs (0.085 mg collagenase per mg Au), and were allowed react overnight at 4°C. Collagenase-labeled AuNPs were sedimented by centrifugation and washed 3 times with 0.1 m sodium phosphate buffer, pH 7.4, and then passed through a 0.22 micron syringe filter.

Characterization of AuNPs

AuNP samples were diluted in Millipore water and deposited on 200 mesh carbon coated copper grids (Polysciences, Warrington, PA) for TEM imaging using a JEOL 1010 transmission electron microscope operating at 80 kV. The hydrodynamic diameters were measured by dynamic light scattering using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK). Concentration was determined with a Genesis ICP-OES (Spectro Analytical Instuments GMBH; Kleve, Germany) in the Department of Earth and Environmental Sciences, University of Pennsylvania, PA, USA and with a Cary Bio 100 UV visible spectrophotometer (Varian, Agilent).

Measurement of Collagenase Activity

Relative azide-collagenase enzyme activity was determined using a collagenase I assay kit modifying the manufacturer's instructions (Molecular Probes, kit E12055), by using a final concentration of collagenase to be of 0.005–0.01 U/mL. The activity was determined by measuring the fluorescence of each sample using a microplate reader (Tecan, Research Triangle Park, NC) and comparing to unmodified collagenase of equivalent concentration. The instructions were further modified to measure the relative enzyme activity of collagenase-labeled AuNPs by allowing the fluorogenic substrate DQ™ collagen to incubate with the AuNPs at room temperature for 2 hours, and then the AuNP was sedimented by centrifugation. Relative activity was determined by measuring the supernatant of the solution comparing to a negative control of 0.1 M sodium phosphate buffer, pH 7.4, containing the uncleaved fluorogenic substrate.

Cell Culture

The cellular and animal studies described here utilized the human alveolar epithelial adenocarcinoma cell line (A549). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% Penicillin/ Streptomycin (Invitrogen), 1% L-Glutamine (Invitrogen), and cultured in a tissue culture incubator at 37°C and 5% CO_2.

Cell Viability Assay

The viability and proliferation of cells in the presence of collagenase-labeled AuNPs were evaluated using a 3-[4,5-dimethylthialzol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, ATCC) assay. The assay was performed in triplicate in the following manner. A549 cells were seeded into 96-well plates at a density of 10,000 cells per well in 200 μL of media and

grown overnight. The cells were then treated at 37°C with various concentrations of AuNPs (0, 16, 32, 62.5, 125, 250, and 500 μM of Au) for 24 h. The media was aspirated and replaced with MTT reagent and incubated for 1 to 2 hours. The absorbance was measured at 560 nm using a Tecan plate reader (Tecan).

Biodistribution and Toxicity Analysis

All animals were maintained in accordance with the Institutional Animal Care and Use Committee of the University of Pennsylvania. Approximately 6-week-old female nu/nu nude mice (Charles River Laboratory, Charles River, MS, USA) (n = 6, 3 per group) were injected intravenously under anesthesia with either Control AuNPs or collagenase-labeled AuNPs in PBS at dose of 150 mg Au/kg (<12 mg collagenase/kg). Twenty-four hours post-injection, the mice were euthanized by CO_2 and 0.3 mL blood was removed by cardiac puncture from the right ventricle immediately after the cessation of breathing. After the final aliquot of blood was collected the heart, lungs, kidneys, spleen, and liver were removed from each animal. Organ samples were washed with PBS. Tissues were fixed in formalin and embedded with paraffin, then cut in half to allow for both histological analysis and measurement of gold content. The gold content in the organs was analyzed by ICP-OES.[21] To remove extraneous wax, paraffin-embedded organ samples were washed three times in toluene while sonicating at 60°C, for a total of one hour. The organs were then weighed into borosillicate glass tubes with teflon caps (MedSupply Partners, Atlanta, GA, USA) and digested at 60°C with 70% nitric acid to digest the organic material and remaining paraffin. After 2 hours, HCl was added and the digest continued to dissolve the inorganic material. Gold content was calculated as the percent of the injected dose present per gram of tissue.

Blood chemistry analytes (alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, and total bilirubin(TBIL) were analyzed by the diagnostic core laboratories at the University of Pennsylvania School of Veterinary Medicine. Hematoxylin and eosin (H&E) processing, staining, and evaluation of the liver and spleen was carried out by the Cancer Histology Core within the Perelman School of Medicine at the University of Pennsylvania.

Measurement of AuNP Uptake in Tumors

Approximately 6-week-old female nu/nu nude mice (Charles River Laboratory, Charles River, MS, USA) were maintained in accordance with the Institutional Animal Care and Use Committee of the University of Pennsylvania. Mice (n = 6, 3 per group) were anesthetized using isoflurane, and A549 cells were injected subcutaneously into the rear left flank (2×10^6 cells in 0.1 mL of PBS). Tumors were grown until the mean diameter was approximately 4–6 mm. Either collagenase-labeled AuNPs or control AuNPs were injected intravenously at a dose of approximately 3 mg in 200 μL of injected solution. After 24 h the animals were sacrificed and the subcutaneous tumors were extracted and thoroughly washed with PBS and blotted dry to minimize the contribution of any nanoparticles remaining in the bloodstream. The tissues were weighed and digested overnight at 60°C with 70% nitric acid. HCl was then added and the digest continued to dissolve the inorganic material. Samples were diluted with Millipore water and analyzed for gold content using ICP-OES.

Acknowledgements

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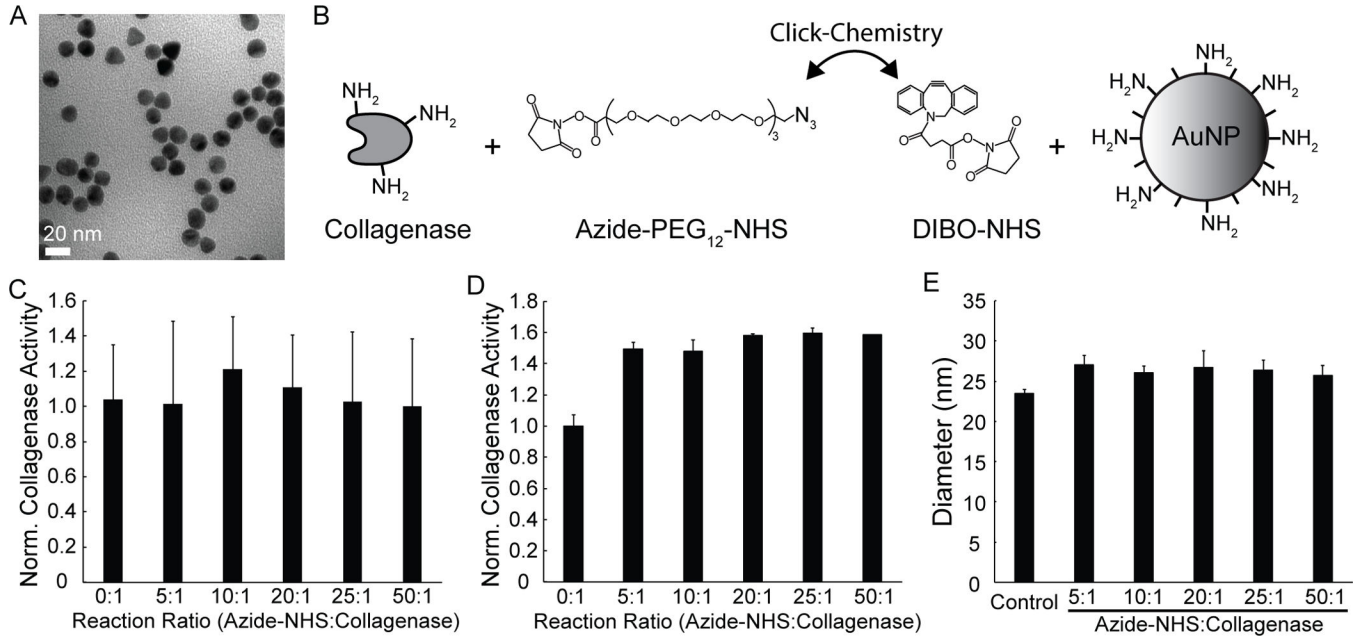

Figure 1.

Schematic and experimental validation of collagenase-AuNP conjugation. (A) Representative transmission electron micrograph of AuNPs, acquired using a JEOL 1010 transmission electron microscope (scale bars = 20nm). (B) Schematic of collagenase-AuNP conjugation strategy. Azide-labeled collagenase was “clicked” to dibenzycyclooctyne (DBCO)-labeled AuNPs. (C) Azide-PEG_{12}-NHS was reacted with collagenase at various molar ratios and collagenase activity was measured. All data was normalized to unreacted collagenase (i.e. 0:1 azide-NHS: collagenase), at an equivalent concentration. (D) Azide-labeled collagenase was reacted with dibenzycyclooctyne (DBCO)-labeled AuNPs. Following removal of unreacted collagenase, collagenase activity was measured. All data was normalized to a PBS control containing the uncleaved fluorogenic substrate. (E) The hydrodynamic diameter of control AuNPs and collagenase-labeled AuNPs was measured by dynamic light scattering, using a Zetasizer Nano-ZS (Malvern Instruments). Each sample was measured three times and the mean diameter ± standard deviation was calculated.

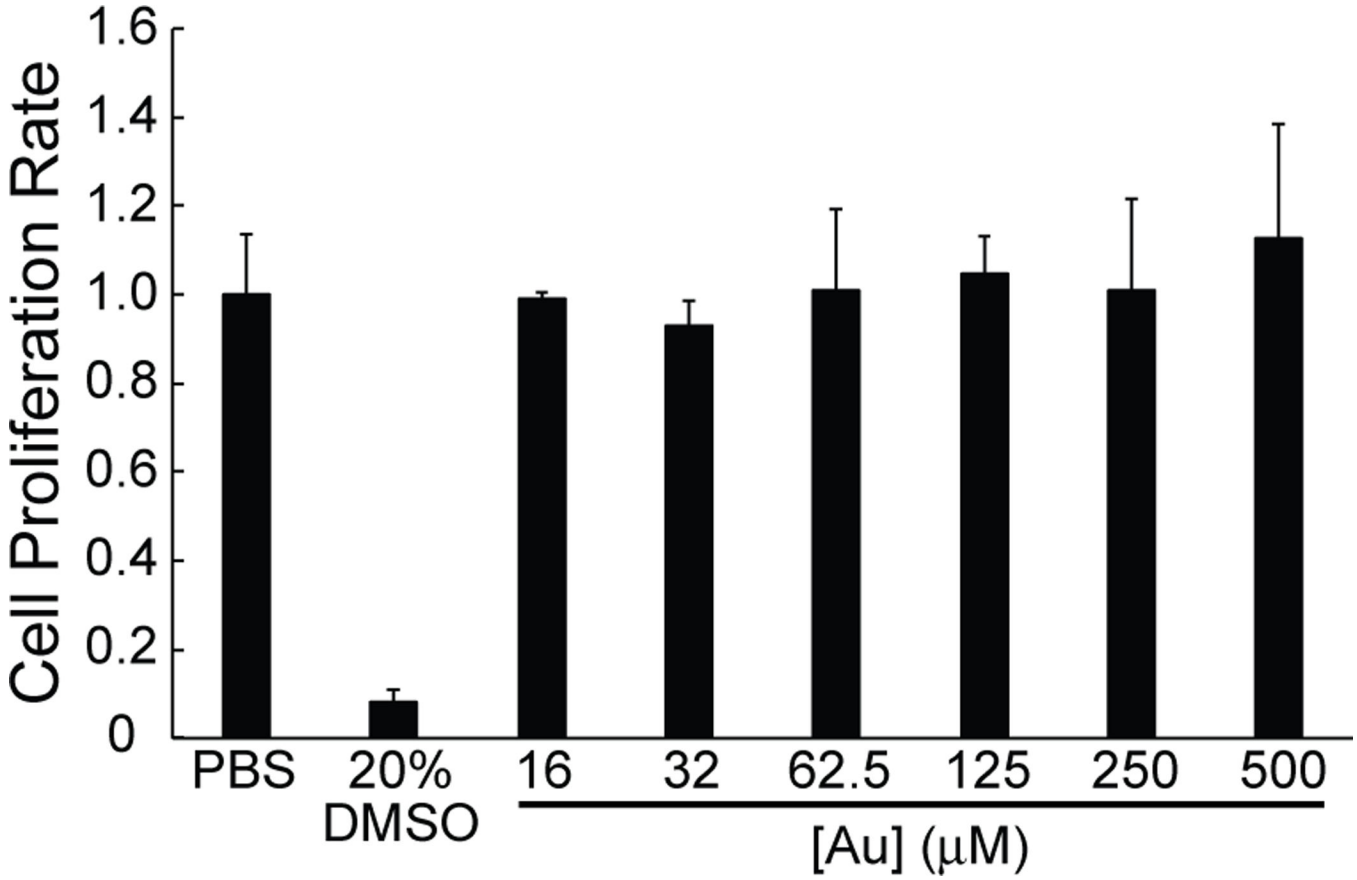

Figure 2.

Effect of collagenase-labeled AuNPs on cell proliferation. The proliferation of A549 cells in the presence of collagenase-labeled AuNPs was evaluated using an MTT assay. The cell proliferation rate was normalized to that of cells treated with PBS. Cells treated with 20% DMSO was used as a negative control. The assay was performed in triplicate.

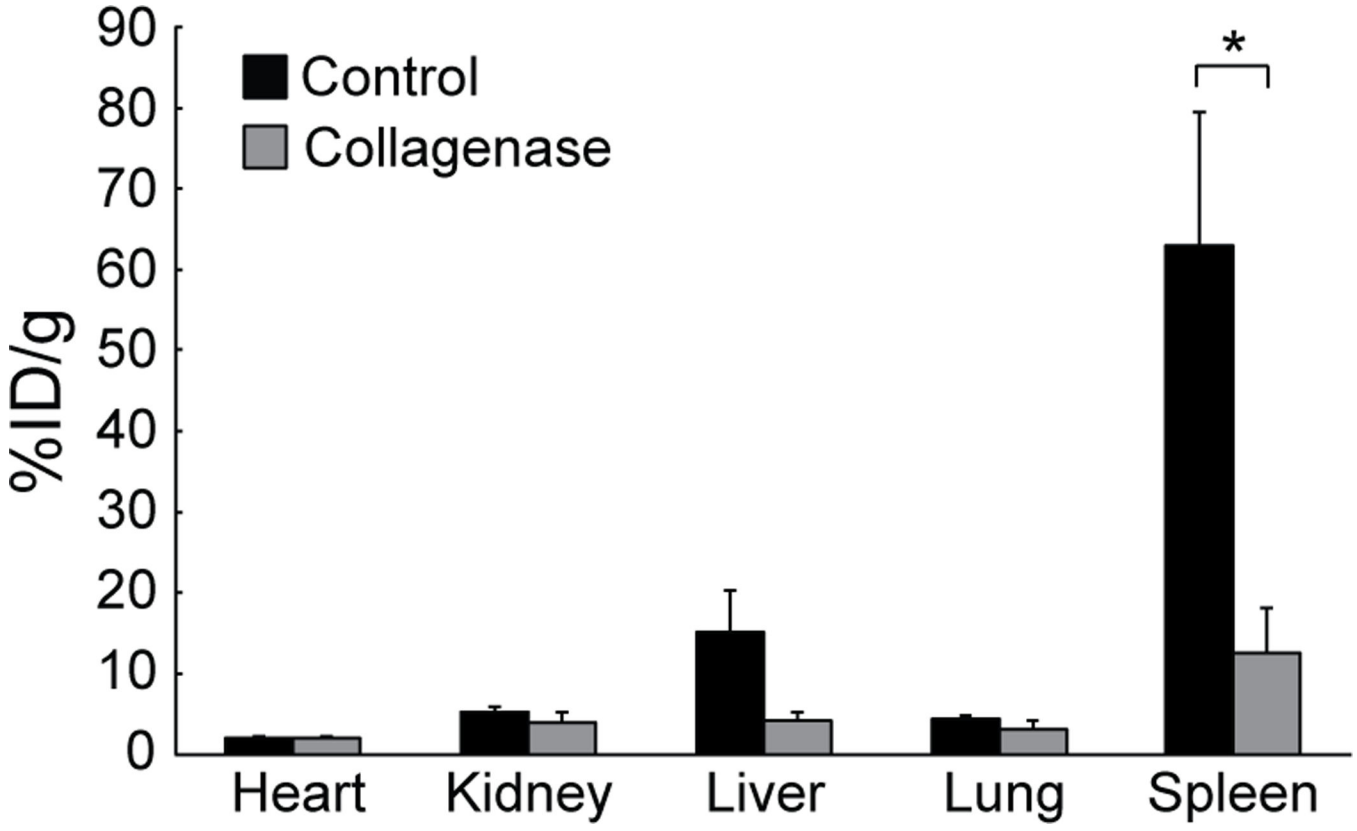

Figure 3.

Biodistribution of control AuNPs and collagenase-labeled AuNPs. The content of gold within various tissues was measured in mice (n=3 per group) 24 hours following the injection of control AuNPs and collagenase-labeled AuNPs (150 mg Au/kg). All measurements of gold were acquired via ICP-OES.

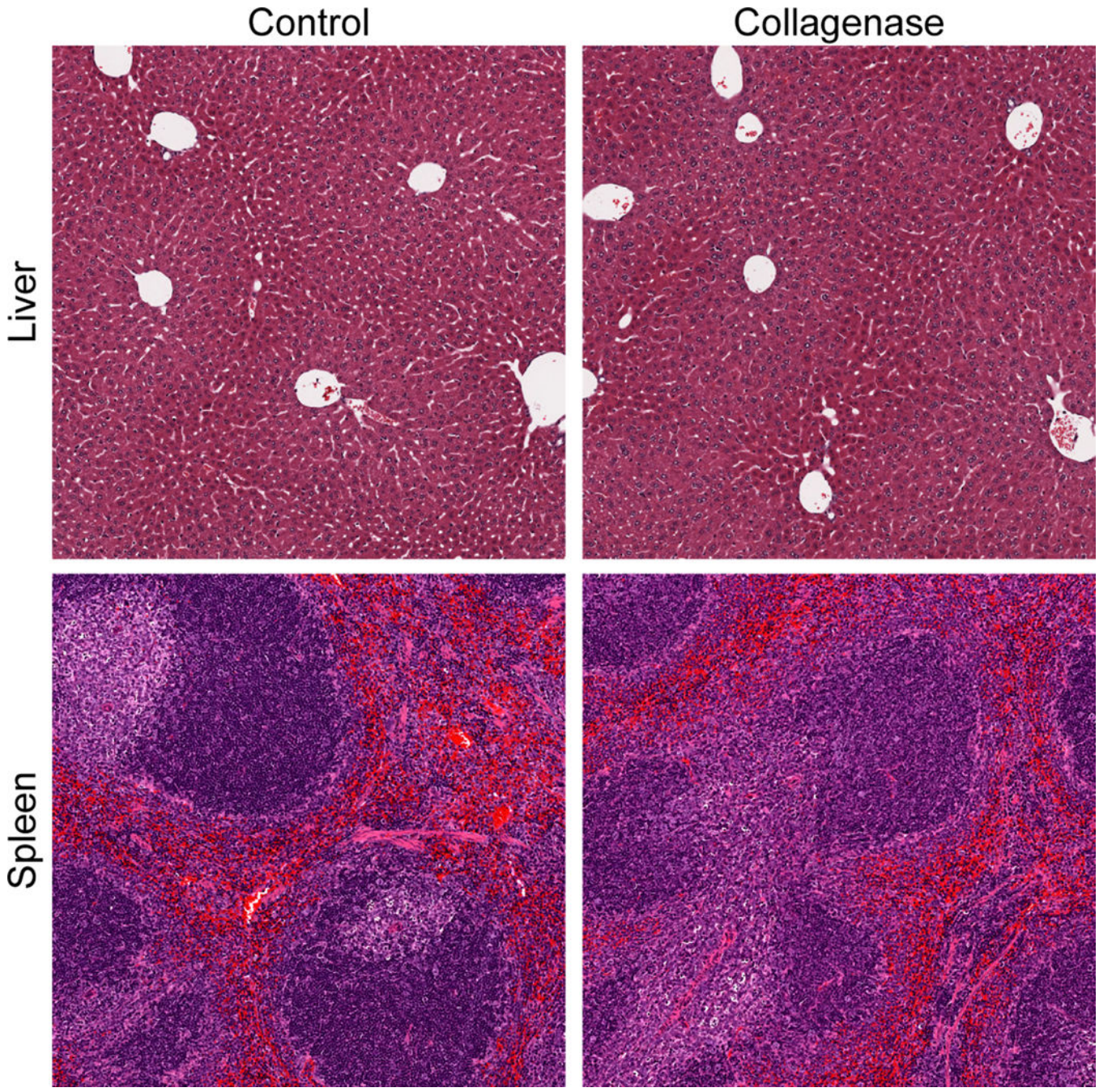

Figure 4.

Histology of liver and spleen for mice treated with AuNPs or collagenase-labeled AuNPs. Mice (n=3 per group) received a single intravenous injection of 0.2 mL of either control AuNPs or collagenase-labeled AuNPs (150 mg Au/kg dose in PBS) followed by dissection of the liver and spleen 24 hours post injection. Sections were stained with H&E and images were acquired via light microscopy at 10× magnification.

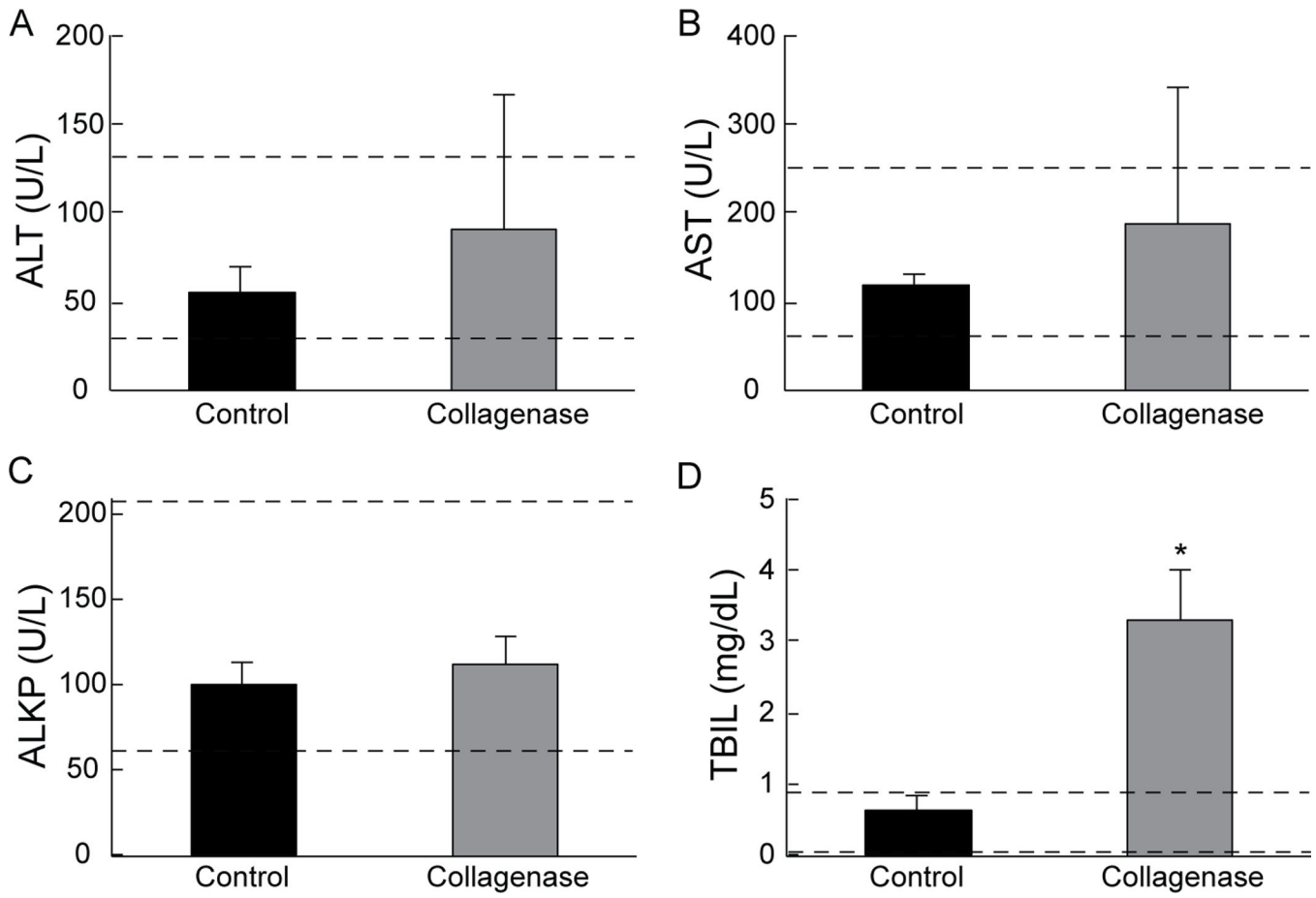

Figure 5.

Hematological analysis of mice treated with control AuNPs or collagenase-labeled AuNPs. Blood enzyme levels of mice were acquired 24 hour post-injection of control AuNPs or collagenase-labeled AuNPs (150 mg Au/kg). Grey dotted lines denote the “normal” analyte levels. The specific enzymes analyzed were (A) alanine transaminase (ALT), (B) aspartate aminotransferase (AST), (C) alkaline phosphatase (ALKP), and (D) total bilirubin (TBIL).

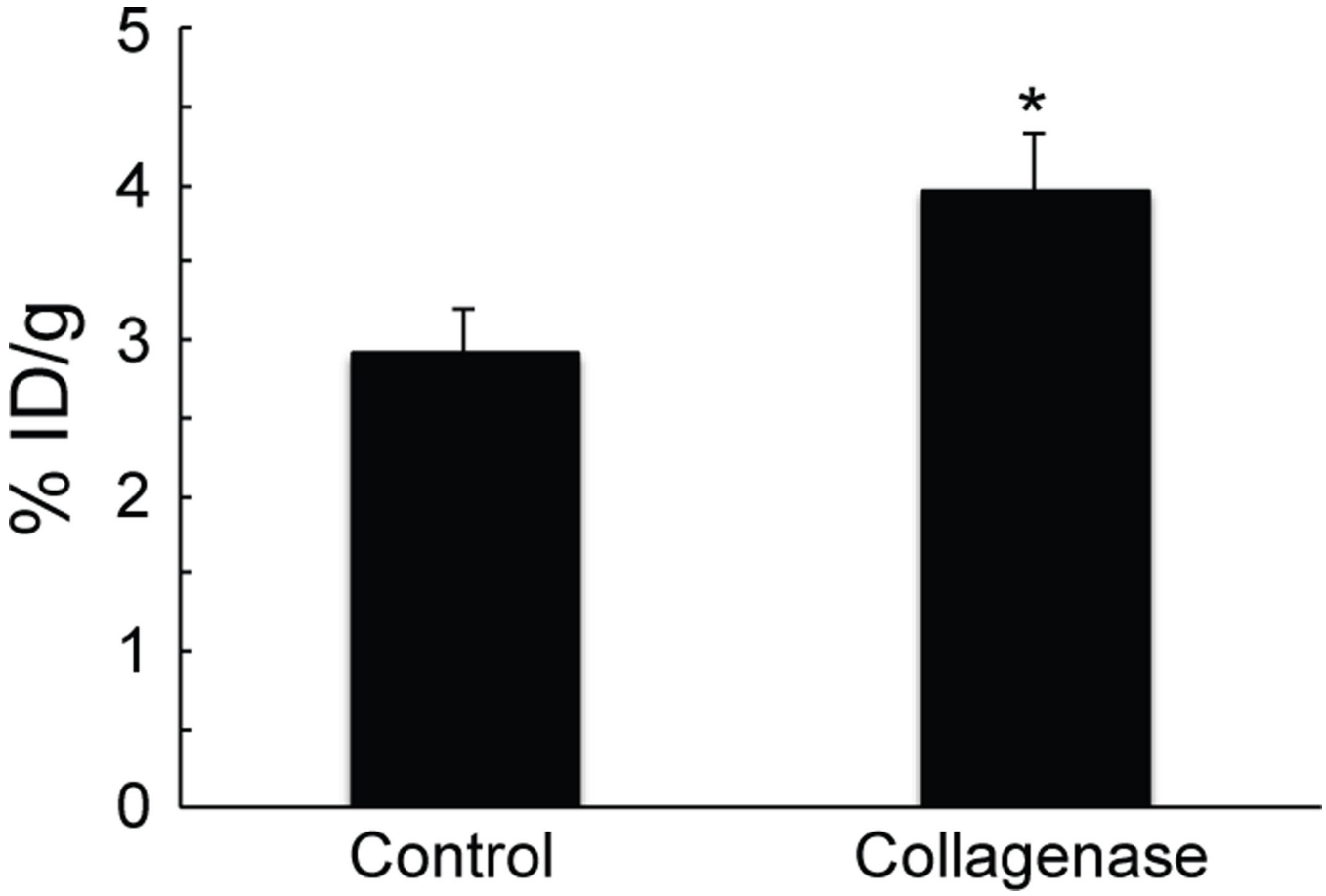

Figure 6.

Measurement of gold accumulation in tumors following injection of AuNPs or collagenase-labeled AuNPs. The percent injected dose of gold per gram of tissue was measured in murine tumor xenografts (n = 3 per group) 24 hours following the intravenous administration of control AuNPs or collagenase-labeled AuNPs (150 mg Au/kg). All measurements of gold were acquired via ICP-OES. Asterisk indicates statistical significance ($p < 0.05$) between groups.