Bioorthogonal radiopaque hydrogel for endoscopic delivery and universal tissue marking

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Dr. Seonki Hong,
Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

Dr. Jonathan Carlson,
Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

Prof. Hakho Lee, and
Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

Prof. Ralph Weissleder
Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, Department of Systems Biology, Harvard Medical School, Boston, MA 02114

## Graphical abstract

We report a novel dual marking hydrogel system for radiological and laparoscopic localization of lesions. Bio-orthogonally crosslinked hydrogel containing both tantalum and india ink can be rapidly formed inside the body after injecting precursors, and stably located for several days as a long-term biocompatible carrier for markers.

Correspondence to: Ralph Weissleder.

Bio-orthogonally crosslinked hydrogel

Dual-localization of lesions

Radiological

Laparoscopic
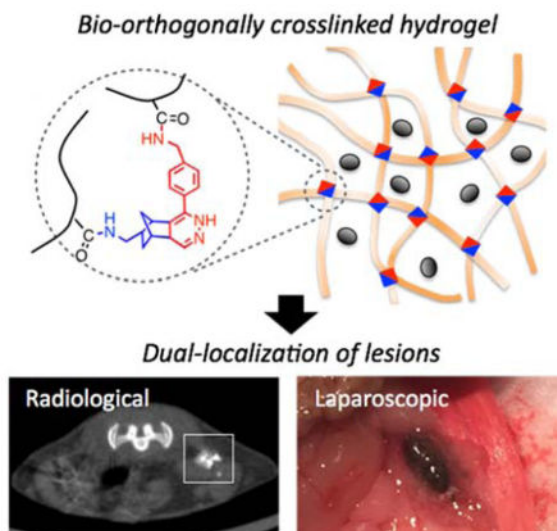
Keywords

hydrogels; medical applications; hybrid materials

Accurate marking of internal anatomic sites is critical to precision delivery of radiation therapy, to enable longitudinal follow-up of lesions under observation, or for multimodal treatment across an array of clinical conditions.[1] Here, we report a new dual marking system for radiological and endoscopic/surgical localization of lesions that addresses these goals. The system is based on a hydrogel carrier, i.e. a polymeric network that forms in aqueous environments and has tunable mechanical properties similar to natural soft tissues. We reasoned that an injectable hydrogel system could form versatile and highly visible markers by entrapping a diverse range of molecular tracers within its network, including contrast media and biocompatible pigments. We used gelatin, a water-soluble protein derived from native collagen, as a substrate, tetrazine ligation chemistry for bioorthogonal cross-linking, and functionalized the gel with tantalum oxide and India ink. The developed marker showed high cell adhesion and proliferation efficiency *in vitro*, excellent visible and radiographic contrast, and maintained stability up to 12 days after successful formation *in vivo*.

Precise localization of enteric lesions is particularly essential for gastroenterologic (GI) and GI oncologic care, where serial examination by endoscopy and durable marking for surgical resection and/or radiation therapy are cornerstones of the treatment process.[2] In this context, an ideal biocompatible tissue marker should be (i) stable/durable (weeks to months); (ii) highly visible both intraluminally (endoscopic) and extraluminally (intraperitoneal); and (iii) amenable to multimodal detection by visual, tactile, and cross-sectional imaging means. Current clinical methods often fail to satisfy these key askpects. India ink tattooing, the clinical gold standard for endoscopic marking, offers excellent tissue stability and facile visual localization,[2] but is invisible to x-ray, CT, MRI or ultrasound.[3] Surgical clips are radio-opaque and can be deployed endoscopically, yet can be difficult to

locate due to their small size and often fall off within a few days due to the intrinsic mechanical activity of the GI tract.[4] Radiographic localization for image-guided radiotherapy (IGRT) uses durably embedded gold fiducial markers. These markers, however, have limited visual detectability, are prone to image artifacts on CT scan, and require specialized equipment for placement.[5] Functionalized PEG-hydrogels are readily imaged by MRI[6] or CT,[7] but appear to require further study of their long term tissue stability/durability.[8]

As a backbone for our universal marking system, we selected gelatin, which is ideally suited for *in vivo* marking for short term applications, as it contains natural cell-binding motifs (e.g., Arg–Gly–Asp) and degrades in response to matrix metalloproteinase (MMP) activity.[9] For longer term applications one may consider natural polymers with slower degradation rates such as chitosan, hyaluronic acid, alginate, and heparin,[10] or irrespective of the backbone, chemical modifications to enhance hydrogel stability. The intrinsic properties of natural polymers can have additional advantages in modulating tissue adhesiveness through electrostatic interactions. Anionic polymers such as hyaluronic acid and alginate have been used to develop non-sticky biomaterials,[11] while cationic polymers such as chitosan have been reported as raw materials for tissue adhesives.[12] For the gelatin we selected, chemical modifications have been widely used to improve the tissue adhesiveness and *in vivo* stability/durability in biomedical applications. Methacrylation is often used to prepare photo-crosslinked hydrogels for long-term tissue regeneration from days to months *in vivo*,[13] and enzyme-mediated conversion of tyrosine to DOPA in gelatin highly improved tissue adhesiveness.[14] As one example of this approach, we adopted a bioorthogonal ("click") reaction for cross-linking and prepared tetrazine- and norbornene-conjugated gelatins (Gel-Tz and Gel-NB) (Figure 1A), leveraging chemistry that effectively occurs inside living systems.[15] Among a growing variety of bioorthogonal methods,[16] tetrazine ligation has been widely used in live cells due to its robust biocompatibility, rapid kinetics and catalyst-free conditions.[17] Gel-Tz and Gel-NB were synthesized through amide coupling between the carboxyl groups of the gelatin and amine-functionalized tetrazine/norbornene tags (Figure S1A). The degree of conjugation of Gel-Tz and Gel-NB was approximately 4–5 functional groups per each gelatin backbone (275 Bloom, from porcine skin), as determined by nuclear magnetic resonance (NMR) analysis (Figure S1B).[18]

The sol-to-gel transition occurred within 1 min by the simple mixing of two solutions (1:1 volume ratio) containing 10% Gel-Tz and Gel-NB in phosphate buffered saline (PBS, pH 7.4), respectively, as measured with the typical vial-tilting method.[19] Gelation time depended on the concentration of each solution, decreasing from 4 minutes to 30 seconds as the concentration of each solution was increased from 5% to 15% (w/v), regardless of the presence of added radiopaque agents (Figure 1B). To explore the impact of cross linking kinetics, we also prepared gelatin conjugated to transcyclooctenol, which is widely used as a bioorthogonal partner for tetrazines on account of its extreme reactivity (3–4 orders of magnitude faster than NB). Gel-TCO exhibited rapid gelation immediately after combining with Gel-Tz (1–2 sec), preventing homogenous mixing and too fast to be used as an injectable system.

The gel point and the mechanical moduli changes of the Gel-NB/Tz hydrogel were further characterized by rheometry. The gel point detected with a rheometer was similar to the gelation time measured with the vial-tilting method (Figure S2), and the overall crosslinking reaction was completed within 20 min, which was confirmed by the stability of the final storage and loss moduli (Figure 1C). The storage modulus of the formed hydrogel was tunable as a function of the Gel-Tz/NB precursor concentrations across a 40-fold range, spanning 100 to 4000Pa (Figure 1D, Figure S3). This modulus is comparable with various natural soft tissues/organs: ~200 Pa for lung, <1000 Pa for brain, ~2000 Pa for fat, and >5000 Pa for smooth muscle, allowing matching to target tissues for optimal integration.[20] Tactile handling and topical application of the fresh gel to a range of organic and inorganic materials revealed no qualitative stickiness or measurable adhesivity.

We evaluated the biocompatibility of Gel-NB, Gel-Tz, and crosslinked Gel-NB/Tz hydrogel. We first incubated cells (3T3 fibroblasts) with each precursor (24 h), and analyzed the cell viability. As shown in Figure S4A, the viability of the 3T3 cells was not affected by adding up to 10 mg/mL of soluble Gel-NB or Gel-Tz in the liquid media. We next tested the growth of cells in media that had been pre-incubated with crosslinked hydrogel for 24 hours to elute any leachable products;[21] no reduction in cell viability after 24 hours of growth in the media (Figure S4B) was observed. We further examined the cell attachment and proliferation on the crosslinked hydrogel containing radiopaque agents. One day after the seeding, 3T3 cells were well attached on the Gel-NB/Tz hydrogel and on the gel impregnated with radiopaque iopamidol, gold, or Ta_2O_5. Cell proliferation was robust after 4 days in all cases (Figure 2A and Figure S5), and quantitative image analysis of the live cell fraction on the surface of the radiopaque hydrogels (Live/Dead staining, Invitrogen™) revealed viability >95% (Figure 2B). Cell density on day 1 and day 4 was equivalent to the control gel (Figure 2C), confirming the excellent biocompatibility of radiopaque Gel-Tz/NB hydrogels.

We next quantitated the radiopacity of the gels on CT imaging. Scans were performed immediately after gel formation as well as after 1 hour of incubation in PBS, pH 7.4 (Figure 2D). The Gel-NB/Tz hydrogel itself had no significant radiopacity (~70 Hounsfield units (HU)), whereas the CT value of the radiopaque gels was linearly correlated to the number of encapsulated Iodine-containing organic molecules (iopamidol) or metallic particles in the hydrogel. The absolute radiopacity of the iopamidol-containing gel (I-gel) was lower than that of the Gold- or Ta_2O_5-containing gels (Au-gel or Ta-gel), with the I-gel containing 10% (w/v) iopamidol peaking at ~2000 HU, while the Au-gel and the Ta-gel containing 10% (w/v) metals both achieved densities greater than 3500 HU (Figure 2E). This corresponds to an even stronger signal in CT images than the typical bones (700 for cancellous bone to 3000 for dense bone). As shown in Figure 2E, even the 5% (w/v) Ta-gel showed densities above 1000 HU, which can be effectively distinguished by CT *in vivo*.[22] On repeat imaging, the CT value of the I-gel had decreased dramatically after 1 h of incubation in the PBS buffer (pH 7.4, 37 °C) by the release of iopamidol from the gel. However, the metallic particles were stably encapsulated in the gel, with no detectable decline in radiopacity after incubation on this timescale. We selected the Ta-gel as the most promising and assessed its gelation and imaging performance with chicken breast as a model tissue *in vitro*. Freshly mixed Ta-gel was applied topically at an incision site or injected into the tissue through an

18G needle (Figure S6). In both cases, the gel was well formed and localized on the cutting area or in the middle of the chicken breast without any evident diffusion into surrounding tissue.

To achieve a dual marking system for radiological and visual (e.g. endoscopic, laparoscopic or surgical) localization, Ta-gel (5% Ta_2O_5) was further functionalized by addition of India ink. We employed a 1:100 (v/v) dilution, matching the dilution currently used clinically in endoscopic tattooing to avoid causing any inflammatory responses *in vivo*.[23] India ink is a suspension of micron-sized particles of carbon black pigment,[24] and we hypothesized that it would be stably trapped within the Ta-gel. We tested this hypothesis *in vitro*, using a Ta-gel containing India ink. The gel was incubated it in PBS for 12 days, with no leaching of the black pigment from the gel observed (Figure S7). Intramural injection of a freshly mixed sample of Ta-gel-black into the wall of a fresh porcine colon specimen (Animal Technologies, Tyler, Texas, USA) produced a palpable ovoid nodule within the tissue, with black pigment readily visible on examination of the site from either the intraluminal (mucosal) or extraluminal (serosal) aspect (Figure 3A).

Finally, we tested the *in vivo* performance of injectable radiopaque gels in mouse experiments. Because the small scale of the mouse colon makes intramural injection impractical, we chose to inject the gel into the mouse peritoneum, where it would be exposed to the same anatomical compartment as a stringent test of biocompatibility for the proposed endoscopic applications. Gel-Tz mixed with Ta_2O_5 and india ink was combined with Gel-NB, mixed, and then immediately injected intraperitoneally (IP) through a 25G needle. We selected the 10% gelatin concentration formulation of Ta-Gel to balance the dual priorities of adequate handling time for preparation/injection IP and rapidity of gelation *in vivo*. In parallel, a control mouse was injected IP with a matching Gel-Tz and Ta_2O_5 suspension that had been mixed with unmodified gelatin instead of Gel-NB. The initial CT scan showed the bioorthogonal hydrogel formed at the mid-peritoneum injection site (Figure 3B); the contrast material had spread diffusely throughout the peritoneum in the control animal. On serial scans, the cross-linked gel remained physically intact within the intraperitoneal compartment, with high radiopacity confirmed for a minimum of 12 days. With IP injection, the gel was deliberately not entrapped within fixed tissue planes, and mobility of the gel deposit within the peritoneal space was observed/expected across the series of scans due to the regular movement of bowel. This lack of stickiness improves tractability for handling and injection, desirable given both practical and safety considerations for minimizing the risk of bowel adhesions as a complication.[25] The mouse was euthanized after 12 days and the gel was located and then removed from the peritoneum. The black color of the India ink remained highly visible, and was stably incorporated in the gel with no pigmentation evident in the surrounding tissues (Figure 3B, at lower right).

In summary, we have developed an easily injectable hydrogel marker that can be administered through image guided delivery, endoscopic application, or during surgery. The material allows encapsulation of markers giving it multimodal detection capabilities often desired in clinical care. The system exploits the gel as a biocompatible space-filling scaffold to embed both contrast agents and pigment within a solid and highly biocompatible matrix.

The gelatin-based hydrogel was successfully formed *in vivo* and stably located up to 12 days, and was highly detectable by CT imaging, with excellent radiopacity and no streak artifacts observed. Our future work would include exploration of other polymer systems with different degradation rates to match with different clinical needs.

Experimental Section

Synthesis of Gel-NB/Gel-Tz

First, 700 mg of 275 Bloom gelatin from porcine skin (Gelita®, pharmaceutical grade) were dissolved in 70 mL of 50 mM MES buffer, pH 6 (Sigma-Aldrich) at 50 °C. Next, 370 mg of N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, Sigma-Aldrich) and 274 mg of N-Hydroxysuccinimide (NHS, Sigma-Aldrich) were sequentially added to the gelatin solution at 50 °C, followed by 98 mg of 1-Bicyclo [2.2.1] hept-5-en-2-ylmethanamine (Matrix Scientific) or 89 mg of (4-(1,2,4,5-Tetrazin-3-yl)phenyl)methanamine hydrochloride (Tetrazine amine, Conju-Probe), which were added to the mixture, respectively, to synthesize a Gel-NB or Gel-Tz conjugate. With vigorous stirring, the temperature of the mixture cooled to 37 °C, at which point the mixture was kept for 5 hrs at 37 °C in room air. After the reaction, the mixture was purified by dialysis (MWCO: 12,000–14,000 Da, SpectraPor, USA) against distilled water containing 20 mM of sodium chloride for two days at 37 °C, followed by distilled water for 5 hrs at 37 °C. The final product was achieved by lyophilization and stored at −80 °C until needed for further use.

^{1}H-NMR and UV-vis spectrum

The degree of conjugated norbornene and tetrazine on gelatin were determined by 1H-NMR following the method previously described for quantifying the degree of methacrylation on gelatin.[18] The ^{1}H-NMR study was performed by using a Bruker AscendTM 400 spectrometer in deuterium oxide (Sigma-Aldrich). The peak at 0.9 ppm corresponded to the hydrophobic alkyl side chains of valine, leucine, and isoleucine on gelatin. The composition of each side chain was announced by the manufacturer (0.025 mol Val, 0.027 mol Leu, 0.012 mol Ile in 100 g gelatin); therefore, the integration of this peak (total 18 protons) was considered to be 0.384 mol/100 g gelatin. The amount of conjugated moiety (mol/100g gelatin) can be calculated by comparing the integration of the newly formed peak to this methyl peak at 0.9 ppm, as shown below:

$$\text{Conjugated moiety (mol/100 g gelatin)} = (I_{new}/\#\text{ of equiv protons})/I_{0.9} * 0.384 \text{ (mol/100 g gelatin)}$$

Thus, the degree of conjugated moiety can be represented as the number of moiety on one gelatin molecule.

Hydrogel preparation and characterization

For the preparation of 10% (w/v) hydrogel, 10 mg of Gel-NB and Gel-Tz were each dissolved in 100 μL of phosphate buffered saline (pH 7.4, 10 mM) at 37 °C. The two mixtures were then mixed in a 1:1 volume ratio and kept at room temperature until the gel formed. Gelation time was determined by the typical vial-tilting method.[19] For radiopaque

hydrogels, 10 mg of gold powder (Alfa Aesar, 1.5–3 μm), Tantalum oxide (Ta_2O_5, Sigma Aldrich, < 20 μm) or Iopamidol (AK Scientific, Inc) were pre-mixed with 100 μL of 10% (w/v) Gel-Tz solution. Finally, 100 μL of 10% (w/v) Gel-NB solution were added to the mixture.

Mechanical characterization

Oscillatory rheometry, using a rotating rheometer (Discovery Hybrid Rheometer with a parallel 20 mm plate, TA Instruments, USA), was used to determine the gelation kinetics and mechanical strength of the Gel-Tz/NB hydrogel. First, 100 μL of both precursors were applied together on the bottom plate of the machine, followed by immediate closure of the upper plate to begin measurement. However, approximately 30 sec was inevitably needed to get an initial data point from the mixing. Modulus change of the mixture was monitored by time sweep at 1 Hz of frequency with 1 % of the strain at 37 °C up to 20 min from the mixing of the two components, Gel-Tz (containing Ta_2O_5 or not) and Gel- NB solution. In addition, a strain sweep was performed at 37 °C with a single frequency of 1 Hz after 20 min from the gelation to confirm the mechanical strength of the hydrogel when the crosslinking was complete. Gap size was 300 μm, and mineral oil surrounded the sample to prevent its dehydration during measurement.

Cell culture

NIH 3T3 fibroblasts were cultured in Dulbecco's modified eagle medium (DMEM, Gibco®), which contained 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2. Cells were passaged approximately two times per week, and media were changed every two days.

Cytotoxicity of precursors

Cytotoxicity of the conjugated polymers (Gel-NB and Gel-Tz) was quantitatively measured by the PrestoBlue® (Invitrogen) cell viability assay. NIH 3T3 fibroblast cells were seeded in a 96-well culture plate (1×10^4 cells/well) and incubated for 24 hrs prior to use. Then, the media of the cells were replaced with media containing various concentrations (10, 3, 1, 0.3, 0.1 mg/mL) of conjugated polymers (Gel-NB, Gel-Tz, and gelatin as a control). The cells were incubated for another 24 hrs, followed by analysis via the PrestoBlue® assay, according to the manufacturer's instructions. Briefly, the PrestoBlue® reagent (20 μL) was directly added to each well after incubation and reacted for 40 min at 37 °C. The absorbance at 570 nm of wavelength was measured by using a Tecan Safire2 plate reader (Männedorf, Switzerland), and 600 nm of wavelength was used as a reference. The viability was normalized to ensure that the cells in the fresh media did not contain any conjugated polymers cultured in the same way.

Leachable products from the hydrogel

Cytotoxicity of the released product from the crosslinked gel was also quantitatively evaluated by the PrestoBlue® (Invitrogen) cell viability assay, as shown above. For collecting leachable products, 30 mg of the crosslinked gel (prepared by the above-mentioned method, kept for 30 min at 37 °C prior to use) was incubated in 3 mL of fresh

media for 24 hrs in the cell culture incubator. Next, the media were filtered by a 0.22 μm-sized syringe filter to remove hydrogel fraction, followed by dilution to 1:1, 1:3, 1:10, 1:30, 1:100 in fresh media. Cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and incubated for 24 hrs prior to use. For the viability assay, the media of the cells were replaced with the media containing leachable products and its diluted ones, followed by incubation for another 24 hrs in the cell culture incubator. Finally, the PrestoBlue® assay was performed.

Cell attachment test

For two-dimensional (2D) seeding of cells on the surface of the hydrogel, 100 μL of 10% (w/v) Gel-NB and 100 μL of 10% (w/v) Gel-Tz, containing radiopaque agents, were mixed in a 48-well plate and incubated for 30 min at 37 °C to make a fully crosslinked radiopaque hydrogel, which covered the entire exposed area of each well. NIH 3T3 cells (3×10^4 cells/well) were seeded on top of the hydrogel in a 48-well plate and incubated at 37 °C in the cell culture incubator. Cell adhesion and proliferation were investigated via a fluorescence-based LIVE/DEAD® viability/cytotoxicity kit for mammalian cells (Invitrogen™), according to the manufacturer's instructions. Briefly, cells were stained in DPBS containing 0.5 μL/mL of Calcein AM and 2 μL/mL of ethidium homodimer-1 (EthD-1) for 15 min at 37 °C in the culture incubator. After two washings with DPBS, stained cells were observed with a fluorescence microscope (Nikon Eclips TE2000S), and the number of live cells stained green was counted by ImageJ software. Assays were performed on days one and four from the seeding of the cells, and quantitative data of cell density were collected from the average of three independent experiments.

CT imaging

First, 5 mg of Tantalum oxide (Ta_2O_5) and 1 μL of India ink (Speedball® Super Black™) were pre-mixed with 50 μL of 10% (w/v) Gel-Tz solution, and 50 μL of 10% (w/v) Gel-NB solution was added to the mixture at 40 C. For control, unmodified gelatin was used instead of Gel-NB conjugate. Next, Mice were placed under anesthesia (isoflurane 1.5%; O_2 2 L/minute) and an intraperitoneal injection was given with either the hydrogel or control agent (50 μL of pre-mixed solution). Computed tomographic images of the mice, under similar anesthesia, were taken immediately after IP injection, 4 hrs, and 12 days using the Siemens Inveon system with an x-ray tube of 80 kVp and 500 μA over 360 degrees with 1 projection per degree with 125 mm detector. Images were reconstructed into 110 μm isotropic voxels using the modified Feldkamp cone beam reconstruction algorithm (COBRA Exxim). Images were visualized and analyzed using OsiriX (The OsiriX Foundation, Geneva, Switzerland).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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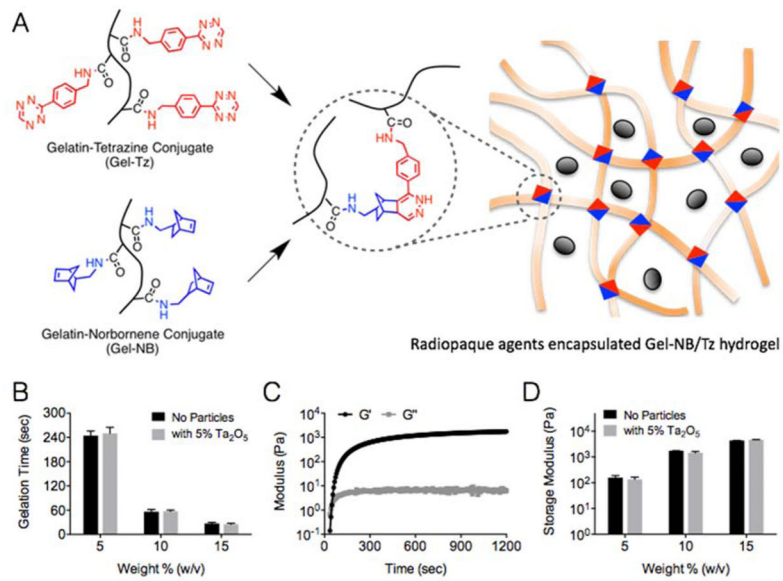
Figure 1.
Bio-orthogonally crosslinked gelatin-based hydrogel preparation. (A) A schematic description of the inverse electron demand Diels-Alder reaction between tetrazine and norbornene forming the hydrogel. (B) Gelation time of 5, 10, and 15% Gel-NB/Tz hydrogel with and without Ta_2O_5 particles measured by the typical vial-tilting method. (C) Storage and loss modulus changes during initial state of gel formation up to 20 min. (D) Storage moduli of 5, 10, and 15% (w/v) Gel-NB/Tz hydrogel with and without encapsulated Ta_2O_5 particles.
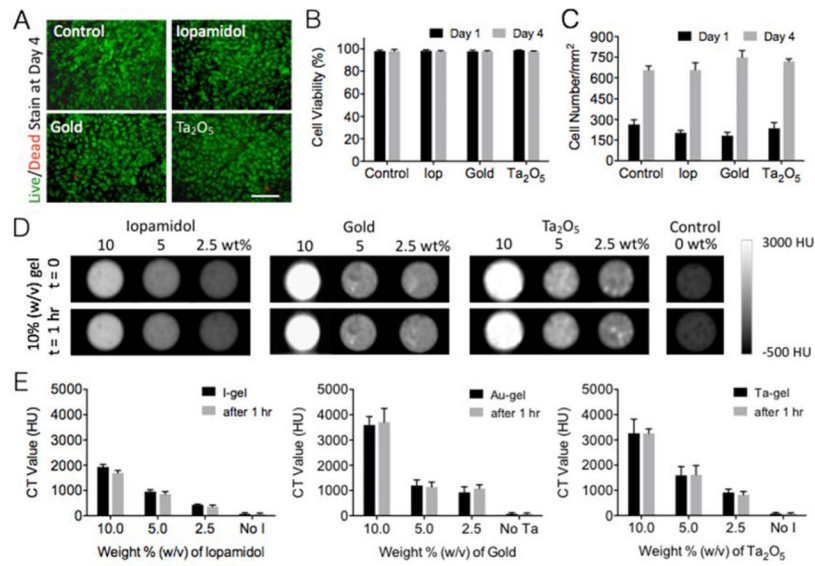
Figure 2.
2D culture of 3T3 cells on the surface of Gel-NB/Tz hydrogels with incorporated radiopaque agents and their radiopaque properties. (A) Representative images of Live/Dead stained cells after 4 days of seeding on 10% (w/v) Gel-NB/Tz hydrogels containing 5% (w/v) of various radiopaque agents (scale bar = 200 μm). (B–C) Quantification of cell viability and cell density from Live/Dead images. (D) *In vitro* CT contrast images of 10% (w/v) Gel-NB/Tz hydrogels containing Iopamidol, Ta_2O_5 microparticles, and gold powders, and (E) CT values quantitatively analyzed from CT images.
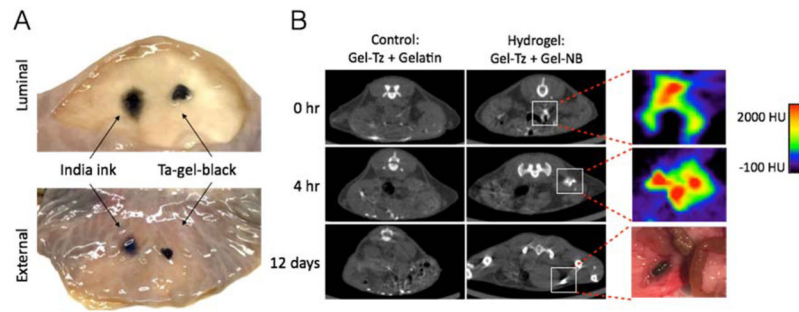
Figure 3.
Ex vivo colonic localization and *in vivo* CT imaging. (A) *Ex vivo* experiment of localization on a porcine colon specimen showing the efficiency of the gel as a visible marker. (B) Serial CT images (axial sections) of a live mouse following injection of 10% (w/v) Gel-NB/Tz hydrogel containing 5% Ta_2O_5 particles into the peritoneum, with comparison to photographic image of the gel in situ post euthanasia (lower right).