



Indocyanine green-loaded injectable alginate hydrogel as a marker for precision cancer surgery

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Background: Accurate identification of tumor sites and boundaries is of paramount importance during minimally invasive surgery. Although laparoscopic resection is being increasingly and widely performed for early gastric and colorectal cancers, the detection of tumors located inside the stomach and intestine is difficult owing to the lack of tactile sensation. Here, we propose the application of an indocyanine green (ICG)-loaded alginate hydrogel system as a fluorescence surgical marker for precise laparoscopic operations.

Methods: A physical complex of ICG and human serum albumin (HSA) was mixed with sodium alginate to form an injectable hydrogel system. Calcium carbonate and D-gluconic acid (GA) were added to the gel to control its strength and gelation time, respectively. The optimal conditions for the preparation of injectable hydrogels were determined by analyzing the fluorescence spectra and sol-gel transition time of the prepared samples at various concentrations and compositions. Next, the aqueous solutions of ICG, ICG-HSA, and ICG-HSA-loaded alginate were subcutaneously injected into nude mice (three mice per group), and near-infrared (NIR) fluorescence images of the mice ($\lambda_{\text{ex.}}=780$ nm, $\lambda_{\text{em.}}=845$ nm) were obtained at different points in time for 8 days. Then, fluorescence intensities at the injection sites, target-to-background ratio, and areas of ICG fluorescence were analyzed. Finally, the potential utility of ICG-HSA-loaded alginate hydrogel as a surgical marker was evaluated in a porcine model. The ICG-HSA-loaded alginate solution was injected into three sites in the submucosal space of the porcine stomach via a catheter. A fluorescent laparoscopic system was installed on the abdomen of the pig 3 days post-injection, and the fluorescence signal generated from the fluorescence surgical marker located inside the stomach was evaluated using the fluorescence laparoscope system ($\lambda_{\text{ex.}}=785$ nm, $\lambda_{\text{em.}}=805$ nm).

Results: The optimal concentration of ICG-HSA complex was determined to be 30 μM , and maximum fluorescence intensity of the complex was obtained at a 1:1 mole ratio of HSA to ICG. The subcutaneous injection of ICG or ICG-HSA solution in mice resulted in the rapid spread of the fluorescence signal around the injection site in 3 h, and a weak fluorescence was detected at the injection site 24 h post-injection. In contrast, the fluorescence detection time was effectively prolonged up to 96 h post-injection in the case of ICG-HSA-loaded alginate gel, while diffusion of the injected ICG from the injection site was effectively prevented. In the laparoscopic operation, injection sites of the hydrogel in porcine stomach could be accurately detected in real time even after 3 days.

Conclusions: This alginate hydrogel system may be potentially useful as an effective surgical marker in terms of accuracy and persistence for laparoscopic operation.

Keywords: Alginic acid; hydrogel; indocyanine green (ICG); laparoscopy; surgical marker

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Introduction

Laparoscopic surgery, a minimally invasive procedure, reduces the impact on the patient's body and allows surgeons to perform operations through small holes (usually 0.5–1.5 cm long) as compared to traditional operations (usually through 24–36 cm long holes) (1). Past several decades have noticed development in laparoscopic surgery owing to advantages such as less bleeding and pain, rapid recovery rate, and shortened hospitalization period as compared with traditional open surgery (2). However, palpation of suspicious regions for tumor detection is impossible with laparoscopic operation unlike conventional surgery, and it is difficult to detect small soft lesions or polypectomy sites (3). Therefore, the incision range may vary according to the skills of the operator. To reduce this error and improve the accuracy of operation, it is imperative to preoperatively mark the surgical sites. To improve tumor detection, several marking methods have been developed, including fluoroscopy location (4), barium imaging (5), marking with metal clips (6,7), and tattooing using dyes such as methylene blue or India ink (8). However, each of these methods suffer from limitations. Intraoperative fluoroscopy is expensive and time-consuming, may need bowel distension, and increases contamination rate (9). Barium imaging demand the use of radiation (5) and may be unreliable (10). Metal clips are expensive and invisible outside, and may dislodge before or during surgical operation (5,8). Direct injection of a dye into the bowel may result in its diffusion and spilling into the peritoneal cavity and cause fat necrosis, focal perforation, or peritonitis (11,12). Therefore, development of a new surgical marker and an imaging method is desirable.

Fluorescence dyes with absorption and emission peaks in the near-infrared (NIR) wavelength range (650–900 nm) have been considered to serve as excellent agents to mark tumor sites (13). NIR fluorescence imaging allows detection of fluorescence signals in deep tissues by minimizing autofluorescence in the NIR wavelength range (14,15). Moreover, fluorescence signals may be sensitively detected using only a small amount of dye (nanomolar concentrations) *in vivo* (16). Indocyanine green (ICG), an FDA-approved NIR dye, is widely used as an imaging agent in medical diagnosis. However, its applications as a surgical marker are limited owing to the low fluorescence intensity resulting from the concentration-dependent coagulation, instability in aqueous solutions, and rapid diffusion at the site of injection (17).

Herein, we propose an injectable alginate hydrogel containing an ICG-human serum albumin (HSA) complex as a new surgical marker for minimally invasive surgery (*Figure 1*). Alginate is a polysaccharide resembling glycosaminoglycan as an extracellular matrix. It is a natural polymer extracted from brown algae; the excellent biocompatibility and hydrophilic characteristic have extended the use of alginate for biomedical applications, including soft tissue engineering and biomolecular delivery (18). As alginate is a polyanionic polymer (19), it can easily form a gel through ionic cross linking with divalent ions (e.g., Ca^{2+}) (18,20), while it is not cleaved in mammals owing to the lack of suitable enzymes (21,22). In addition, the gelling time may be modulated by adjusting concentrations and types of ions and additives added to alginate solution (19).

We expected the following improvements through this system. First, as ICG exhibits high affinity for proteins (23), it may easily bind to HSA and show higher fluorescence efficiency and stability than free ICG. Second, as a scaffold for the ICG-HSA complex, alginate hydrogel would prevent the rapid diffusion of the injected dye and prolong the fluorescence detection time.

Methods

Materials

Sodium alginate was obtained from KIMICA Incorporation (Tokyo, Japan, viscosity 500–600 mPa·s). Calcium carbonate (CaCO_3), D-gluconic acid (GA) solution, HSA, and ICG were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dialysis membrane (MW cut-off: 50 kDa) was supplied by Spectrum Laboratories (Laguna Hills, CA, USA).

Determination of optimal conditions for ICG-HSA complex formation

The optimal concentration of ICG-HSA complex was determined. ICG (1.5 mg) was dissolved in 3 mL deionized (DI) water to obtain a concentrated ICG solution. To prepare ICG-HSA complex, 1 mL of concentrated ICG solution was added to 42.89 mg HSA at a 1:1 (ICG:HSA) molar ratio. The concentrated ICG-HSA solution was stirred at 25 °C for 1 h for stabilization. The ICG-HSA solution was then diluted with DI water to 2–100 μM range. Fluorescence spectra of ICG-HSA solutions ($\lambda_{\text{ex.}} = 720 \text{ nm}$, $\lambda_{\text{em.}} = 740\text{--}850$) were measured with a multifunctional

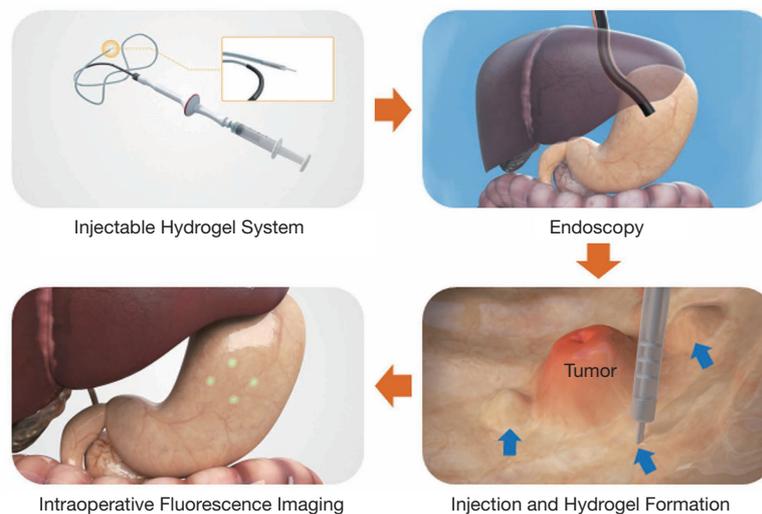


Figure 1 Schematic illustration of marking surgical sites with an ICG-HSA-loaded alginate hydrogel and subsequent fluorescence detection of the marking sites in real-time.

Table 1 The compositions of alginate hydrogels in a dual syringe

Sample	#1	#2	#3
Solution A			
Alginate (wt/v%)	1.0	1.0	1.0
CaCO ₃	0.2x	0.4x	0.6x
Solution B			
Alginate (wt/v%)	1.0	1.0	1.0
D-Gluconic acid	0.4x	0.8x	1.2x

x, Number of moles of carboxylic acid in 1.0 wt/v% alginate solution.

microplate reader (Safire 2, Tecan, Männedorf, Switzerland).

The optimal ratio of ICG versus HSA to obtain maximum fluorescence intensity was evaluated. ICG concentration in DI water was fixed to 30 μM , and ICG was mixed with various molar ratios of HSA. The fluorescence spectra of the solution ($\lambda_{\text{ex.}} = 720 \text{ nm}$, $\lambda_{\text{em.}} = 740\text{--}850$) were obtained and compared.

Analysis of gelation time for alginate hydrogels containing CaCO₃-GA

Sodium alginate was dissolved in DI water, dialyzed for 2 days (MWCO =50 kDa), and then freeze dried.

Sodium alginate in DI was mixed with different amounts of CaCO₃ to obtain solution A (see Table 1 for the

concentration of sodium alginate and CaCO₃). Solution B was prepared by mixing sodium alginate solution with different concentrations of GA solution. The molar ratio of GA to CaCO₃ was adjusted to 2 for the neutralization of the pH of the final solution (22). Solutions A (0.35 mL) and B (0.35 mL) were separately charged into a dual syringe and simultaneously injected into a 5 mL glass vial to prepare an in situ gel. Sol-gel transition time of alginate/Ca²⁺-GA composite solutions was determined by the test-tube inverting method (24) by inverting the tube every 1 min after injection. The solution state was determined by flow (sol) and non-flow state (gel).

In vivo evaluation of alginate hydrogels in a mouse model

The potential utility of ICG-HSA-loaded alginate hydrogel

as a surgical marker was evaluated in a mouse model. ICG, ICG-HSA solution, and ICG-HSA-loaded alginate/ Ca^{2+} -GA composite system (alginate hydrogel) were subcutaneously injected into male nude mice (6 weeks, $n=3$ per group). The final concentrations of sodium alginate, ICG-HSA, CaCO_3 , and GA in the injected gel were 1 wt/v %, 30 μM , 0.3 \times , and 0.6 \times , respectively. The injection volume of the sample solution was 100 μL . NIR fluorescence images ($\lambda_{\text{ex.}}=780$ nm, $\lambda_{\text{em.}}=845$ nm) of mice were obtained using the IVIS Lumina XR system (Xenogen Corp., Alameda, CA, USA) at different time points for 8 days. The animal experiment protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Cancer Center (approval No. NCC-18-451) of Korea.

For quantitative analysis of fluorescence intensities in the region of interest (ROI), target-to-background ratio (TBR) and area of ICG fluorescence were determined using an imaging software. TBR was determined as follows: $\text{TBR} = \text{ROI (target)}/\text{ROI (background)}$ (25).

In vivo evaluation of alginate hydrogels in a porcine model

A healthy male landrace pig (45 kg) was obtained from Cronex Co., Ltd. (Hwaseong, Korea). All experiments were conducted in KNOTUS Co., Ltd (Incheon, Korea) and approved by the IACUC of KNOTUS (approval No. 19-KE-358).

The pig was fed with a semifluid diet for 48 h prior to experiment inception and fed with a polyethylene glycol solution on the day prior to endoscopy. General anesthesia was administered with an injection of Zoletil 50 (VIRBAC, France, 5 mg/kg) and xylazine (Rompun[®], Bayer AG, Germany, 2.5 mg/kg). The pig was intubated and anesthesia was maintained with 1–2% isoflurane added to the oxygen gas by a ventilator. The pig was administered a 50-cc saline enema through the anus until all residual fecal material was evacuated. An endoscope with an attached cap was advanced into the stomach of the pig.

ICG-HSA-loaded alginate solution was then injected into three sites (1 mL solution per site) of the submucosal space in the porcine stomach via catheter (23G needle Clear-Jet injector, Finemedix, Daegu, Korea). To mark injection sites, hemostatic clips were clipped right beside the injection sites. After 3 days of hydrogel injection, a fluorescent laparoscopic system was installed on the abdomen of the pig, and the fluorescence signal generated from the fluorescence surgical marker located inside the stomach was evaluated in real time using a fluorescence laparoscope system. The pig

was euthanized, and the stomach was dissected to verify that the position identified as the gel injection site by the fluorescence image was accurate (26).

NIR fluorescence laparoscopic system

Fluorescence light source is composed of white and infrared light (Figure 2). White light has a color temperature of 5,500 K at a maximum output of 2,600 lm and an infrared wavelength of 785 nm at a maximum output of 3.5 W. Two light sources are mixed with a 5 mm diameter fiber bundle and irradiated onto the image target using a laparoscope ($\lambda_{\text{ex.}}=785$ nm, $\lambda_{\text{em.}}=805$ nm). Laparoscopes are designed to target images 50 mm apart with a 10 mm diameter and 70° field of view.

The eyepiece of the laparoscope was attached to a camera head to simultaneously acquire color and fluorescence images. The camera head comprised a coupler lens and an image-accepting sensor. The coupler lens clarifies the focus of the image targeted by the laparoscope and delivers the image to the sensor. The image-accepting sensor passes the data to an imaging system for image construction.

Results

Optimal condition for ICG-HSA complex formation

To determine the optimal concentration of ICG-HSA, fluorescence spectra were obtained after mixing ICG and HSA at different concentrations and mole ratios. The ICG-HSA complex (1:1 mole ratio) showed a continuous increase in the fluorescence intensity in the concentration range of 2–30 μM (Figure 3A,B). The maximum fluorescence intensity remained unchanged above 30 μM . Further, the change in the fluorescence intensity of ICG-HSA complex was analyzed by varying the mole ratio of HSA to ICG (from 0 to 2; Figure 3C,D). As a result, the fluorescence intensity of the complex solution increased as the amount of HSA in the ICG-HSA complex increased. In particular, the fluorescence signal increased until the two substances were combined at a one-to-one ratio, while only a little change was observed with any further increase in HSA. Therefore, we selected the one-to-one combination of ICG-HSA in subsequent experiments.

Gelation time of alginate hydrogels containing CaCO_3 -GA

To prepare alginate hydrogel with an adequate gelation time

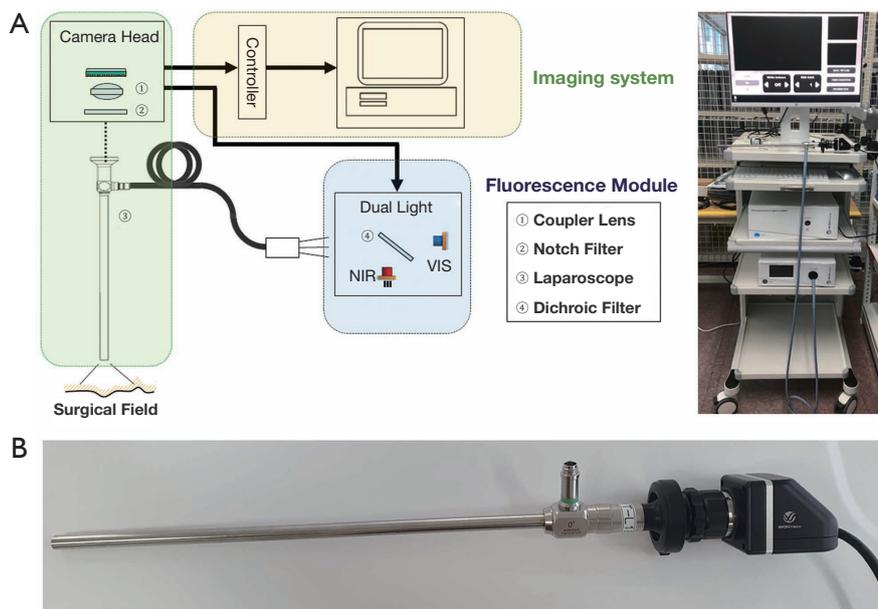


Figure 2 Real-time NIR fluorescence laparoscopic system. (A) Schematic diagram and the assembled system for laparoscopic fluorescence imaging; (B) the constructed laparoscope with coupling lens and camera head.

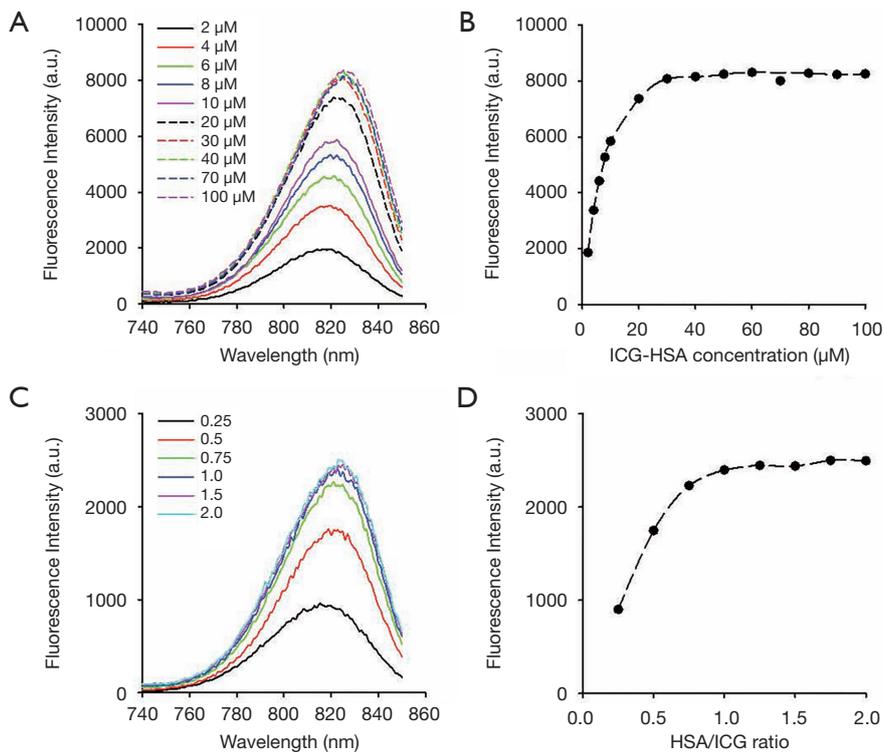


Figure 3 *In vitro* fluorescence data. (A) Fluorescence spectra of ICG-HSA complexes ($\lambda_{ex.} = 720$ nm) at various concentrations; (B) peak fluorescence intensity (molar ratio of ICG:HSA = 1:1; $\lambda_{ex.} = 720$ nm, $\lambda_{em.} = 823$ nm) versus ICG-HSA concentration; (C) fluorescence spectra ($\lambda_{ex.} = 720$ nm) of ICG-HSA complexes at different HSA/ICG ratios; (D) peak fluorescence intensity (ICG = 30 μM; $\lambda_{ex.} = 720$ nm, $\lambda_{em.} = 823$ nm) versus HSA/ICG molar ratio. ICG, indocyanine green; HSA, human serum albumin.

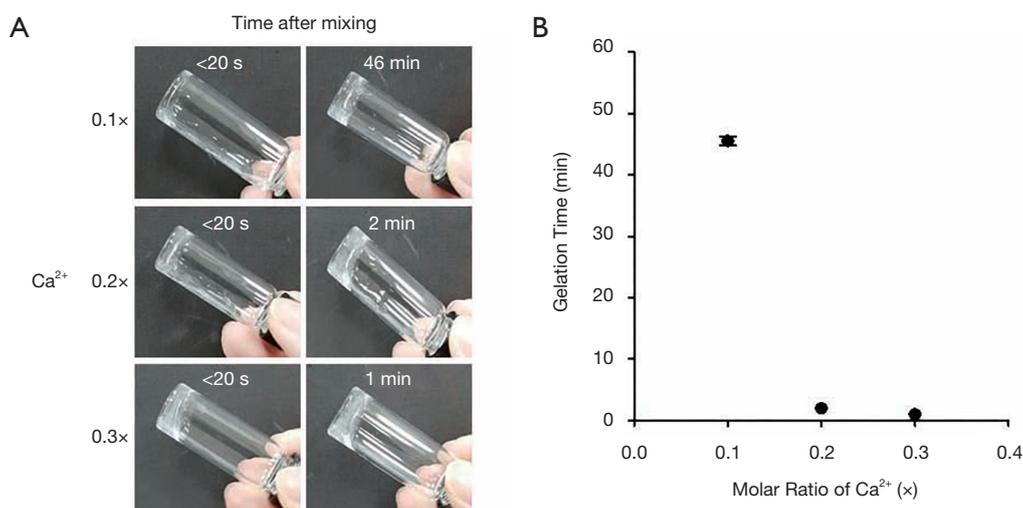


Figure 4 Effect of calcium ions on gelation time. (A) Images of gels formed at different time points after injection with a dual syringe; (B) gelation time of alginate hydrogels as a function of Ca²⁺ concentration (x indicates the mole number of carboxylic acids in 1.0 wt/v% alginate solution).

as a surgical marker, different concentrations of calcium ions were mixed with the alginate solution using a dual syringe (Table 1). Gelation time of the alginate solution was evaluated.

As shown in Figure 4A, the alginate solution containing 0.1x calcium ion remained in its liquid state (sol) for about 46 min after injection before forming a solid gel. Under 0.2x calcium concentration, the solution was in a slightly liquid state (sol) immediately after injection and changed to the solid gel state after about 2 min. The solution exposed to 0.3x calcium concentration formed a weak gel immediately after injection and changed to a solid gel state after about 1 min. The gelation time is shown in Figure 4B according to the final concentration of calcium ions in the injected gel. The gelation time of alginate was shortened as the concentration of calcium ion increased from 0.1x to 0.3x. In particular, the alginate solution treated with 0.3x calcium existed in a solid state (gel) immediately after injection. Therefore, 0.3x calcium concentration was selected for further experiments.

In vivo evaluation of alginate hydrogels in a mouse model

We next evaluated the ICG-HSA-loaded alginate hydrogel as a surgical marker *in vivo*. The aqueous solutions of ICG, ICG-HSA, ICG-HSA-loaded alginate were subcutaneously injected into nude mice using a dual

syringe (Figure 5A). Then, NIR fluorescence images and photographs of the mice were periodically obtained (Figure 5B,C). As a result, the fluorescence intensities at ICG and ICG-HSA injection sites rapidly decreased with time, and most of the fluorescence signals disappeared after 24 h from injection. Quantitative analyses of the fluorescence intensity and area of fluorescence emission with time (Figure 6) showed that most of the injected ICG and ICG-HSA complex rapidly diffused into the surrounding tissues and disappeared from the injection sites within 24 h. Only 16% and 12% fluorescence signals were retained at ICG and ICG-HSA injection sites, respectively, relative to the signal detected at the start. In contrast, the mice receiving alginate hydrogel retained 61% fluorescence signal 48 h post-injection, while significant NIR fluorescence persisted at the injection sites for about 4 days. The area of fluorescence emission was maintained at low values for several days, indicating that the alginate hydrogel effectively prevented the diffusion of the ICG-HSA complex from the injection site.

Figure 5C shows the images of the injection site of ICG, ICG-HSA solution, and alginate hydrogel in nude mice. In particular, the injection sites for ICG and ICG-HSA solutions appeared slightly swollen immediately after injection, and the solutions spread and became invisible after about 24 h. On the other hand, protrusions formed immediately after injection in the alginate hydrogel-treated mice, and were retained for 5 days.

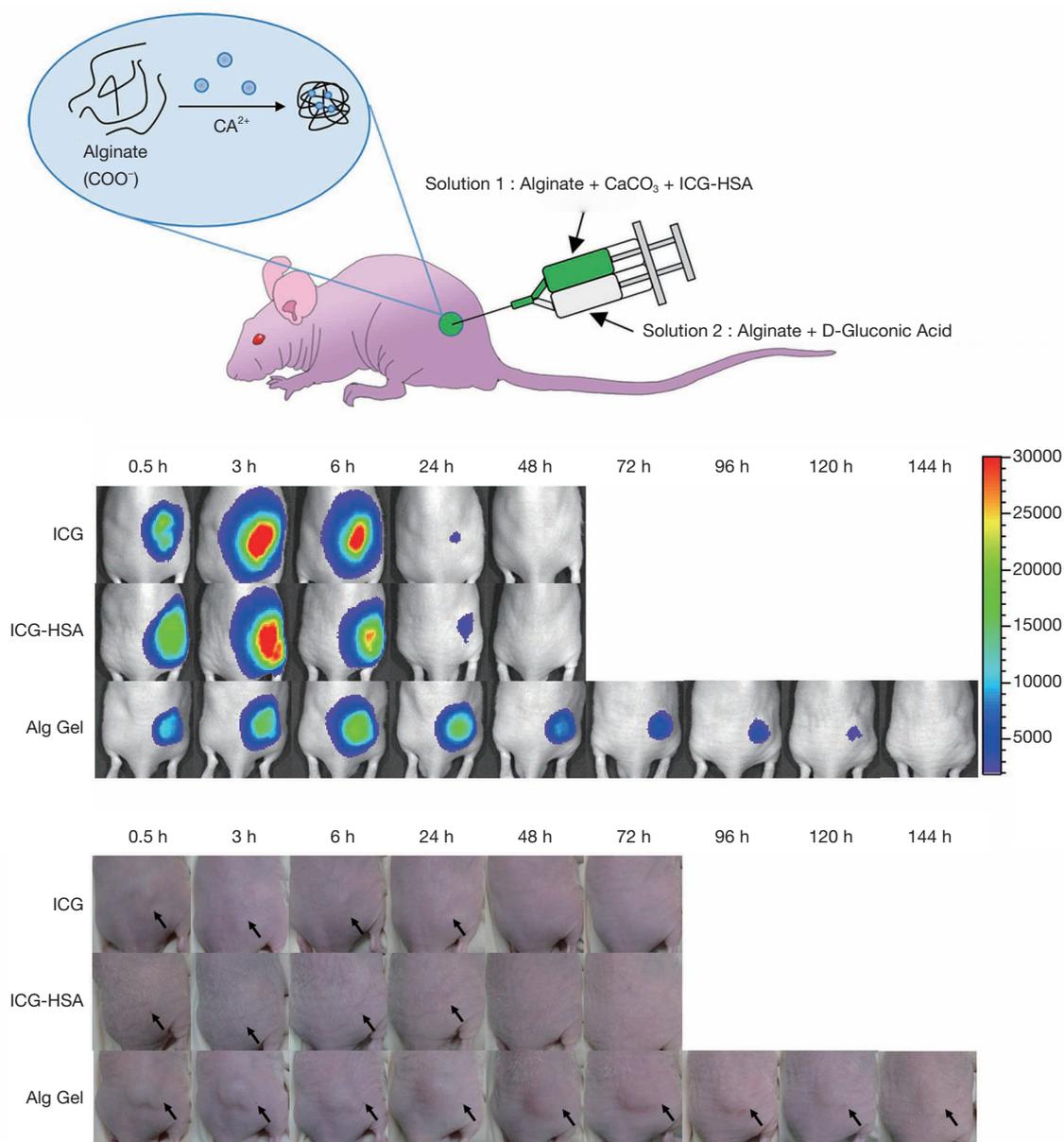


Figure 5 *In vivo* evaluation of ICG-HSA alginate hydrogel in a mouse model. (A) Scheme of the subcutaneous injection of ICG-HSA-loaded alginate solution into a nude mouse using a dual syringe; (B) NIR fluorescence images of mice obtained using the IVIS Lumina XR system ($\lambda_{ex} = 780$ nm, $\lambda_{em} = 845$ nm) and (C) images of injection sites in nude mice at defined time intervals. Arrows indicate the injection sites of ICG, ICG-HSA, and ICG-HSA-loaded alginate hydrogel (Alg Gel) in mice. ICG, indocyanine green; HSA, human serum albumin; NIR, near-infrared.

In vivo evaluation of alginate hydrogels in a porcine model

The potential application of ICG-HSA-loaded alginate hydrogel as a surgical marker was evaluated in a porcine model. The solution was injected into the submucosal space

of the porcine stomach using an injector. Hemostatic clips were clipped right beside the injection sites. After 3 days from injection, NIR fluorescence imaging was performed for the injected sites using a fluorescence laparoscopic system (Figure 7A). As a result, the location and margins

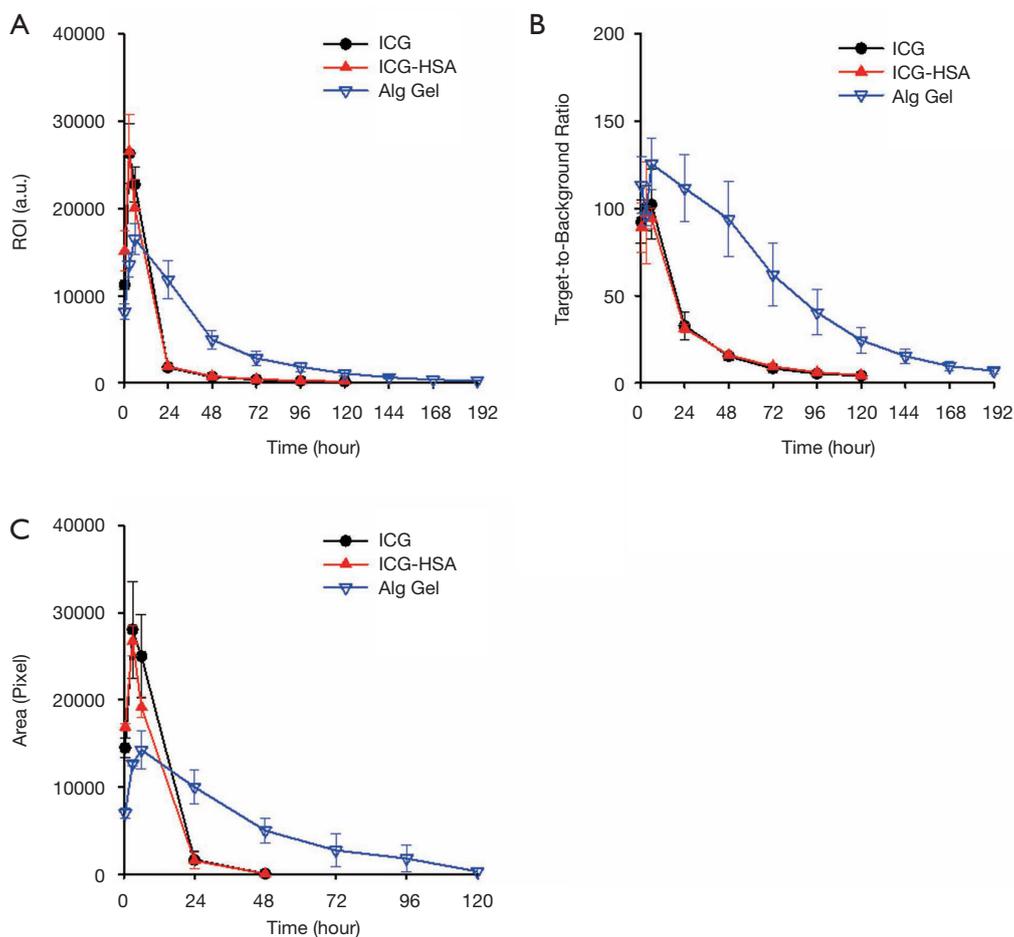


Figure 6 Quantitative analyses of NIR fluorescence images. (A) Region of interest, (B) target-to-background ratio, and (C) area of fluorescence emission in nude mice at defined time intervals. NIR, near-infrared.

of injection sites could be clearly distinguished in real time using NIR fluorescence images (Figure 7B).

To ensure that the fluorescence signal was emitted from the injected hydrogel, porcine stomach was opened and the fluorescence was evaluated *ex vivo* (Figure 7C). Strong fluorescence signal was detected right beside the hemostatic clip, confirming that the NIR fluorescence signal detected using fluorescence laparoscopic system was derived from the injection sites of ICG-HSA-loaded alginate hydrogels.

Discussion

The incidence of early gastrointestinal cancers has been increasing, resulting in the increase in popularity of the use of the laparoscopic system for related surgeries. However, real-time detection of small lesions is difficult due to the

lack of tactile sensation using the laparoscopic system. Therefore, ICG-HSA-loaded alginate hydrogels were developed as a novel surgical marker for the non-invasive and real-time detection of cancer sites, enabling precision cancer surgery.

As depicted in Figure 5B, the ICG-HSA complex initially emits strong fluorescence signals at the injection site; however, the signals rapidly decrease within 24 h due to dye diffusion from the injection site to the surrounding tissues. Alginate was mixed with both ICG-HSA and an ion crosslinker (e.g., Ca^{2+}) to form a hydrogel, successfully inhibiting the diffusion of the ICG dyes from the injection site. Because the solution is injected through a catheter, it is essential to form a gel after injection to prevent jamming. Calcium chloride (CaCl_2) has been widely used for the preparation of hydrogels; however, gel formation after

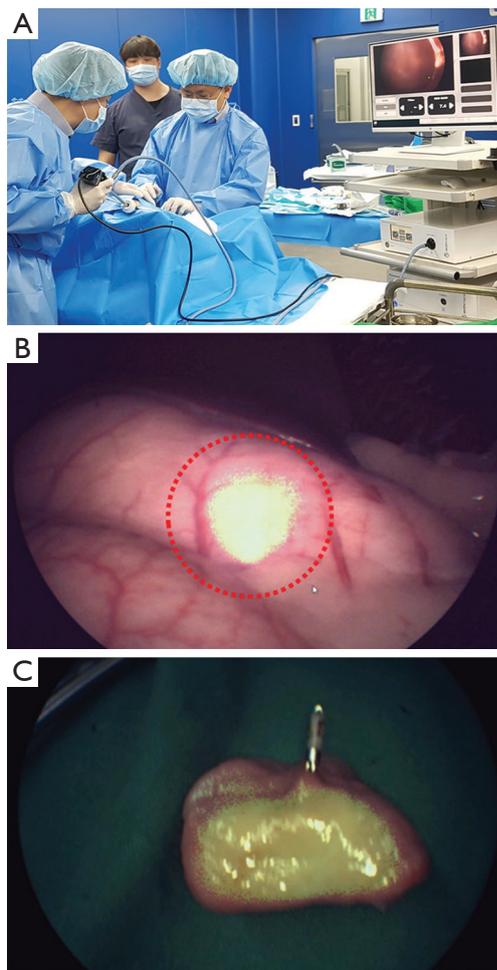


Figure 7 *In vivo* evaluation of ICG-HSA-loaded alginate hydrogels in a porcine model. (A) Image of the intraoperative identification of the injection site using laparoscopic fluorescence imaging system; (B) *in vivo* NIR fluorescence image obtained with fluorescence laparoscopy; (C) *ex vivo* NIR fluorescence image of the stomach tissue 3 days after injection. Clip is located right beside the injection site. ICG, indocyanine green; HSA, human serum albumin; NIR, near-infrared.

CaCl₂ addition is excessively rapid. It is also difficult to form a uniform gel and the injector line gets easily clogged. Therefore, calcium carbonate (CaCO₃) was used in this study as a source of calcium ions to delay the gelation time (21). GA in solution B was added to facilitate CaCO₃ dissolution at the injection site.

In vivo animal studies in mouse and porcine models confirmed that dye diffusion from the injection sites could be effectively prevented using the ICG-HSA-loaded

alginate hydrogel. Thus, strong fluorescence at the injection sites was maintained for more than 3 days, while the fluorescence emission area remained narrow.

In conclusion, here we demonstrate the application of ICG-HSA-loaded alginate hydrogel as a new surgical marker for noninvasive and real-time identification of tumor sites. *In vivo* animal studies in mouse and porcine models revealed promising results of this hydrogel system for preivision surgery of gastrointestinal tumors. This hydrogel system is expected to mark lesions using various contrast agents and serve as a drug delivery system following incorporation of suitable therapeutics.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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