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Evaluation of Injectable Chitosan-based Co-cross-linking Hydrogel for Local Delivery of ^{188}Re-LIPO-DOX to Breast-tumor-bearing Mouse Model

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Abstract. *Background/Aim: An injectable chitosan-based co-cross-linking thermosensitive hydrogel combining ^{188}Re- and doxorubicin-encapsulated liposomes (C/GP/GE/^{188}Re-LIPO-DOX) was developed for the prevention of locoregional recurrence after mastectomy. Materials and Methods: The hydrogel properties, in vitro drug release characteristics, and in vivo scintigraphy imaging attributes were investigated. Results: The gelation time of the hydrogels can be controlled to be within 5 min. Results from Fourier-transform infrared spectroscopy, scanning electron microscopy, and dynamic mechanical analysis showed that a covalent cross-linking reaction between chitosan and genipin occurred and that the hydrogel's mechanical strength and chemical stability were improved. In vitro drug release studies showed that the hydrogel can prolong the release of doxorubicin by several weeks (51.5%±5.3% at 21 days). In addition, in vivo scintigraphy results suggested high retention rates (43.1%±1.0% at 48 h) of the radiopharmaceutical compound at the tumor injection site. Conclusion: The preliminary results indicated that the C/GP/GE/^{188}Re-LIPO-DOX radiopharmaceutical hydrogel is a potential candidate for further in vivo therapeutic evaluation.*

Breast cancer is the most common cancer and the second leading cause of cancer-related death for women in the United States (1). The standard treatment method is mastectomy followed by adjuvant chemotherapy or radiotherapy. However, locoregional recurrence (LRR) and metastasis after mastectomy remain a challenging clinical problem resulting in poor prognosis for breast cancer recovery (2). LRR must be avoided in the early stages for patients who have undergone breast-conserving therapy (3). Although several treatments for breast cancer are available clinically, none of them completely eradicates breast cancer to date. Therefore, novel treatment strategies that can efficiently prevent LRR after mastectomy are needed to reduce breast cancer mortality rates.

Injectable chitosan-based hydrogels have been widely used as drug delivery systems for biomedical applications and have been reviewed in the literature (4-8). An injectable chitosan-based thermosensitive hydrogel for biomedical applications, chitosan/β-glycerophosphate (C/GP), was first proposed by Chenite *et al*. and had been claimed in US patent 6344488 (9, 10). Chitosan, a natural amino-polysaccharide derived from the deacetylation of chitin, is obtained from the exoskeleton of shrimp or crab shells (11). C/GP is biodegradable, nontoxic, and biocompatible within the human body (10, 12). Its physicochemical characteristics and behavior in drug delivery have also been well investigated (13, 14). Currently, it is one of the most widely used hydrogel systems for drug delivery applications and tissue engineering (6, 15-17).

C/GP systems are usually designed for easy delivery into a lesion site *via* various pathways followed by the local delivery of various therapeutic agents (4). Once injected into the body, the system undergoes a sol-gel transition in response to environmental temperature changes and rapidly forms a gel *in situ* at the injection site. The major benefit of

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this technique is that it can sustainably deliver high amounts of cytotoxic agents to the lesion site but minimize the side effects to healthy tissue or organs. Subsequently, the formation of a highly porous three-dimensional scaffold with a highwater content transforms it into a powerful drug reservoir for the sustained release of drug molecules. Some disadvantages of C/GP systems include low mechanical integrity and a high degradation rate. Their application and development are limited by weak ionic cross-linking (physical cross-linking) (4, 18). In 2011, Moura *et al.* proposed a novel chitosan-based co-cross-linking (ionic/covalent) hydrogel formed *in situ* obtained by introducing a natural "genipin (GE)" cross-linker (18) into the conventional C/GP system. The novel system (C/GP/GE) not only retained its original injectable and thermosensitive properties but also exhibited significantly increased mechanical strength and chemical stability (19). Furthermore, the *in vivo* inflammatory response of C/GP/GE hydrogels has been evaluated, and they demonstrated similar biodegradation and biocompatibility to C/GP hydrogels (20). Additionally, C/GP/GE hydrogels have been studied for local drug delivery and tissue engineering (21, 22).

^{186}Re/^{188}Re-BMEDA-liposome, a nanoparticle-based radiopharmaceutical compound, was first proposed by Bao *et al.* (23) and its applications for the treatment of various cancers have been reviewed in the literature (24-26). ^{186}Re and ^{188}Re have physical half-life values of 90 and 17 h, respectively. They are theranostic isotopes that simultaneously emit γ-radiation (E_γ=137 and 155 keV, respectively) and β-radiation ($E_{\beta max}$=1.07 and 2.12 MeV, respectively) (27). Liposomes are small vesicles with a phospholipid structure similar to cell membranes and have been used for fundamental research as well as potent clinical pharmaceutical agents since their discovery by Bangham in the 1960s (28). Liposomes have excellent ability to encapsulate a wide variety of anticancer drugs including hydrophilic and lipophilic molecules. The ^{186}Re/^{188}Re-liposome formulation was designed not only to encapsulate theranostic radionuclides such as ^{186}Re and ^{188}Re but also to load chemotherapeutic molecules such as doxorubicin (DOX) in their core for the treatment of cancers (29-32). Therefore, it constitutes a promising means of improving the therapeutic efficacy of cancer treatments. Recently, ^{186}Re/^{188}Re-liposomes have been investigated for local drug delivery for the treatment of cancers where surgery is difficult or impossible, such as head and neck squamous cell carcinoma (33, 34), glioblastoma multiforme (35), and hepatocellular carcinoma (36). They have achieved outstanding results in the inhibition of tumor growth.

C/GP thermosensitive hydrogels containing a ^{188}Re-Tin colloid radiopharmaceutical agent with or without DOX (C/GP/DOX/^{188}Re-Tin colloid) have been studied for local drug delivery in cancer treatment (36-38). The results showed that the hydrogel not only achieves the sustained release of the chemotherapeutic drug but also confines the radiopharmaceutical agent at the injection site without appreciable diffusion up to 48 h after the injection. This significantly inhibited tumor growth compared with a control group. In addition, C/GP systems have been studied for the sustained release of paclitaxel at tumor sites to prevent LRR. The results showed that intratumoral injection of C/GP/paclitaxel had therapeutic efficacy for the inhibition of tumor growth similar to that of the intravenous injection of Taxol®, but was less toxic (39). However, the low mechanical strength of C/GP hydrogels and the poor ability to deliver hydrophilic chemotherapeutic drugs limits its efficacy in cancer treatment (18, 40).

In the present work, an injectable chitosan-based co-cross-linking C/GP/GE hydrogel was prepared and combined with a nanoparticle-based ^{188}Re-LIPO-DOX radiopharmaceutical agent to produce a novel injectable hydrogel radiopharmaceutical compound for local delivery to breast tumors in mice. The physicochemical characteristics and morphology of the C/GP/GE hydrogel were studied. The *in vitro* controlled drug release and *in vivo* scintigraphy properties of the hydrogel were also investigated. The novel injectable hydrogel radiopharmaceutical compound could achieve maximum tumor inhibition by localizing the radiopharmaceutical agent around the lesion site and prolonging the release of the chemotherapeutic drug. The strategy proposed here focuses on the adjuvant treatment of breast cancer at the reception site and is expected to inhibit LRR after mastectomy and decrease the mortality rate from breast cancer in the near future.

Materials and methods

Materials. Medical-grade chitosan (Ultrasan®) with a molecular weight (MW) of 292,000 g/mol and 91% degree of deacetylation was supplied by BioSyntech, Inc. (Quebec, Canada). GP (MW: 306 g/mol) was obtained from Fluka through UNI-ONWARD Corp. (New Taipei, Taiwan). GE (MW: 226 g/mol) was obtained from Challenge Bioproducts Co., Ltd. (Yunlin, Taiwan). *N,N*-bis(2-mercaptoethyl)-*N',N'*-diethylethylenediamine (BMEDA) was procured from ABX (Radeberg, Germany). The LIPO-DOX® anticancer drug (DOX concentration: 2 mg/ml; approximately 100 nm) was a gift from Taiwan Liposome Company, Ltd. (Taipei, Taiwan). The PD-10 column was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Dialysis tubes (Spectra/Por®) with a 10,000 Da cutoff were obtained from Merck Taiwan (Taipei, Taiwan). The isotope source of the carrier-free ^{188}Re-perrhenate solution ($Na^{188}ReO_4$; 185-740 MBq) was prepared in house using an alumina-based ^{188}W/^{188}Re generator from the Isotope Application Division of the Institute of Nuclear Energy Research (INER, Longtan, Taiwan), while the original ^{188}W radionuclide was supplied by Oak Ridge National Laboratory (Oak Ridge, TN, US). All other chemicals and reagents were obtained from Merck Taiwan (Taipei, Taiwan) and used as received.

Preparation of ^{188}Re-liposome and ^{188}Re-LIPO-DOX. A neutral PEGylated nanoliposome (approximately 100 nm) was prepared using the lipid film hydration-extrusion method with repeated freezing-

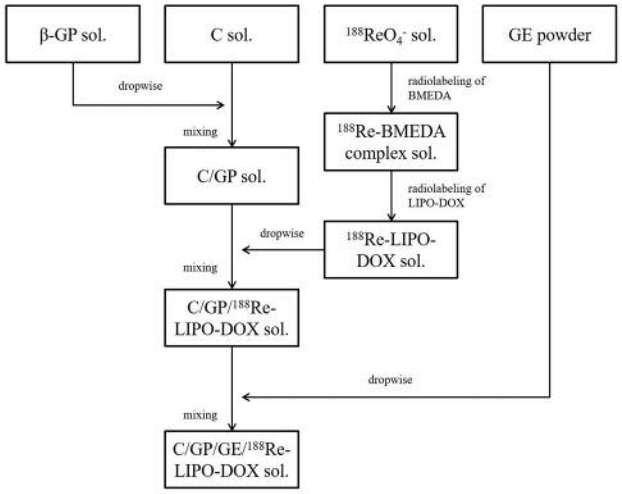

Figure 1. *Experimental flowchart for the preparation of the injectable thermogelling C/GP/GE/^{188}Re-LIPO-DOX radiopharmaceutical compound. All procedures were carried out in an ice bath.*

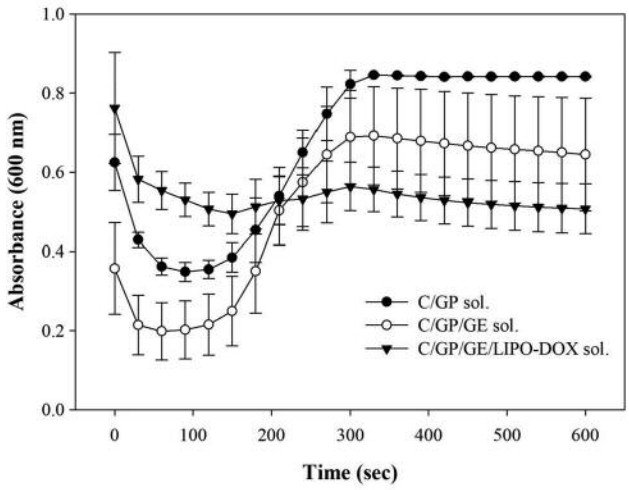

Figure 2. *Absorbance evolution at 37°C for various hydrogel samples during the sol- gel transition. (●) C/GP solution; (○) C/GP/GE; (▼) C/GP/GE/LIPO-DOX (DOX concentration: 2 mg/ml), (mean±SD, n=3).*

thawing cycles as described in previous reports (41-43). Radiolabeling of ^{188}Re-liposome or ^{188}Re-LIPO-DOX was performed in accordance with previously published protocols (30, 43) with some slight modifications. Briefly, a preformed ^{188}Re-BMEDA complex (activity of ^{188}Re: 185-740 MBq/ml) and a liposome or LIPO-DOX solution (DOX concentration: 2-6 mg/ml) were mixed in a vial (1 ml:1 ml). Then, the mixture was incubated in a 60˚C water bath with shaking at 80 rpm for 30 min. The ^{188}Re-liposome or ^{188}Re-LIPO-DOX was purified from free $Na^{188}ReO_4$ and/or ^{188}Re-BMEDA by using a PD-10 size exclusion column through elution with normal saline buffer. The radiochemical yields were calculated from the radioactivity of the collected ^{188}Re-liposome or ^{188}Re-LIPO-DOX fractions divided by their original total radioactivity.

Preparation of the injectable chitosan-based co-cross-linking hydrogel radiopharmaceutical compound. The injectable chitosan-based co-cross-linking hydrogel (C/GP/GE) was prepared according to our previously published method (36, 37), but with some modifications (19). First, 2% (w/w) chitosan and 45% (w/w) GP were dissolved in 0.1 N acetic acid and deionized water, respectively, and cooled in an ice bath for 15 min. Then, the GP solution was added dropwise into the chitosan solution to obtain the C/GP solution. Subsequently, GE powder was carefully added into the C/GP solution in the ice bath under stirring for 5 min to yield the final C/GP/GE solution (containing 0.036% (w/w) GE). The hydrogel radiopharmaceutical compound was prepared according to the flowchart shown in Figure 1. Briefly, the preformed ^{188}Re-LIPO-DOX solution was added dropwise into the C/GP solution under stirring in an ice bath for 5 min. Then, the GE powder was added under stirring for another 5 min. The final solution of C/GP/GE/^{188}Re-LIPO-DOX showed a ^{188}Re activity of 37 MBq/ml and a DOX concentration of 1 mg/ml. Similarly, the LIPO-DOX solution was substituted for the ^{188}Re-LIPO-DOX solution to prepare C/GP/GE/LIPO-DOX (DOX concentration: 2 mg/ml).

Characteristics of the hydrogels. Gel properties such as gelation time (T_g), cross-linking, morphology, and mechanical strength were investigated. The gelation time was obtained as described previously (36, 44) using the absorbance kinetics of the sol-gel transition. Aliquots of 2 ml of preformed C/GP, C/GP/GE, and C/GP/ GE/LIPO-DOX, were loaded into a transparent cuvette and placed inside a UV/visible spectrophotometer. The time-dependent absorption at 600 nm was recorded every 30 sec for 600 sec at 37˚C. To examine the cross-linking reaction and the morphology, preformed hydrogel solutions, namely C/GP, C/GP/GE, C/GP/LIPO-DOX, and C/GP/GE/^{188}Re-LIPO-DOX, were incubated at 37˚C overnight for the sol-gel transformation to occur. For scanning electron microscopy (SEM) measurements, the gels underwent an additional 6 h of exposure to phosphate-buffered saline (PBS) at 37˚C before freeze drying. Subsequently, the gel samples were carefully frozen in liquid nitrogen and freeze-dried for 48 h. After freeze-drying, the samples were analyzed through Fourier-transform infrared spectroscopy (FTIR) (FTIR-4100 spectrometer, JASCO International Co., Ltd., Tokyo, Japan) and SEM (Hitachi 3000 N, Japan). To study the mechanical strength of the hydrogels, cube-shaped aliquots of 4 cm^3 were prepared and analyzed using a dynamic mechanical analyzer (DMA-2980, TA Instruments, Inc., DE, USA) at 37˚C.

Controlled in vitro drug release. The controlled *in vitro* drug release of DOX from different formulations was investigated using a dialysis tube (MW cutoff: 10,000 g/mol). Aliquots of 0.5 ml of the drug solutions with free DOX (DOX concentration: 2 mg/ml), LIPO-DOX (DOX concentration: 2 mg/ml), and C/GP/GE/LIPO-DOX (DOX concentration: 2 mg/ml) were carefully loaded into the dialysis tube and placed into 500 ml of PBS (0.01 M, pH 7.4) maintained at 37˚C by a kinetic thermostat to release DOX. For each data point at 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 2 days, and until 21 days, aliquots of 5 ml were sampled from the release tank and then replaced with the same volume of PBS. Subsequently, the sampled solutions were

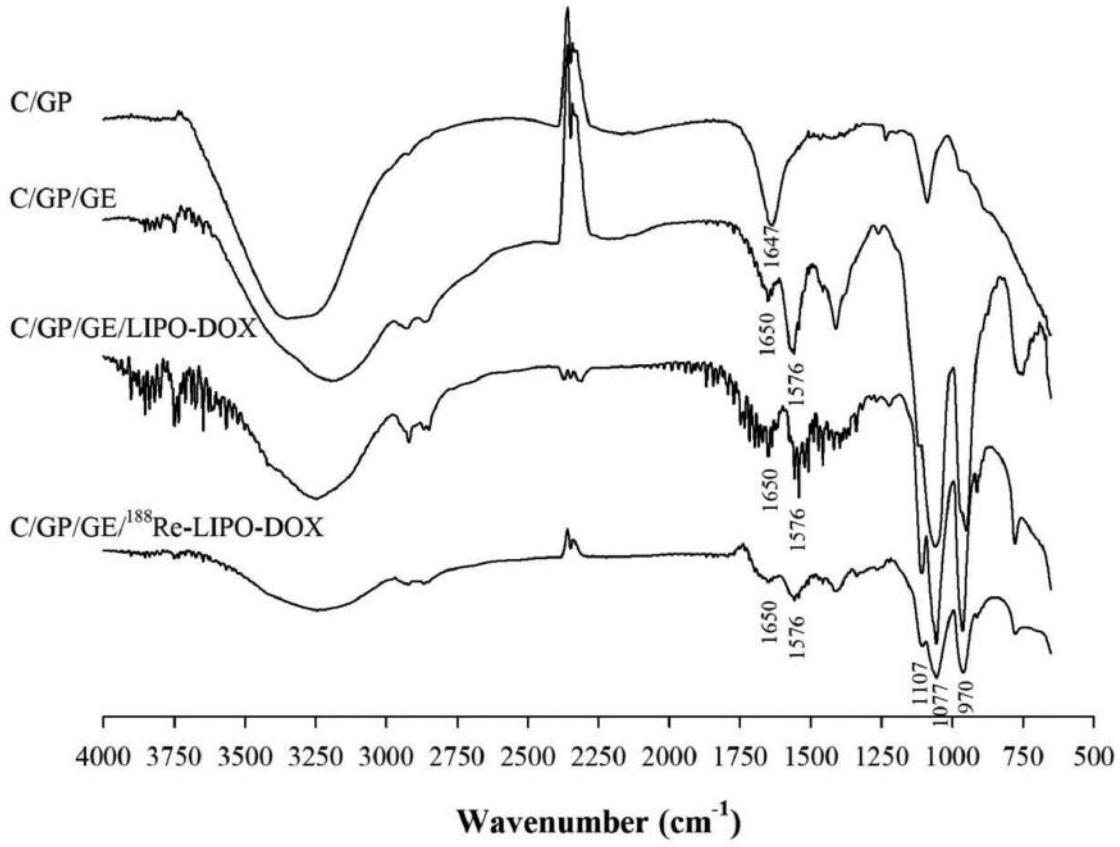

Figure 3. *Time-dependent FTIR spectra for various chitosan-based injectable thermosensitive hydrogels, namely C/GP, C/GP/GE, C/GP/GE/LIPO-DOX, and C/GP/GE/^{188}Re-LIPO-DOX (reacted at 37°C for 1 h for sol-gel transition).*

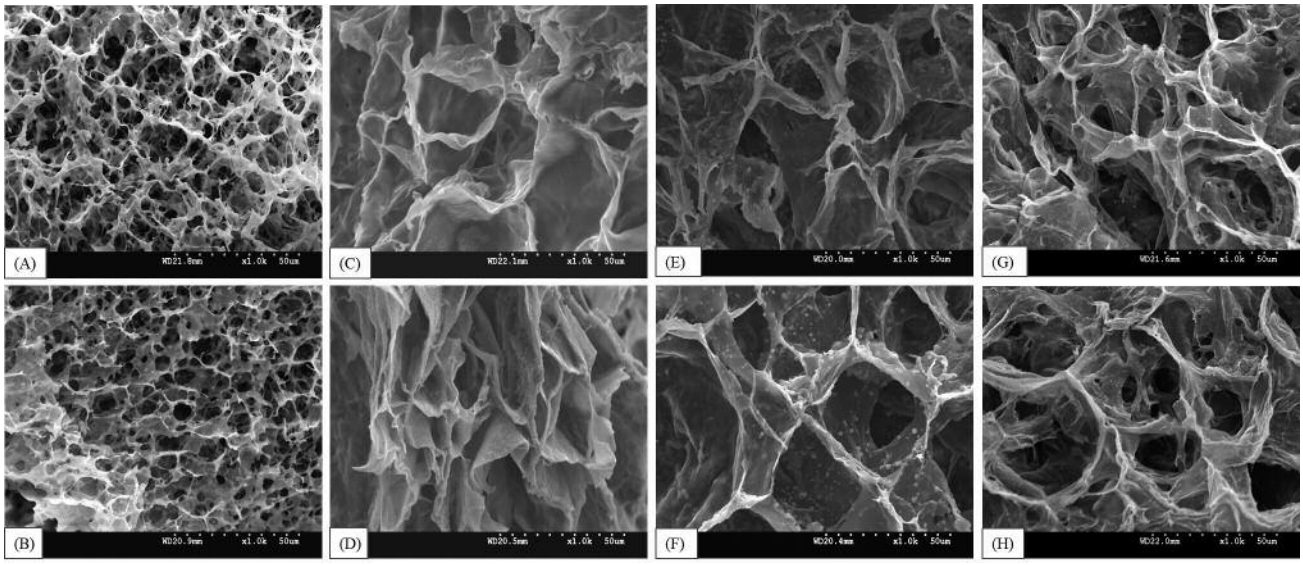

Figure 4. *Scanning electron micrograph of chitosan-based injectable thermosensitive hydrogels at ×1,000 magnification. (A) C/GP surface; (B) C/GP section; (C) C/GP/GE surface; (D) C/GP/GE section; (E) C/GP/GE/LIPO-DOX surface; (F) C/GP/GE/LIPO-DOX section; (G) C/GP/GE/^{188}Re-LIPO-DOX surface; (H) C/GP/GE/^{188}Re-LIPO-DOX section.*

analyzed through fluorometry (Hitachi F-2500, Japan) at 475/580 nm (excitation/emission), and the DOX concentrations from each sample were calculated using a pre-established DOX calibration curve (0.01-5 μg/ml). All experiments involving DOX were performed under light protection to avoid photodegradation.

Cell culture and animal tumor model. The MDA-MB-231 human breast adenocarcinoma cell line was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan), and cells were cultured in 90% Leibovitz L-15 medium with 2 mM L-glutamine, 10% fetal bovine serum, and 100 units/ml penicillin. The cells were incubated at 37˚C in a humidified environment without CO_2.

BALB/C nu/nu athymic (nude) mice (4 weeks old, 18-20 g, female) were obtained from the National Laboratory Animal Center (Taipei, Taiwan), housed in a sterilized environment, and fed sterilized food and water *ad libitum*. To establish a tumor-bearing animal model, the MDA-MB-231 cells were harvested, resuspended in a serum-free medium, and plated on ice before use. Aliquots of the medium with a concentration of 4×10^6 cells/0.1 ml were prepared and inoculated into the nude mice's right hind leg with a 27-gauge needle. After 4 weeks of inoculation, mice with tumor sizes of approximately 100 mm^3 were subjected to scintigraphy studies. All animal experiments were approved by the Institutional Animal Care and Use Committee of Central Taiwan University of Science and Technology.

Scintigraphy studies. A total of nine breast-tumor-bearing mice were randomly divided into three groups (three mice per group). After anesthetization through intraperitoneal injection of a Zoletil® 50 and Balanzine 2% mixture at a 5:2 volume ratio and 1 ml/kg mouse body weight, three groups of mice were administered preformed $^{188}ReO_4^-$ (activity of ^{188}Re: 37 MBq/ml), ^{188}Re-liposome (activity of ^{188}Re: 37 MBq/ml), and C/GP/GE/^{188}Re-LIPO-DOX (activity of ^{188}Re: 37 MBq/ml; DOX concentration: 1 mg/ml), respectively, through intratumoral injection with a 26-gauge needle. Whole-body planar scintigraphy images were acquired 1, 24, and 48 h after injection using a gamma camera (Siemens e.cam). Imaging was performed using a low-energy collimator at 155 keV, a window of 10, a zoom factor of 2, and a preset time of 60 sec. The retention rate of the radiopharmaceutical compound in the injection site was calculated according to a previously published formula (37) by drawing a region of interest (ROI) on the scintigraphy images using specialized software (ICON workstation, Siemens, IL, USA).

Statistical analysis. Data were expressed as mean±standard error of the mean (SEM). The unpaired *t*-test was used for group comparisons. Values of $p<0.05$ were considered significant.

Results

^{188}Re-liposome and ^{188}Re-LIPO-DOX. The size of the PEGylated liposome prepared was 83.4±3.5 nm (n=3), and the surface zeta potential was −1.6±2.1 mV (n=3). The concentration of phospholipids in the PEGylated liposome prepared was 20.13±0.22 μmol/ml (n=3). The radiolabeling yield of ^{188}Re-liposome and ^{188}Re-LIPO-DOX was 76.6% and 71.2%, respectively.

Physicochemical properties and morphology of the hydrogels. Experimental measurements of the gelation time showed that the C/GP, C/GP/GE, and C/GP/GE/LIPO-DOX solutions underwent a sol-gel transition at body temperature with similar T_g values of 5.5, 5, and 5 min, respectively (Figure 2). The results of the present study show no significant change in the thermogelling process induced by the introduction of GE or LIPO-DOX into the C/GP hydrogel system. In addition, all hydrogel solutions can be injected using a 26-gauge needle. Figure 3 shows the time-dependent FTIR spectra for C/GP, C/GP/GE, C/GP/GE/ LIPO-DOX, and C/GP/GE/^{188}Re-LIPO-DOX. Figure 4 shows the SEM micrographs of different chitosan-based hydrogel formulations under ×1,000 magnification. The average pore diameter in the C/GP system (Figure 4 A and B) was in the 5-20 μm range, and similar to that measured in previous investigations of C/GP hydrogels (36, 37). Dynamic mechanical analysis measurements showed that the mechanical strength of the hydrogels was 1.4±0.5, 35.6±15.7, and 12.7±7.6 kPa for C/GP, C/GP/GE, and C/GP/GE/^{188}Re-LIPO-DOX, respectively (n=3). The mechanical strength of the GE-containing hydrogel (C/GP/GE) was 25-fold higher than that of the C/GP hydrogel.

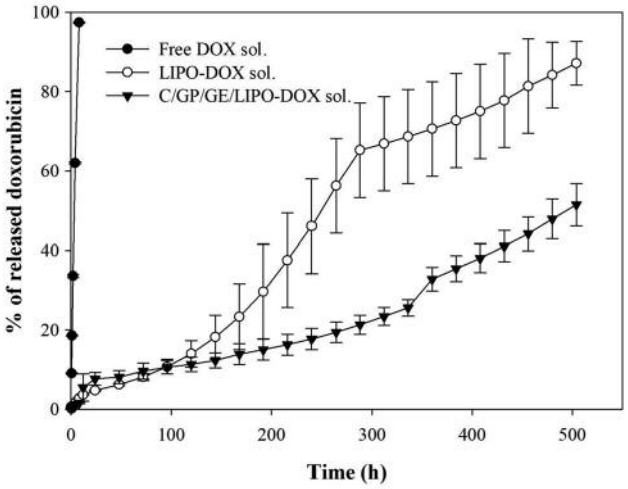

Figure 5. *Controlled in vitro release of DOX from different groups in PBS at 37˚C. (●) Free DOX solution (2 mg/ml); (○) LIPO-DOX solution (2 mg/ml); (▼) C/GP/GE/LIPO-DOX solution (2 mg/ml), (mean±SD, n=3).*

Controlled drug release. *In vitro* release profiles of DOX from the different formulations are shown in the Figure 5. The release of DOX from both LIPO-DOX and C/GP/GE/LIPO-DOX occurred over several weeks, whereas in the control group, free DOX was exhausted within a few hours. The LIPO-DOX group released 65.2%±11.9% and 87.2%±5.5% of the total DOX content within 12 and 21 days, respectively (n=3). Except for an initial burst, the C/GP/GE/LIPO-DOX group released 21.3%±2.4% and 51.5%±5.3% of the total

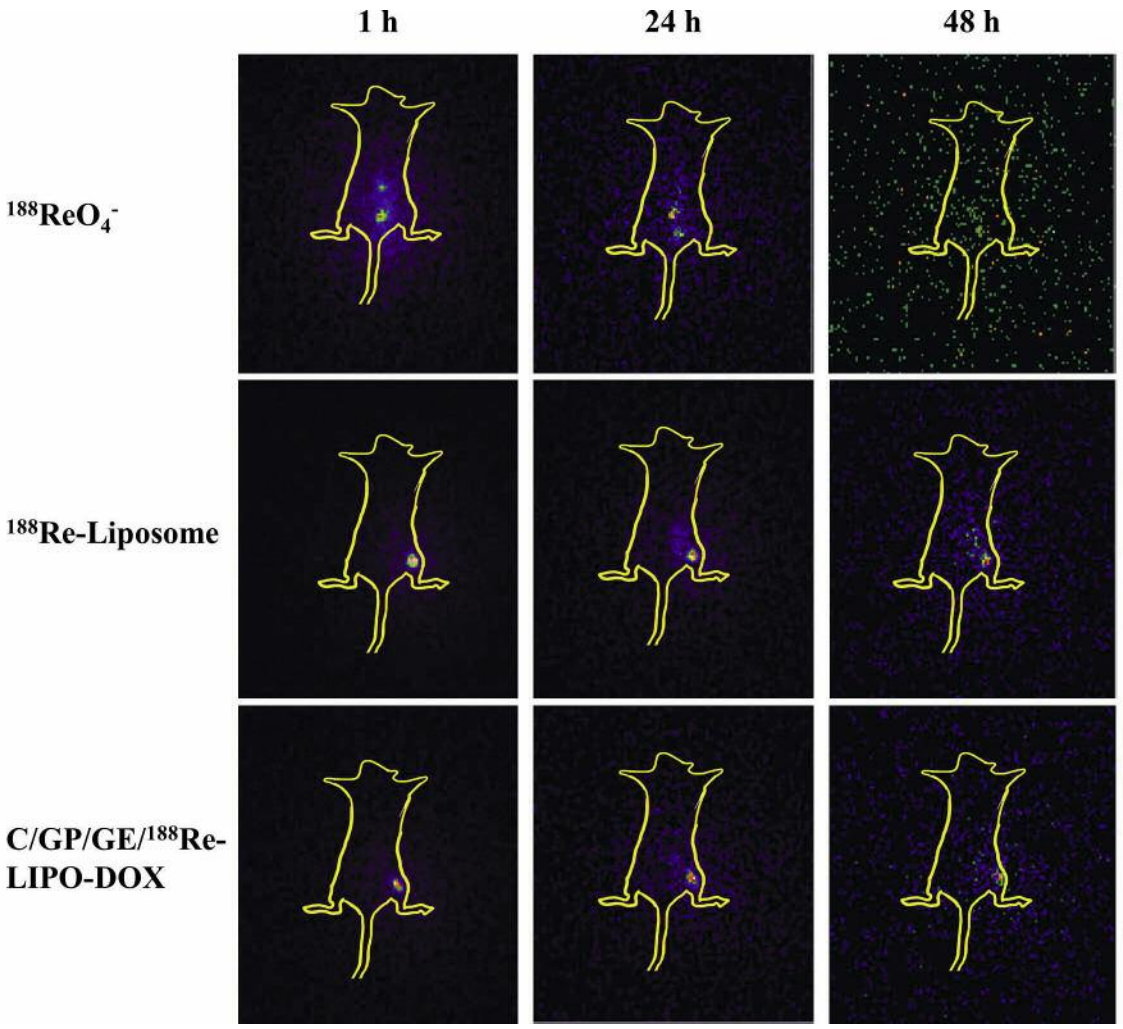

Figure 6. *Planar scintigraphy images 1, 24, and 48 h after intratumoral injection of different groups of radiopharmaceutical agents in breast-tumor-bearing mice. Upper: $^{188}ReO_4^-$ (activity of ^{188}Re: 37 MBq/ml); middle: ^{188}Re-liposome (activity of ^{188}Re: 37 MBq/ml); bottom: C/GP/GE/^{188}Re-LIPO-DOX (activity of ^{188}Re: 37 MBq/ml; DOX concentration: 1 mg/ml), (mean±SD, n=3).*

DOX content within 12 and 21 days, respectively (n=3). Results from the controlled release study revealed that the C/GP/GE/LIPO-DOX hydrogel can significantly prolong the release of hydrophilic DOX molecules for several weeks.

Scintigraphy studies. Figure 6 shows the evolution over time of *in vivo* planar scintigraphy images of breast-tumor-bearing mice after intratumoral injection of various radiopharmaceutical compounds. The images clearly show that the radioactivity in the ^{188}Re-liposome and C/GP/GE/^{188}Re-LIPO-DOX groups was mostly localized within the injection site for up to 48 h after the injection, whereas the radioactivity in the control groups ($^{188}ReO_4^-$) rapidly dispersed from the injection site once the drug was administered. According to the ROI analysis (Table I), the retention rate of the radiopharmaceutical agent at the injection site 48 h after injection was 36.8%±2.6% and 43.1%±1.0% for ^{188}Re-liposome and C/GP/GE/^{188}Re-LIPO-DOX, respectively (n=3).

Discussion

The physicochemical properties and radiolabeling measurements for the preparation of ^{188}Re-liposome and ^{188}Re-LIPO-DOX were carried out according to previously published methods and results were obtained similar to previous studies (30, 42, 43). Factors such as the MW, degree of deacetylation, concentration of GP, and reaction temperature have been reported to influence the thermogelling process of C/GP, and the mechanisms have been previously described in detail (45-47). The absorbance kinetics signal related to sol-gel transition

Table I. *Retention rate of the radiopharmaceutical compound at the injection site (mean±SD, n=3).*

Group	Retention rate of radiopharmaceutical (%)		
	1 h	24 h	48 h
$^{188}ReO_4^-$ (control)	2.6±0.1	2.0±0.1	1.1±0.2
^{188}Re-Liposome	55.7±0.8	47.4±2.0	36.8±2.6
C/GP/GE/^{188}Re-LIPO-DOX	57.0±0.9	50.3±1.8	43.1±1.0

in the C/GP/GE/LIPO-DOX group was weaker than those in the other groups but still corresponded to a thermogelling process (Figure 2). This could be due to high optical background of the encapsulated DOX, which can influence the absorbance measurements (48).

The FTIR analysis (Figure 3) revealed that covalent cross-linking occurred in the C/GP/GE hydrogel given the presence of secondary amide (-CONH) bands at 1650 cm^{-1}, which was correlated with the reaction between the amino groups of chitosan and the carboxymethyl groups of GE (49). Other characteristic stretching vibrations identified in the FTIR spectra included 1576 cm^{-1} and 1647 cm^{-1} (C=O) from the C/GP hydrogel (50), 970 cm^{-1} (P-O) and 1077 cm^{-1} (P-OH) from GP (51), 1107 cm^{-1} (PO_2^-) from LIPO-DOX (52-54), and 2800-2900 cm^{-1} (C-H) and a broad band at 3000-3600 cm^{-1} (N-H and O-H) from the C/GP-based hydrogel (13, 49). Results from SEM micrographs (Figure 4), the pore diameter was slightly larger, in the 20-30 μm range, when GE was added to the hydrogels for C/GP/GE (Figure 4 C and D), C/GP/GE/LIPO-DOX (Figure 4 E and F), and C/GP/GE/^{188}Re-LIPO-DOX (Figure 4 G and H). Furthermore, the pore walls in the GE-containing hydrogel groups were significantly thicker and stronger than those in the C/GP hydrogels, which can be attributed to co-cross-linking (ionic and covalent cross-linking) within the C/GP/GE hydrogels. Similar results have been previously discussed (19). This result corroborates the SEM observations, where the pore walls of the GE-containing hydrogels were thicker and stronger than those of the GE-free C/GP system. Our results are in accordance with previously published findings on chitosan hydrogels cross-linked with GE (19, 20).

For the studies of controlled drug release (Figure 5), this result is similar to that in a previous investigation where chitosan-based hydrogels containing nanoliposomes extended the delivery of a hydrophilic drug by a month (55). Since it can extend the release of an anticancer drug *in vitro*, the C/GP/GE-LIPO-DOX formulation could be potentially used for *in vivo* local drug delivery in cancer treatment. In addition, the C/GP/GE/^{188}Re-LIPO-DOX group had a higher retention rate than that of ^{188}Re-liposome alone. This could be attributed to the slower release of the encapsulated nanoliposome within the three-dimensional scaffold structure of the hydrogel. Once the radiopharmaceutical agent is localized within the tumor region, β-emission from the ^{188}Re radionuclide can destroy the cancer cells around the injection site through a "cross fire" effect (42). Moreover, the hydrogel can sustain the release of smaller hydrophilic drug molecules (such as DOX) locally at the same time. To summarize, the results of *in vitro* and *in vivo* drug delivery studies demonstrated that the C/GP/GE/^{188}Re-LIPO-DOX hydrogel can control the release of the chemotherapeutic drug for several weeks, while the therapeutic radionuclide was localized within the tumor. Therefore, it is suitable for use in inhibiting LRR after mastectomy and reducing systemic side effects in breast cancer treatment.

Conclusion

A novel breast cancer treatment method using the co-cross-linking of injectable chitosan-based hydrogels to deliver radiotherapeutic and chemotherapeutic drugs locally was developed and evaluated. The results demonstrated that the C/GP/GE/^{188}Re-LIPO-DOX formulation not only localized the therapeutic radiopharmaceutical agent at the injection site but also prolonged its release. These results indicate that C/GP/GE/^{188}Re-LIPO-DOX has some potential for further *in vivo* therapeutic evaluation as a promising option for the prevention of LRR after the surgical treatment of breast cancer.

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