Remodeling of Tannic Acid Crosslinked Collagen Type I Induces Apoptosis in ER+ Breast Cancer Cells

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Abstract. *Background: The naturally-occurring phytochemical tannic acid (TA) has anticancer properties. We have demonstrated that estrogen receptor-positive (ER^+) breast cancer cells are more sensitive to effects of TA than triple-negative breast cancer cells and normal breast epithelial cells. In the present study, cells were grown on TA-crosslinked collagen beads. Growing cells remodel collagen and release TA, which affects attached cells. Materials and Methods: The ER^+ breast cancer cell line MCF7 and the normal breast epithelial cell line MCF10A were grown on TA-crosslinked collagen beads in roller bottles. Concentrations of TA in conditioned media were determined. Induced apoptosis was imaged and quantified. Caspase gene expression was calculated by real-time polymerase chain reaction (PCR). Results: Both cell lines attached and grew on TA-crosslinked collagen beads where they remodeled collagen and released TA into surrounding medium. Released TA induced caspase-mediated apoptosis. Conclusion: TA induced apoptosis in a concentration-dependent manner, with ER^+ MCF7 cells displaying more sensitivity to effects of TA.*

The study of natural dietary compounds to block or delay the onset of cancer is generating promising results (1-5). There are many purposes of administering these purported chemopreventative agents, such as to suppress cancer cell proliferation, to induce cancer cell differentiation, or to initiate apoptosis in cancer cells. Many natural phytochemicals containing phenolic compounds also demonstrate anticancer capacities (6-8). Phytochemical polyphenols have anticancer functions both *in vitro* and *in vivo* (9-14). Tannic acid (TA) comprises a pentagalloylglucose core, esterified at all functional hydroxyl groups with gallic acid molecules, and belongs to the class of hydrolysable tannins (15). TA is found in coffee, black and green tea, nuts, red wine, and most vegetables (16-18). Apoptotic activity is increased in breast cancer and prostate cancer cells in response to exposure to tannin extracts (19-22).

Collagen type I is commonly used as a tissue-engineering scaffold due to its bioactive and biodegradable characteristics. Collagen is naturally derived and is enzymatically degraded when uncrosslinked (21, 23). TA can function as a collagen crosslinking agent. TA crosslinks collagen by inducing hydrogen bonding and hydrophobic effects; thus, as TA-crosslinked collagen is remodeled, TA is released (24). Since TA crosslinks collagen and has anti-tumor properties, this combination could prove to be an effective agent to induce localized cancer cell apoptosis when introduced in tumor environments. If used as a biomaterial for tissue engineering purposes, TA-crosslinked collaged type I would not only serve as an attachment scaffold for cells but also function as an extended release anti-cancer treatment.

Our previous studies determined that TA preferentially targets estrogen receptor-positive (ER^+) breast cancer cells compared to triple-negative breast cancer cells and normal breast epithelial cells (20). The cells were grown in conventional two-dimensional (2D) culture conditions where TA-crosslinked collagen type I beads were placed in Transwell inserts suspended above the cells. This system allowed for the diffusion of trapped, uncrosslinked TA to induce effects on the cells below. In the current study, normal breast epithelial cells and ER^+ breast cancer cells were grown on TA-crosslinked collagen type I beads in three-

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dimensional (3D) culture conditions. This allowed the direct interaction of cells and beads. Since previous studies indicated that triple-negative breast cancer cells were not sensitive to the effects of TA, ER^+ breast cancer cells were the focus of the current study. We found that TA-induced cell death in the ER^+ breast cancer cells is significantly greater than death induced in normal breast cells.

Materials and Methods

TA crosslinked collagen bead preparation. Crosslinked collagen type I beads were prepared as described previously (20). Briefly, a 1 mg/ml collagen type I solution was prepared from a stock solution of 3.1 mg/ml (Advanced BioMatrix, Poway, CA, USA), as previously described (25). The TA cross-linked beads were prepared using a Nisco Encapsulation Unit VAR V1 electrostatic syringe pump loaded with a 60:40 ratio of 1 mg/ml collagen:1.2% alginate solution in MilliQ water (Sigma-Aldrich, St. Louis, MO, USA); the syringe pump was programmed to pump at 10 ml/h into a 1.5% wt/v $CaCl_2$ solution (Fisher, Pittsburgh, PA, USA) in water. One hour after formation, the beads were filtered out using a mesh strainer and placed in TA crosslinking solution comprising TA (10%, 1.0%, or 0.1% wt/v TA/ MilliQ water), 1.5% $CaCl_2$, 0.15 M NaCl (Sigma-Aldrich) and 1.1% wt/v N-cyclohexyl-2-aminoethanesulfonic acid (Sigma-Aldrich) buffer overnight (26). Twelve hours later, the TA-crosslinked beads were placed in a 50 mM sodium citrate solution (Fisher) for 3 hours, washed with deionized H_2O and stored in phosphate buffered saline (PBS) at 4˚C.

2D cell culture. The normal human breast epithelial cell line MCF10A and the MCF7 cell line isolated from human breast cancer (both from ATCC, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Atlanta Biologicals, Atlanta, GA, USA) with 10% fetal bovine serum (Corning Life Sciences, Corning, NY, USA), 1% antibiotic/antimycotic (Life Technologies, Grand Island, NY, USA) and 0.5% fungizone (Life Technologies). The cultures were maintained at 37˚C with 5% CO_2.

3D Cell Culture. Confluent 2D cultures of MCF7 or MCF10A cells were trypsinized and counted. For each experiment 100,000 cells were added to 50 ml roller tubes with 10 ml medium containing TA-crosslinked collagen type I beads. The roller tubes were rotated at 3 rpm and were maintained at 37˚C with 5% CO_2.

LIVE/DEAD assay. Cells grown on TA-crosslinked collagen type I beads were subjected to LIVE/DEAD assay according to manufacturer's guidelines (Life Technologies). Cells were imaged using a Zeiss Axiovert 40 CFL inverted microscope and collected using a Zeiss AxioCam camera and Axiovision software (Zeiss, Pleasanton, CA, USA).

Quantification of soluble TA in culture medium. Medium samples were collected at various timepoints and stored at –20˚C until analysis. TA concentrations were determined by Folin-Ciocalteu (FC) analysis. A 1% (mass/vol) TA standard was serially diluted so the lowest concentration was 1/1000 in DMEM. Collected media samples and TA standards were diluted 75:25 in water and 100 μl of each was added to 200 μl of 10% (vol/vol) FC reagent (Sigma-Aldrich) in water and mixed thoroughly. To each sample, 800 μl of 700 mM sodium carbonate was added and incubated for 2 h at room temperature. Samples were vortexed and 200 μl of each was added to individual wells of a 96-well plate. Absorbance was read at 765 nm.

Table I. *Prime time qPCR primers used for real-time PCR.*

Gene	Sequence
GAPDH	1: 5'-TGTAGTTGAGGTCAATGAAGGG-3'
	2: 5'-ACATCGCTCAGACACCATG-3'
Caspase 3	1: 5'-GTTTGCTGCATCGACATCTG-3'
(*CASP3*)	2: 5'-CTCTGGAATATCCCTGGACAAC-3'
Caspase 7	1: 5'-CTTCTCCATGGCTTAAGAGGATG-3'
(*CASP7*)	2: 5'-GTGCCAAGATGCAAGATCTG-3'
Caspase 8	1: 5'-TCTGAAATCTGATAGAGCATGACC-3'
(*CASP8*)	2: 5'-AGACTGGATTTGCTGATTACCTAC-3'
Caspase 9	1: 5'-GTCCTCAAACCTTCCTGGAAC-3'
(*CASP9*)	2: 5'-GCCCAAGCTCTTTTTCATCC-3'

RNA extraction and real-time PCR. Total mRNA was collected from cells grown on TA-crosslinked collagen type I beads using Trizol (Ambion, Carlsbad, CA, USA) according to manufacturer's instructions and stored at –80˚C until analysis. RNA was reverse-transcribed to cDNA using the Ambion Retroscript kit two-step protocol. The quantity and quality of the RNA was determined using the Nanodrop 2000. The cDNA was analyzed using the QuantiTech SYBR Green RT-PCR Kit (Qiagen, Valencia, CA, USA) on the Step One Plus (Applied Biosystems, Grand Island, NY, USA). The markers analyzed were *GAPDH*, caspase 3 (*CASP3*), caspase 7 (*CASP7*), caspase 8 (*CASP8*), and caspase 9 (*CASP9*). The primer sequences can be seen in Table I. Relative expression was determined using the ΔΔCt method with GAPDH as the reference gene and the cells treated with 0.1% TA as the control cells.

Computation and statistical analyses. Each experiment was performed a minimum of three times with at least three replicates performed within each experiment. A minimum of 500 cells was counted for all observations. One-way analysis of variance (ANOVA) was used to analyze cell counts and the Student's *t*-test was used to analyze TA concentrations.

Results

Growth of cells on TA-crosslinked collagen type I beads. The ER^+ breast cancer cell line MCF7 and the normal breast epithelial cell line MCF10A were cultured in roller tubes with TA-crosslinked collagen type I beads that were crosslinked using various concentrations of TA (10%, 1%, and 0.1%). Samples were collected after 5 and 9 days of culture. In all instances, cells of both cell lines were able to attach to the TA-crosslinked collagen type I beads within 5 days (Figures 1A and B). LIVE/DEAD assays were performed to assess the effects of TA on the cells. As demonstrated in Figures 1A and 1B the highest concentration of TA resulted in the highest level of dead cells in both cell lines. After 5 days, TA induced cell death in MCF7 cells in

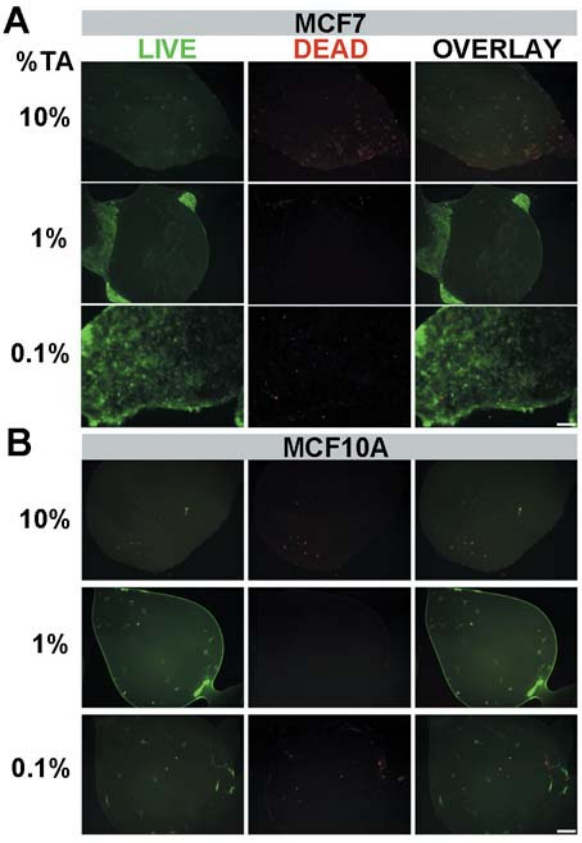

Figure 1. *LIVE/DEAD assay on A) MCF7 and B) MCF10A cells grown on 10%TA-, 1% TA-, or 0.1% TA-crosslinked collagen type 1 beads for 5 days in roller tubes. Green staining indicates live cells and red staining indicates dead cells. Scale bars=100 μm.*

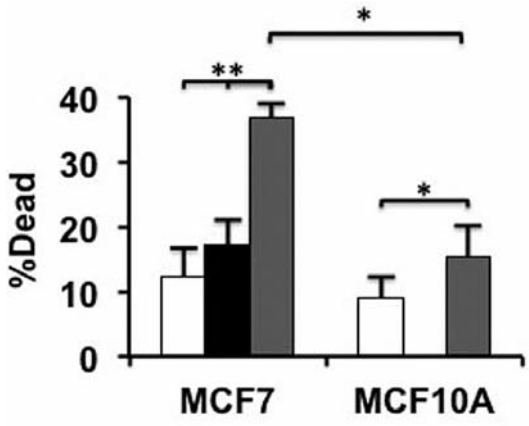

Figure 2. *LIVE/DEAD assay on MCF7 and MCF10A cells grown on TA-crosslinked collagen type 1 beads for 5 days in roller tubes. Live and dead cells were counted and % of dead cells was determined. Bars: White, 0.1% TA; Black, 1% TA; Gray, 10%TA. n=3, *p<0.05, **p<0.001. Error bars=standard deviation.*

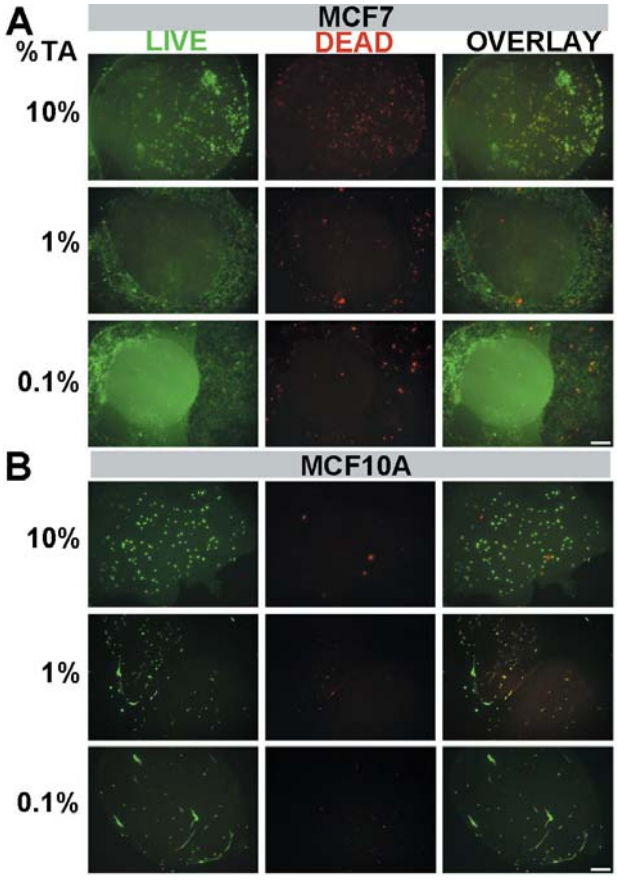

Figure 3. *LIVE/DEAD assay on A) MCF7 and B) MCF10A cells grown on 10%TA-, 1% TA-, or 0.1% TA-crosslinked collagen type 1 beads for 9 days in roller tubes. Green staining indicates live cells and red staining indicates dead cells. Scale bars=100 μm.*

a concentration-dependent manner (Figure 2). The greatest concentration of TA (10%) induced a significant difference ($p<0.05$) in the number of MCF10A dead cells after 5 days of growth on TA-crosslinked collagen type I beads of varying TA concentrations (Figure 2). The two highest concentrations of TA (10% and 1.0%) induced significantly higher cell death ($p<0.05$) in MCF7 cultures than in MCF10A cultures.

After 9 days in culture, many dead MCF7 cells were detected on the TA-crosslinked collagen type I beads (Figure 3A), while very few dead MCF10A cells were still attached to collagen beads (Figure 3B). The highest percentage of dead cells was observed on the 10% TA-crosslinked collagen type I beads. The TA-induced killing of the MCF7 cells was again TA concentration-dependent (Figure 4). The percentage of dead MCF7 cells remained similar after 4 and 9 total days in culture (Figures 2 and 4). After 9 days in culture, very few dead cells were observed in MCF10A cultures where the cells were growing on 1% or 0.1% TA-collagen beads. Less than 10% of the MCF10A cells counted, which were attached to

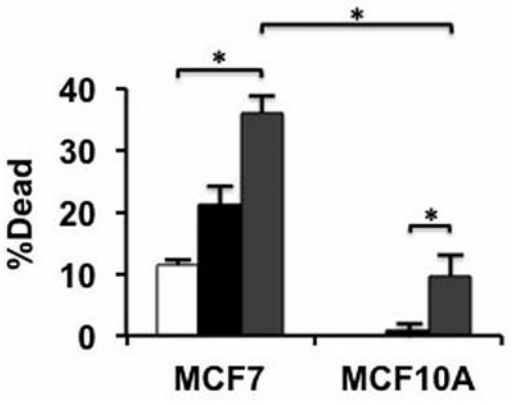

Figure 4. *LIVE/DEAD assay on MCF7 and MCF10A cells grown on TA-crosslinked collagen type 1 beads for 9 days in roller tubes. Live and dead cells were counted and %Dead was determined. Bars: White, 0.1% TA; Black, 1% TA; Gray, 10%TA. n=3, *p<0.05. Error bars=Standard deviation.*

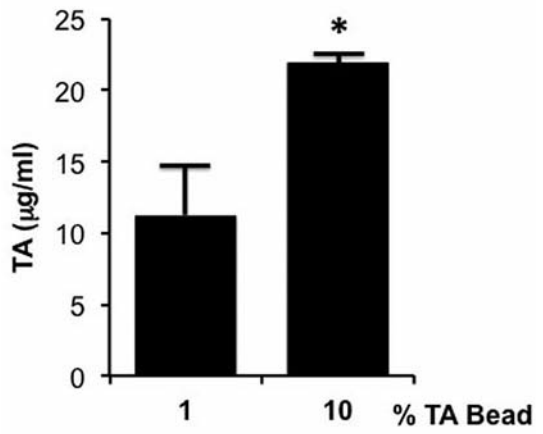

Figure 5. *Quantitation of TA levels in the conditioned media collected from cultures of MCF7 and MCF10A cells grown on TA-crosslinked collagen beads using FC analysis. TA was only detected in conditioned media from MCF10A cells grown on 10% and 1% TA-collagen beads after 9 days in culture. n=3, *p<0.05.*

the 10% TA-beads, were dead. The overall percentages of dead cells found in MCF7 cultures remained constant over the 9-day culture period, while the percentages of dead MCF10A cells dropped significantly ($p<0.05$) even at the highest concentration of TA (Figures 2 and 4). This observation suggests that MCF10A cells are more resistant to the effects of TA than MCF7 cells.

Remodeled collagen releases TA into surrounding media. To test the theory that cell attaching and growing on the TA-crosslinked collagen type I beads would remodel the collagen, thus releasing TA, we measured TA levels in conditioned media over time. The only detectable levels of TA were found in conditioned medium collected after 10 days of MCF10A cells grown on 10% and 1% TA-collagen beads (Figure 5). The two conditions in which TA was detectable in the surrounding medium are the two conditions that were the longest in time, had the highest initial starting concentrations of TA and had the most cells attached to the beads remodeling the collagen and releasing the previously crosslinked TA. This result indicates that very low local concentrations of TA induce apoptosis in MCF7 cells.

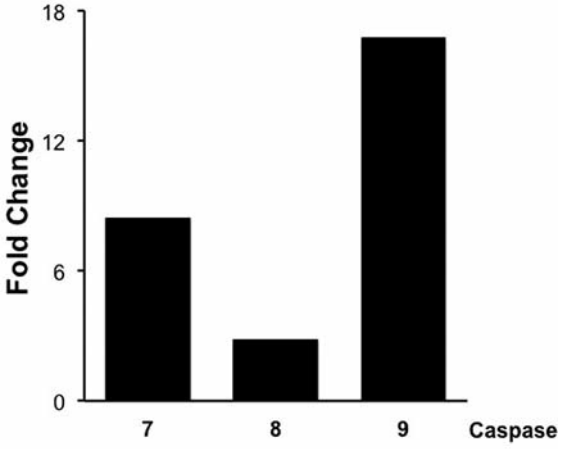

Figure 6. *Real-time PCR results (fold change) on caspase 7/8/9 expression. MCF7 and MCF10A cells were grown on 1.0% TA-crosslinked beads for 4 days and mRNA collected.*

TA induces caspase-dependent apoptosis in 3D cultures. We have previously demonstrated that TA-induced cell death is through a caspase 3/7-mediated apoptotic mechanism (20). MCF7 and MCF10A cells were grown on TA-crosslinked collagen type I beads. RNA was collected from cells grown on beads for 4 days. Gene expression of caspases 3, 7, 8 and 9 was examined (Figure 6). Expression of caspase 7 was determined to be two-fold higher in MCF7 cells compared to its expression in MCF10A cells and the expression of caspase 9 was nearly five-fold higher in MCF7 cells than in MCF10A cells. This result clearly demonstrates that TA is inducing caspase 7- and caspase 9-mediated apoptosis in the ER^+ breast cancer MCF7 cells, to a greater degree than in the normal breast epithelial cell line MCF10A.

Caspase 3 expression was absent in RNA collected from MCF7 cells. MCF7 cells do not express caspase 3 (27) and very little expression of caspase 3 was found in the MCF10A cells. No difference in expression of caspase 8 was found between cancer and normal cells. This matches previous observations of cells exposed to TA in culture (20).

Discussion

In the present study, the effects of TA that has been released from remodeled collagen type I on MCF7 and MCF10A cells were investigated. Our previous study indicated that triple-negative breast cancer cells were less sensitive to the effects of TA than ER^+ breast cancer cells. We decided not to continue experiments involving the triple negative cells and instead focused solely on ER^+ breast cancer cells. We found that both ER^+ breast cancer cells and normal breast epithelial cells initially attached and grew on TA-crosslinked collagen type I beads and that released TA from the remodeled beads induced caspase-mediated apoptosis in both cell lines in a TA concentration-dependent manner.

The highest concentration of TA in the conditioned media was found in cultures with the most surviving cells after the longest period in culture, and with the highest starting concentrations of crosslinked collagen. This finding suggests that low local concentrations of TA are inducing apoptosis in MCF7 cells. As MCF10A cells are more resistant to the effects of TA, this result is not surprising. More MCF10A cells are attached and growing on the beads after 10 days compared to MCF7 cells. Therefore, more TA is being released, as the greater number of cells are remodeling the collagen to a greater extent than is occurring in the MCF7 cultures where the cells are dying-off at a faster rate.

Many tissue-engineering techniques, including those used for breast reconstruction following trauma or anti-cancer surgical mastectomy, use collagen type I due to its availability, intrinsic bioactivity, and biodegradability (21). Collagen type I is unstable when not crosslinked; however, when crosslinked, its mechanics are altered, resulting in decreased thermal and enzymatic degradability. Tumor recurrence is a leading cause of breast cancer-related death, even after surgical removal of tumors by mastectomy or lumpectomy (28, 29). The biomaterial used in these studies comprises-collagen type I crosslinked by the natural anticancer agent TA. The long-term goal is to demonstrate that TA is released from the crosslinked collagen beads when cells attach to and remodel the beads. The released TA will induce apoptosis in any residual cancer cells that happen to remain following lumpectomy. Indeed, the TA-crosslinked beads are stable at body temperature (20) and the released TA induces apoptosis in ER^+ breast cancer cells (Figures 1-4). TA-crosslinked collagen sheets have a capacity to decrease wound-healing time (30). Taken together, these properties make TA-crosslinked collagen type I a biomaterial with great potential in the fight against breast cancer recurrence.

Epidemiological studies suggest that vegetable and fruit heavy diets have inherent anticancer advantages (1-6, 9-10). Anti-cancer substances found in vegetables and fruits initiate apoptosis in cancer cells. Such high vegetable and fruit diets are potential cancer prevention strategies and aid in anticancer treatments due to the increased concentration of antioxidants and anti-cancer agents (31-34).

Previous results demonstrated that TA induces apoptosis in MCF7, MCF10A and MDA-MB-231 cells via a mechanism that is mediated by caspases 3/7 and 9, but not caspase 8 (20). No involvement of caspase 8 was detected in the previous studies or the current study (Figure 6). Both caspase 3 and 7 are downstream of caspase 9. Activation of caspases 3, 7 and 9 can occur due to extracellular stress. Caspase 9 is an initiator caspase that, once activated, cleaves and activates downstream caspases, such as 3 and 7. Peroxisome proliferator-activated receptor gamma (PPARγ) has been shown to be involved in TA-induced apoptosis (19, 22-23). Both caspase 3 and 7 cleave PPARγ (35). An explanation for the increased sensitivity of the ER^+ MCF7 cells to TA-induced apoptosis is that MCF7 cells do not express caspase 3 (27). Caspase 3 and caspase 7 are activated by caspase 9 (36). Caspase 3 acts as a regulator of caspase 9 through feedback inhibition (37). Therefore, the lack of feedback inhibition would allow for unchecked activation and signaling of caspase 9 and caspase 7 leading to apoptosis. A future goal is to investigate additional ER^+ breast cancer cells to determine if the lack of caspase 3 in MCF7 cells is the only determining factor in their increased TA sensitivity.

The results from the current study demonstrate the feasibility for combining the natural anticancer agent TA with the biomaterial collagen type I. This combination could help clinicians and breast cancer patients by serving as a growth scaffold for tissue engineered breast reconstruction following lumpectomy while, simultaneously, helping to prevent breast cancer recurrence.

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