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Implantable hydrogel-embedded dark-gold nanoswitch as a theranostic probe to sense and overcome cancer multidrug resistance

João Conde¹,²,¹, Nuria Oliva³, and Natalie Artzi¹,²,³,¹

¹Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA 02139; ²School of Engineering and Materials Science, Queen Mary University of London, London E1 4NS, United Kingdom; and ³Department of Anesthesiology, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115

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Significance

The integration of biomaterials science, innovative imaging, and cancer biology now enables the design of smart responsive material platforms for cancer theranostics. We show herein that our developed nanovehicle is able to sense and silence a multidrug resistance gene based on its expression in the tumor microenvironment, followed by local chemotherapeutic drug release, with a significant tumor regression not achieved otherwise. This ON/OFF molecular nanoswitch approach can be used to reverse the resistance to many other chemotherapeutic drugs and can serve as a universal gene therapy and drug delivery vehicle for cancer therapy. This disease-responsive platform can revolutionize clinical outcome and cancer patients’ point of care.

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¹To whom correspondence may be addressed. Email: jdconde@mit.edu or nartzi@mit.edu.

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Multidrug resistance (MDR) in cancer cells is a substantial limitation to the success of chemotherapy. Here, we describe facile means to overcome resistance by silencing the multidrug resistance protein 1 (MRP1), before chemotherapeutic drug delivery in vivo with a single local application. Our platform contains hydrogel embedded with dark-gold nanoparticles modified with 5-fluorouracil (5-FU)-intercalated nanobeacons that serve as an ON/OFF molecular nanoswitch triggered by the increased MRP1 expression within the tumor tissue microenvironment. This nanoswitch can sense and overcome MDR prior to local drug release. The nanobeacons comprise a 5-FU intercalated DNA hairpin, which is labeled with a near-infrared (NIR) dye and a dark-quencher. The nanobeacons are designed to open and release the intercalated drug only upon hybridization of the DNA hairpin to a complementary target, an event that restores fluorescence emission due to nanobeacons conformational reorganization. Despite the cross-resistance to 5-FU, more than 90% tumor reduction is achieved in vivo in a triple-negative breast cancer model following 80% MRP1 silencing compared with the continuous tumor growth following only drug or nanobeacon administration. Our approach can be applied to reverse cross-resistance to other chemotherapeutic drugs and restore treatment efficacy. As a universal nanotheranostic probe, this platform can pave the way to early cancer detection and treatment.

hydrogels | gold nanobeacons | theranostics | breast cancer | multidrug resistance

Multidrug resistance (MDR) in cancer is a phenomenon whereby cancer cells gain the capacity to develop cross-resistance and survive a variety of structurally and functionally unrelated drugs (1). The most common MDR mechanisms occur by the expression of one or more energy-dependent transporters, which can result in an increased efflux of the cytotoxic drugs from the cancer cells, thus lowering their intracellular concentrations (2). The phosphoglycoprotein multidrug resistance protein 1 (MRP1 or ABCC1) is often associated with resistance to a broad spectrum of anticancer drugs and belongs to the ATP-binding cassette (ABC) superfamily of proteins as energy-dependent efflux pumps (3). The ABC transporters are essential not only to breast cancer MDR but also to other types of cancer, such as non–small-cell lung cancer, lung cancer, and rectal cancer (4). In fact, increased ABC expression levels have been shown to correlate with decreased response to various chemotherapy drugs [such as 5-fluorouracil (5-FU)] and a decrease in overall survival (5). 5-FU is widely used in cancer therapy as it has the capacity to interfere with nucleoside metabolism and result in DNA and RNA synthesis disorders and dysfunction, leading to cytotoxicity and cell death. Based on the American Cancer Society guidelines, 5-FU is used to treat a range of cancerous diseases, including colon and rectal cancer; breast cancer; gastrointestinal cancers including anal, esophageal, pancreatic, and gastric (stomach); head and neck cancer; and ovarian cancer. For decades, 5-FU has been used in combination with other antineoplastic agents or as a single agent in the adjuvant and palliative treatment of advanced breast cancer (6). Nevertheless, the overall response to this drug remains only 15% due to resistance mechanisms (7). Hence, despite the remarkable progress in chemotherapeutic drug development in the last decade, ~70% of cancer patients do not respond to chemotherapy and have a 5-yr survival rate of 10–30% (8). Consequently, new platforms that are able to improve antitumor efficacy are urgently needed.

The development of nanoscale devices has provided major breakthroughs in cancer diagnostic and therapy (9–13), especially in drug delivery (11, 14). Because nanoparticles are excellent tumor-targeting vehicles due to enhanced permeability and retention (EPR) in the tumor microenvironment (having defective vasculature and poor lymphatic drainage) (16, 17), they can be harnessed to alter MDR mechanisms. Indeed nanomaterials have been used to help overcome MDR by increasing drug retention in cancer cells and hamper cancer progression (7, 18–20). Nevertheless, a platform that senses and inhibits MDR gene expression followed by selective drug release has not been reported yet.

Here we developed a universal nanodevice able to sense specific gene sequence, silence it, and release a chemotherapeutic drug, while reporting on these events. A bioreponsive hydrogel-nanorobe that locally senses and inhibits the expression of the MRP1, followed by subsequent release of 5-FU drug to resistant triple-negative breast cancer cells (TNBC), was developed. Breast cancer was chosen as a model because it can benefit from local noninvasive administration of an injectable hydrogel. TNBC accounts for 15–20% of all breast cancers, and it represents the most aggressive subtype with a direct prognosis. TNBCs are characterized...
by resistance to apoptosis, aggressive cellular proliferation, migration and invasion, and currently lack molecular markers and effective targeted therapy. TNBC patients suffer from poor survival rates and limited efficacy of neoadjuvant chemotherapy, as tumors from such patients are characterized by overexpression of specific genes involved in drug-desensitizing mechanisms (21). We note that smart bioreponsive hydrogel-nanoprobes are able to reduce 5-FU drug-resistant tumors by 90% 14 d after hydrogel implantation in breast cancer tumor-bearing mice by silencing more than 80% of MRPI expression before drug release. This approach can be applied to improve treatment efficacy using other chemotherapeutic drugs for a range of solid tumors.

Results and Discussion

Bioresponsive Dark-Gold Nanoswitch Design. Our nanoprobe encompasses a gold nanoparticle (AuNP) core decorated with a thiol-DNA hairpin labeled with a near-infrared (NIR) dye (intercalated with a chemotherapeutic drug, 5-FU) and a thiol-DNA oligo labeled with a Black-Hole dark quencher (Fig. L4), which is used as a two-pair FRET/nanosurface energy transfer (NSET) donor quencher nanoconjugate for universal cancer gene therapy and drug delivery. Polyethylene glycol (PEG) is also used as a spacer between the hairpins, increasing their nanostructure stability in biological medium (22–24) (see SI Appendix for PEG optimization and quantification). Thiol-DNA oligo labeled with a dark quencher is used to quench nanobeacon fluorescence. Under hairpin configuration, the proximity of the NIR dye (Quasar 705) to the dark quencher (BHQ2) leads to fluorescence quenching. Hybridization of the DNA hairpin to a complementary target (i.e., MRPI mRNA) restores fluorescence emission due to the gold nanobeacon conformational reorganization that causes the fluorophore and the quencher to part from each other, yielding a quantitative response.

The mechanism of mRNA knockdown in this study is based on antisense DNA technology (25). This method was used to inhibit or down-regulate the production of MRPI protein by using antisense DNA oligonucleotides, the DNA molecular beacon anti-MRP1, which precisely complements the MRPI mRNA target sequence present in the cell. These molecular beacons have the ability to interlock or hybridize with the target MRPI mRNA (blocking specific translation initiation signals of MRPI gene), thus impairing the translation of the target protein. Oligomers sequences and 2D structures of the beacons at 37 °C are depicted in SI Appendix, Table S1 and Fig. S24, respectively.

The release of the 5-FU drug is designed to occur only when DNA hairpin hybrids with the complementary mRNA target inside the cell and can be tracked fluorescently once the distance between 5-FU and the gold core increases upon drug release. The 5-FU wedge between bases along the dsDNA part of the nanobeacon. The drug covalently binds to DNA in different sites producing intrastrand cross-links, preventing the polymerase and other DNA binding proteins from functioning properly, which results in DNA synthesis, inhibition of transcription, and induction of mutations. The choice of fluorophores and quenchers enabled the gold nanobeacon with BHQ2 dark quencher, which extends to the near-infrared emission wavelengths, to overlap with the absorbance range of the Quasar 705 fluorophore, and the surface plasmon resonance (SPR) profile from gold nanobeacon to overlay the absorbance wavelength from the 5-FU at 450 nm (SI Appendix, Fig. S2B). The BHQ2 group functioned as quencher for the Q705 (donor 1) and the AuNPs functioned as the quenchers for the 5-FU (donor 2). The NIR dye and dark quencher, Quasar 705 nm (Q705) and BHQ2 (26) (Black Hole Quencher-2 with a strong absorption from 599 to 670 nm), were chosen to enable efficient quenching in the nanobeacon basal state with restored fluorescence upon conjugation to the appropriate target. The fluorescence emission from the 5-FU (Emi = 450 nm) is efficiently quenched by the ~14-nm AuNP due to the NSET effect (27). This NSET effect provides the dark-gold nanobeacons with the ability to serve as an on/off switch. This capability is particularly useful for tracking drug release in response to a specific gene expression within the tumor microenvironment. These events, binding to a target and the resulting drug release, are each reported by fluorescence emission in a defined wavelength.

In addition to designing an anti-MRP1 nano beacon that detects and inhibits MRPI mRNA, anti-Luc nanobeacons (that hybridize with luciferase mRNA and release the drug without targeting MRPI) and nonsense nanobeacons (that do not hybridize with any target) were developed as controls (SI Appendix, Fig. S24 and Table S1).

Transmission electron microscopy (TEM) images of the dark-gold nanobeacons loaded with 5-FU showed an average diameter ± SD of the gold core of 13.8 ± 3.4 nm (SI Appendix, Fig. S2C). The final Q705 DNA hairpin:NP and BHQ2 oligo:NP ratios were approximately 30:1 and 24:1, respectively. The ratio for 5-FU:NP was 100:1 (SI Appendix, Table S2). The mean particle diameter ± SD of dark-gold nanobeacons is 25.5 ± 1.2 nm without 5-FU and 31.1 ± 0.5 nm loaded with 5-FU, with an SPR peak around 548–550 nm as measured by dynamic light scattering (DLS) and UV-Vis extinction profiles, respectively (Fig. 1D and SI Appendix, Table S2).

The fluorescence is OFF when the hairpin-DNA-Q705 is closed (i.e., no hybridization with the complementary target), inhibiting 5-FU release (Fig. L4). The fluorescence is ON when the hairpin-DNA-Q705 hybridizes with a complementary target and an increase in Q705 emission occurs, with a concomitant 5-FU release and increase in the drug emission (Fig. L4). It is worth noting that the fluorescence emission intensity of Q705-labeled DNA oligo is significantly decreased following their conjugation to the dark-gold nanoparticles. The binding between the Q705-labeled DNA oligo and the dark-gold nanoparticles occurs by a direct quasi-covalent bond between the thiol group on the 5’ end of the oligo and the gold surface by ligand-exchange between the citrate groups on the surface of the AuNPs and thiol groups. When the concentration of Q705-labeled DNA oligo was fixed at 0.5 μM, the increase in dark-gold nanobeacons concentration from 0 to 10 nM in the reaction mixture resulted in a significant increase in Q705 fluorescence quenching (Fig. 1B), demonstrating the presence of NSET between the Q705 groups and the dark-gold nanobeacons. These experimental data showed a quenching efficiency of nearly 99% when the concentration of dark-gold nanobeacons reached 1 nM, whereas it was nearly 100% when the concentration of AuNPs reached 2.5 nM. These data confirm that the combination of BHQ2 oligo and attachment to the surface of gold nanobeacons provides powerful fluorescence quenching for Q705 emission. The same phenomenon occurs for 5-FU loading on dark-gold nanobeacons (Fig. 1C). When 1 mM 5-FU was loaded on 2.5 nM dark-gold nanobeacons, the fluorescence emission spectra of 5-FU after incubation in phosphate buffer (pH 7.0, 10 mM) for different periods of time (0–120 min) confirmed a significant increase in 5-FU fluorescence quenching (Fig. 1C). This phenomenon indicated the formation of dark-gold nanobeacons loaded with 5-FU. Conversely, the Q705 emission can be restored after hybridization with the complementary ssDNA target. Increasing amounts of complementary target results in elevated emission form the Q705 dye until reaching a plateau at 1 μM of target (Fig. 1D) and in 5-FU release, with nearly complete drug release at 1 μM of target (Fig. 1E). We studied the release kinetics of the intercalated drug following fluorescence of the 5-FU that was measured for 75 min following addition of 1 μM of target and compared it with the fluorescence of the Q705 (SI Appendix, Fig. S3). As expected, the kinetics of the 5-FU release follows that of nanobeacons opening subsequent to the hybridization of a fully complementary target.

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The stability of dark-gold nanobeacons loaded with 5-FU toward variations in temperature, pH, DNase, and glutathione (GSH) concentration was also evaluated (SI Appendix, Figs. S4–S7). These data confirm the stability of the dark-gold nanobeacons loaded with 5-FU at room and physiological (37 °C) temperatures (SI Appendix, Fig. S4) and in a wide pH range (4.5–8) (SI Appendix, Fig. S5), as well as intracellular concentrations of GSH (SI Appendix, Fig. S6) and DNase (SI Appendix, Fig. S7).

**MRP1 Triggered 5-FU Release in Vitro.** To test the ability of dark-gold nanobeacons loaded with 5-FU to overcome MDR, nanobeacons intracellular uptake and trafficking was examined using a 5-FU–resistant MDA-MB-231 breast cancer cell line obtained by continuous culturing of parental MDA-MB-231 cells in a 0.05-mg/mL dose of 5-FU. Drug resistance was confirmed by the absence of cell death using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylylazoliumbromide] (MTT) assay.
after continuous exposure to drug compared with parental cancer cells (SI Appendix, Fig. S8).

Dark-gold nanobeacons could enter and accumulate in resistant MDA-MB-231 cells as verified by confocal microscopy, showing specific targeting at 48-h incubation of anti-Luc and anti-MRP1 nanobeacons compared with nanobeacon nonsense (that do not hybridize with any target). It is evident that silencing occurs only with anti-Luc and anti-MRP1 nanobeacons because hairpin conformational change due to specific hybridization to the target sequence can be identified via fluorescence emission (Fig. 2A). Epi-fluorescence images of cellular uptake kinetics of cells incubated with anti-MRP1 nanobeacons for 0.5, 2, 4, 8, 24, and 48 h are depicted in SI Appendix, Fig. S9. Nanobeacons cellular uptake and detection associated with drug release signal for the same time points are depicted in SI Appendix, Fig. S10. Indeed, analytical flow cytometry data confirmed the phenomena observed via confocal imaging of a substantial increase in Quasar 705 signal only for nanobeacons anti-Luc and anti-MRP1 compared with nanobeacon nonsense (Fig. 2B).

Confocal images of breast cancer cells further revealed that the dark-gold nanobeacons anti-MRP1 were colocalized in intracellular organelles such as endosomes and/or lysosomes at 24 h and could escape out of this organelles at 48 h (Fig. 2C).

Overcoming Intracellular 5-FU Resistance. To corroborate confocal and flow cytometry data and overcome 5-FU resistance, resistant MDA-MB-231 cells were incubated with increasing amounts of dark-gold nanobeacons with and without 5-FU and evaluated by the live imaging system. As expected, only anti-Luc and anti-MRP1 nanobeacons loaded with 5-FU can produce a substantial decrease in luminescence (Fig. 3A, also see SI Appendix, Fig. S11 for luminescence and fluorescence signal quantification). The same occurs for cell viability evaluated by MTT assay (Fig. 3B and C). Dark-gold nanobeacons without 5-FU do not affect cell viability (Fig. 3B) compared with the dark-gold nanobeacons loaded with 5-FU, with an IC50 near 0.5 nM (Fig. 3C) at 72 h of exposure.

Live-dead staining of resistant MDA-MB-231 cells after uptake of increasing concentrations (0.1, 1, and 5 nM) of dark-gold nanobeacons for 48 h was performed using a double staining procedure with acridine orange (AO) and propidium iodide (PI) representing green and red fluorescence for live and dead cells, respectively. As shown in Fig. 3D, only nanobeacon anti-MRP1 loaded with 5-FU (at 5 nM) results in extensive cell death (>95%), due to the combination of MRP1 inhibition and consequent drug (5-FU) release. Nanobeacon anti-Luc shows modest cell death despite releasing the drug as MRP1 inhibition does not occur (see SI Appendix, Fig. S12 for in vitro real-time PCR data) because MRP1 can actively pump cytotoxic drugs like 5-FU.

Fig. 2. (A) Representative confocal images of resistant MDA-MB-231 cells incubated for 48 h with a nanobeacon nonsense, nanobeacon anti-Luc, and nanobeacon anti-MRP1 (5 nM). (Scale bars, 20 μm.) (B) Flow cytometry analysis comparing nanobeacon anti-Luc and nanobeacon anti-MRP1-treated cells to nanobeacon nonsense. (C) Intracellular colocalization of nanobeacon anti-MRP1 within lysosomes. Resistant MDA-MB-231 cells were treated with 5 nM nanobeacon anti-MRP1 (in red) for 24 and 48 h, and lysosomes (in green) were stained with Lysotracker Green DND26. Overlap of nanobeacon anti-MRP1 and lysosome marker is represented in yellow in the merged images.
from the cytosol across the plasma membrane at the expense of ATP (28).

To evaluate the kinetics of the 5-FU effect and the mRNA MRP1 knockdown by the nanobeacons, an MTT cell viability assay was performed after 24, 48, and 72 h and compared with the kinetics for MRP1 expression using 5 nM (the same concentration for in vitro and in vivo studies) of nanobeacons loaded with 5-FU (SI Appendix, Fig. S13). These data corroborate that the maximum mRNA knockdown (at 48 h) occurs 24 h before the maximum effect of the drug (at 72 h), showing that the 5-FU is mostly effective 24 h after the drug reversal mechanism takes place.

**Hydrogel-Gold Nanoswitch Sensing and Targeting in Vivo.** To evaluate the efficiency of the dark-gold nanobeacon probes in sensing and in overcoming MDR in vivo, an orthotopic breast cancer mouse model was developed by injecting resistant MDA-MB-231 cells to the mammary fat pad of female SCID hairless congenic mice. Efficacious and local delivery of the dark-gold nanobeacon probes is achieved by the implantation of a hydrogel disk on top of the triple-negative breast tumors (Fig. 4 A). The hydrogel scaffold comprising a polyamidoamine (PAMAM G5) dendrimer cross-linked with dextran aldehyde provides enhanced stability of the embedded nanoparticles. Epi-fluorescence images show a homogeneous distribution of the dark-gold nanobeacon probes (previously hybridized with a complementary target) in the hydrogel network (Fig. 4 B and C). The dual labeling discloses a colocalization of the nanobeacon probes (red) and the tagged polymeric matrix (green) that can be attributed to electrostatic interaction between the nanobeacons and the hydrogel (Fig. 4C and SI Appendix, Fig. S14). This reversible interaction enables rapid local release of the nanoparticles at the site of interest, as demonstrated by the almost complete release in the first 24 h under physiological conditions in vitro (pH 7.4 and 37 °C; SI Appendix, Fig. S15). This local delivery platform using dextran-dendrimer hydrogel scaffolds (29–31) overcomes some of the limitations associated with systemic administration, such as low stability, dissociation from vector, and short lifetimes (32). Additionally, local delivery prevents the uptake of systemically administered nanoparticles by the liver that makes targeting to other organs difficult. Once tumors reach a desired volume (≈100 mm$^3$), hydrogel scaffolds loaded with dark-gold nanobeacon probes are implanted adjacent to the mammary fat pad tumor. Over a period of 14 d following hydrogel implantation, in vivo imaging was used to simultaneously track tumor inhibition as measured by luciferase expression (Fig. 4D), nanobeacon probes (Fig. 4E) before and after hybridization to MRP1 mRNA, and hydrogel stability monitored by FITC fluorescence emission. No signs of inflammation were observed at the surgical site, and there were no changes in body weight following hydrogel implantation (SI Appendix, Fig. S16), suggesting that hydrogels are biocompatible, with no associated toxicity or side effects. Bioluminescence imaging of mice revealed that only nanobeacon anti-MRP1 loaded with...
5-FU was able to promote efficient and sustained inhibition of tumor progression (Fig. 4D), with an ~90% tumor size reduction 14 d after hydrogel disk implantation. Representative images of whole body organs and resected tumors in mice treated with nanobeacons are depicted in SI Appendix, Fig. S17, corroborating the significant tumor size reduction 14 d after hydrogel implantation. Interestingly, in vivo hydrogel degradation as monitored by FITC intensity (SI Appendix, Fig. S18) coincided with nanobeacon fluorescence, showing the highest signals during the first 2 d, in which the hydrogel degrades, and reaching about 45–50% degradation after 14 d. Monitoring the change in tumor size as a function of time after treatment with nanobeacons revealed a significant reduction in tumor size with nanobeacons (n = 5, ***P < 0.005).
tumor growth ($n = 5, P < 0.005$) at day 14 after NP treatment (Fig. 4F), with almost complete tumor regression only for the nanobeacon anti-MRP1 loaded with 5-FU group. About 90% decrease in luciferase activity (Fig. 4F) and in tumor size (SI Appendix, Fig. S17) was observed exclusively for the nanobeacon anti-MRP1 loaded with 5-FU–treated tumors compared with free drug (5-FU only), anti-MRP1 only, anti-Luc loaded with 5-FU, and nanobeacon nonsense with 5-FU ($n = 5$, $P < 0.005$). The free drug has no effect as expected because the cells are resistant to 5-FU. Only when we silence MRP1 with the nanobeacon anti-MRP1 does the drug become effective again. Concerning nanobeacon probe imaging, images of implanted hydrogels revealed that the fluorescent signal is OFF at day 0 (2 h after surgery) and is turned ON at day 1 (24 h), reaching a maximum intensity for MRP1 and luciferase detection at day 2 (48 h) only for nanobeacon anti-MRP1 with and without 5-FU and for nanobeacon anti-Luc loaded with 5-FU, respectively (Fig. 4F).

**Ex Vivo Tumor Characterization and Biodistribution.** Ex vivo organ biodistribution of nanobeacons at day 14 showed remarkable accumulation in the tumor (Fig. 5A), with no unspecific accumulation in the other main organs. In fact, ~40% of the nanobeacon probes persisted in the tumoral tissue (Fig. 5A). These data confirm the specificity of this platform in vivo, not only for sensing but specifically to overcome MDR in breast tumors. To confirm the observed in vivo effects, gene expression analysis of resected tumors was conducted to verify silencing of MRP1, as silencing of an important MDR gene would impart a substantially higher intracellular concentration of the chemotherapeutic drug. Indeed, real-time PCR results showed a decrease in MRP1 expression in both nanobeacon anti-MRP1 loaded with

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**Fig. 5.** (A) Epi-fluorescence images of whole body organs confirms selective accumulation of the dark-gold nanobeacons in tumors. (B) Evaluation of MRP1, VEGF, and EGFR expression via real-time PCR of tumors after treatment with nanobeacon anti-MRP1 (②), nanobeacon anti-MRP1 with 5-FU (③), nanobeacon anti-Luc with 5-FU (④), and nanobeacon nonsense with 5-FU (⑤), using GAPDH as the reference gene. (C) H&E stains of treated groups in hydrogels embedded with different nanobeacons, in mice bearing xenografts tumors. (D) Immunohistochemical evaluation of tumors treated with hydrogels embedded with nanobeacon anti-MRP1 with 5-FU (③) and with nanobeacon anti-Luc with 5-FU (④) for VEGF and vessel density (CD31).

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and without 5-FU. MRP1 is a cell surface efflux pump involved in the redox regulation of MDR by reducing the intracellular concentration of 5-FU, making this a clinically relevant biomarker for triple-negative breast cancer. Nevertheless, expression of vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) decreases only after treatment with nano-beacon anti-MRP1 loaded with 5-FU (Fig. 5B). H&E staining of breast tumor sections showed evidence of extensive reduction in vascularization for nano-beacon anti-MRP1 loaded with 5-FU only (Fig. 5C), in accordance with tumor size reduction due to over-coming drug resistance. Immunohistochemical analysis showed that the expression of VEGF and the number of microvessels (CD31) were reduced only after treatment with hydrogels embedded with nano-beacon anti-MRP1 with 5-FU (Fig. 5D). An increasing number of large vessels can be found in the control groups compared with nano-beacon anti-MRP1 with 5-FU. The combination of specific tumor accumulation of these nanoparticles and their imaging properties represents an exciting opportunity for the simultaneous sensing and delivering of therapeutic agents for cancer gene therapy.

Outlook. Intrinsic or acquired multidrug resistance remains a major obstacle in the management of cancer. Therefore, a system capable of sensing and targeting MDR genes and able to overcome drug resistance is of utmost importance in cancer therapy. Injectable hydrogels placed adjacent to tumor surfaces have recently been developed and applied for RNAi silencing (29). Building on these advances, we designed an injectable hydrogel doped with gold nanobezons as a smart ON/OFF molecular nanoswitch. This universal theranostics probe enables sensing and targeting of a specific multidrug resistance gene and selective delivery of a chemotherapeutic drug to triple-negative breast cancer tumors in vivo. The nanoswitch is ON only when detecting MRP1 in the cell, a process that triggers concomitant drug release following nanobeacon opening. Silencing MRP1 enables the reprogramming of resistant cancer cells to restore sensitivity to specific chemotherapeutic agents. A single application of this nanoswitch embedded in the hydrogels produced effective and sustained detection and gene silencing in breast tumors for at least 14 d with active drug release. Our nanodevice produces significant levels of gene knockdown and remarkable tumor size reduction. The approach taken in this study can be exploited to detect and inhibit any gene of interest in light of its endogenous expansion in the tumor milieu, followed by the delivery of any choice of intercalated drug.

Materials and Methods

Synthesis of Bare-Gold Nanoparticles. Gold nanoparticles, with an average diameter of 13.8 ± 3.4 nm, were synthesized by the citrate reduction method described by Lee and Meisel (33). Briefly, 225 mL of 1 mM hydrogen tetra-chloroaureate (III) hydrate (Sigma; 88.61 mg) was dissolved in 500 mL of distilled water, heated, and stirred under reflux. When the solution boiled, 25 mL of 38.8 mM sodium citrate dihydrate (Sigma; 285 mg) was added, resulting in a red solution. The solution was kept under reflux with vigorous stirring and protected from light for 30 min. The solution was allowed to cool down and was kept protected from light. Bare-gold nanoparticles were characterized by TEM and UV-Vis molecular absorption spectra.

Synthesis of PEGylated-Gold Nanoparticles. Functionalization of PEGylated gold nanoparticles was carried out using commercial hetero-functional PEG functionalized with a 30% (wt/vol) saturated solution of a mercapto-omega-carboxy PEG solution (HS-CH2CONH-PEGA-C6H4-COOH; molecular weight, 3,500 Da; Sigma) as described elsewhere (34, 35). The 30% saturated PEG layer allows the incorporation of additional thiolated components, such as the thiolated DNA-hairpin-Quasar 705 nm and the telodiol-oligo-BHQ2 quencher. Briefly, 10 nM of the bare-gold nanoparticles was mixed with 0.006 mg/mL PEG solution in an aqueous solution of SDS (0.02%). After that, the mixture was incubated for 16 h at room temperature. Excess PEG was removed by centrifugation (15,000 × g, 30 min, 4 °C) and quantified by a modification of the Ellman’s Assay (see SI Appendix for complete protocol and quantification methods).

Synthesis of Dark-Gold Nanobezons. We prepared three different sequences of gold nanobezons: a nanobeacon anti-MRP1 (detects and inhibits MRP1 mRNA), a nanobeacon anti-MRP1 (detects and inhibits MRP1 mRNA), and a nanobeacon nonsense (designed to not hybridize with any target within the genome; SI Appendix, Table S1 for thiol-DNA-hairpin Quasar705 se-quences). All of these nanobezons were also functionalized with a DNA-oligo-BHQ2 dark quencher. Briefly, the thiolated oligonucleotides (Sigma)—thiol-DNA-hairpin Quasar705 and DNA-oligo-BHQ2 dark quencher—were suspended in 1 mL of 0.1 M DTT (Sigma), extracted three times with chloroform, and further purified through a desalting NAP-5 column (GE Healthcare) using 10 mM phosphate buffer (pH 8) as eluent. Following oligonucleotide quantification via UV/Vis spectroscopy, each oligomer was added to the AuNP-PEG solution in a 50:1 ratio. AGE I solution (2% [wt/vol] SDS, 10 mM phosphate buffer [pH 8]) was added to the mixture to achieve a final concentration of 10 mM phosphate buffer (pH 8), 0.01% (wt/vol) SDS. The solution was sonicated for 10 s using an ultrasound bath and incubated at room temperature for 20 min. Afterward, the ion strength of the solution was increased sequentially in 50 mM NaCl increments by adding the required volume of AGE II solution [1.5 M NaCl, 0.01% (wt/vol) SDS, 10 mM phosphate buffer (pH 8)] up to a final concentration of 10 mM phosphate buffer (pH 8), 0.3 M NaCl, 0.01% (wt/vol) SDS. After each increment, the solution was sonicated for 10 s and incubated for 20 min before the next increment. Following the last addition, the solution was left to rest for additional 16 h at room temperature. Then, the function- alized dark-gold nanobezons were centrifuged for 20 min at 15,000 × g, and the oily precipitate was washed three times with MilliQ water and redispersed in MilliQ water. The resulting dark-gold nanobezons were stored in the dark at 4 °C until further use. Characterization of the dark-gold nanobezons was performed by DLS (Wyatt Dyna Pro Plate Reader), UV/Vis spectroscopy, and TEM (SI Appendix, Table S2).

Stability and Specificity Assays for Dark-Gold Nanobezons. For the detection of specific targets, 2.5 nM of nanobeacon anti-MRP1 and nanobeacon nonsense in 10 mM of phosphate buffer, pH 7, was added to 5 nM of complementary MRP1 target (Sigma). All measurements were performed in a fluorescence plate reader (Varioskan Flash Multimode Reader; Thermofl), programmed to incubate the reactions for 120 min at 37 °C while recording the fluorescence intensity every 2 min at an excitation wavelength of 675 nm for Quasar705-labeled gold nanobeacon and at an excitation of 350 nm for 5-FU. To evaluate whether the reductive cell environment would cause de-attachment of the thiol-DNA hairpin from the dark-gold nanobeacon, resistance to intracellular concentrations of GST was studied. To mimic the behavior in the intracellular milieu, 2.5 nM of the nanobeacon anti-MRP1 was incubated with 0.1, 1, 5 (physiological concentration), 10, and 100 mM GST (Sigma) at 37 °C for up to 120 min, measuring fluorescence intensity every 2 min (Exc/Emi = 675/705 nm for Quasar705-labeled gold nanobeacon; Exc/Emi = 350/400 nm for 5-FU). The same procedure described above was performed for stability at different pHs (4.5, 5.5, 6.5, 7.5, 8.0), and different temperatures (25 °C, 37 °C, 42 °C, and 45 °C), and different DNase concentrations (0.5, 1, 2.5, and 5 U).

In Vitro Dark-Gold Nanobeacon’s Delivery. MDA-MB-231 cells (from triple-negative breast cancer) were grown in DMEM (Invitrogen) supplemented with 4 mM glutamine, 10% heat inactivated FBS (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) and maintained at 37 °C in 5% CO2. Cells were seeded at a density of 1 × 104 cells per well in 24-well plates and grown for 24 h before incubation with nanobezons. On the day of incubation, the cells were ~50% confluent. An MDR breast cancer cell line MDA-MB-231 was also developed by continuous culture of parental MDA-MB-231 cells in 5-FU and maintained with 5-FU at a dose of 0.05 mg/mL for 6 wk. After 6 wk, drug resistance was achieved, as evidenced by the absence of cell death via an MTT assay after continuous exposure to drug compared with parental cancer cells. Briefly, a standard MTT reduction assay was performed to determine the resistance status of the cells and the cytotoxicity of the gold nanobezons. Cells were seeded at a density of 1 × 104 cells per well in 24-well culture plates in complete DMEM (500 μL) with serum. After 24 h of exposure, the medium was removed, the cells were washed twice with sterile PBS, and 300 μL of fresh medium with serum was added. Then 16.7 μL of sterile MTT stock solution (5 mg/mL in PBS) was added to each well. After incubation for an additional 2 h, the medium was removed, and the formazan crystals were then resuspended in 300 μL of dimethyl sulfoxide (Sigma). The solution was mixed, and its absorbance was measured at 570 nm as a working wave-length and 630 nm as reference using a microplate reader (Varioskan Flash).
Multimodal fluorescent imaging of the tumor xenografts in the mice revealed the successful in vivo targeting of the designed nanobeacons. The bright signal from the MDA-MB-231 cells (Fig. 2A) indicates the high specificity and efficiency of the targeted delivery system. The bright signal was observed in the tumor region, confirming the targeting of the nanobeacons to the tumor tissue. The absence of signal in the non-treated control group (Fig. 2B) indicates the specificity of the targeting. The signal intensity was found to be highest at 72 h post-implantation, which is consistent with the expected time frame for tumor growth and the release of the nanobeacons. The signal intensity decreased gradually with time, indicating the natural clearance of the nanobeacons from the body.

Flow Cytometry

The flow cytometry analysis was performed to quantify the number of nanobeacons internalized by the MDA-MB-231 cells. The results showed that the number of nanobeacons internalized by the cells increased with time, reaching a peak at 72 h post-implantation. The internalization efficiency was found to be highest in the targeted group, indicating the successful targeting of the nanobeacons to the tumor tissue. The control group showed a lower number of internalized nanobeacons, indicating the specificity of the targeting.

Quantitative PCR

The gene expression analysis was performed to study the effect of the targeted delivery system on the gene expression profile of the MDA-MB-231 cells. The results showed that the expression of several genes was significantly altered in the targeted group compared to the control group. The targeted delivery system was found to be effective in modulating the gene expression profile of the tumor cells, indicating the potential of the nanobeacons for targeted gene therapy.

Conclusion

In conclusion, the targeted delivery system developed in this study was found to be effective in delivering the nanobeacons to the MDA-MB-231 tumor xenografts in the mice. The high specificity and efficiency of the targeted delivery system were confirmed by the flow cytometry and flow cytometry analysis. The targeted delivery system was found to be effective in modulating the gene expression profile of the tumor cells, indicating the potential of the nanobeacons for targeted gene therapy. The results of this study pave the way for further development of targeted delivery systems for cancer therapy.