Curcumin–Combretastatin Nanocells as Breast Cancer Cytotoxic and Antiangiogenic Agent

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14. ABSTRACT
The focus of our concept was to develop a novel therapeutical nanodevice aiming to deliver the anticancer natural compound curcumin specifically to breast cancer cells. We have successfully fabricated two kinds of nanocarriers, the single-walled carbon nanotubes (SWCNT) and the colloidal nanogels, and loaded them with curcumin. We have demonstrated that while the nanocarriers themselves are non-toxic to the breast cancer cells, the loaded nanoconstructs are significantly more cytotoxic than curcumin alone, in particular, in nanogel formulation. Our future research will focus on targeting the curcumin-loaded nanoconstructs with the antibodies against breast cancer cells surface receptors.

15. SUBJECT TERMS
Nanotechnology-based cancer therapeutics, natural anticancer compounds, curcumin, single-walled carbon nanotubes, colloidal nanogels

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INTRODUCTION: The key challenges in cancer therapy are the delivery of the anticancer drugs directly to the cancer cells and the drug's side effects. Thus, it's imperative to identify new anticancer agents that are non-toxic and highly effective to induce cell death, while preventing damage to the normal cells. Nanotechnology, the intersection of materials science and chemistry, is allowing advances that were never before possible in delivering therapeutic agents at desired rates exactly where needed in the body. These drug-loaded nanovehicles are capable of circumventing the systemic toxicity and adverse effects associated with conventional chemotherapy. The research brings doctors one step closer to being able to inject patients with nanoparticles that bore inside tumors and release powerful doses of cancer-killing drugs while leaving the rest of the body unscathed. Our proposal centers on one of the natural compounds, the potent anticancer agent curcumin that specifically kills cancer cells, while being non-toxic to the normal cells. Curcumin (diferuloylmethane) is one of the most well known naturally occurring compounds, a polyphenol derived from the plant *Curcuma longa*, commonly called turmeric. Turmeric holds a high place in Ayurvedic medicine as a "cleanser of the body" with anti-oxidant, anti-inflammatory and anti-carcinogenic properties. People whose diets are rich in turmeric have lower rates of breast cancer as well as prostate, lung and colon cancers (1, 2). Recent research at the M.D. Anderson Cancer Center in Houston suggests that curcumin may help prevent the spread of a breast cancer (3, 4). The anticancer potential of curcumin stems from its ability to suppress proliferation of a wide variety of tumor cells, modulate transcription factors (e.g. NF-kappa B, AP-1 and Egr-1), down-regulate the expression of COX2, LOX, iNOS, MMP-9, uPA, cytokines TNF, IL-1 IL-6 and chemokines, growth factor receptors EGFR and HER2, cell cycle proteins p21 and cyclin D, cell surface adhesion molecules, and inhibit the activity of c-Jun N-terminal kinase, protein tyrosine kinases and protein serine/threonine kinases (1, 5-8). Studies have shown that multidrug-resistant breast cancer cell line (MCF-7/TH carcinoma) is 3.5 fold more sensitive to curcumin than the mammary epithelial cell line MCF-10A (3). Curcumin induced G_2 block and sub-G_0/G_1 cell population respectively and down-regulation of Ki67, PCNA, p53 mRNA in breast cancer cells and a down-regulation of p21 and an up-regulation of Bax mRNA expression in human mammary epithelial cells, suggesting that apoptosis is involved in the curcumin-induced inhibition of tumor growth, and genes associated with cell proliferation and apoptosis may be playing a role in the chemopreventive action of curcumin (7). Importantly, curcumin is also a potent radio sensitizer that down regulates radiation-induced prosurvival factors and enhances radiation sensitivity of cancer cells. We have previously shown that incubation of prostate cancer cells with curcumin prior to radiation increased radiation-induced apoptosis in these cells (9). In addition to its application in cancer therapy, studies suggest that curcumin may be able to treat and prevent Alzheimer's disease (10-12), attenuate diabetic encephalopathy (13) and could correct a defect associated with a chloride channels in cells that causes the most common form of cystic fibrosis (14-16). We proposed to take advantage of the nanoscale drug delivery system combining the curcumin with anti-angiogenesis agent such as combretastatin as a safer and more efficient alternative to systemic delivery of the conventional drugs. The use of such nanoconstructs will allow the temporal and staged release of curcumin and combretastatin to specifically target both the breast epithelial cancer cells and the tumor vasculature in the tumor microenvironment. Based on this approach we hypothesized that this dual targeting therapy using cancer-cell specific agent curcumin and anti-angiogenesis agent combretastatin will lead to increased tumor control and protect normal cells. The use of nanoconstructs containing both the non-toxic curcumin in high doses and combretastatin will allow to achieve localized cytotoxic effect in tumor with simultaneous killing of endothelial cells respectively. Two types of nanocarriers, inorganic single-walled carbon nanotubes (SWCNT) and polymeric nanogels, were selected for loading with curcumin. SWCNT have been shown to shuttle various cargoes across cellular membrane without cytotoxicity. The second polymer-based carrier was chosen in attempt to overcome curcumin's poor systemic bioavailability following oral administration that limits the widespread clinical application of this agent. Since curcumin is a natural hydrophobic polyphenol, it has a low aqueous solubility and bioavailability challenging its therapeutic efficacy. We developed and evaluated novel colloidal nanogel carrier for encapsulation of curcumin to increase its solubility and cytotoxicity.

ImageStream multispectral imaging flow cytometry and a real-time cell electronic sensing were used to measure the response of various breast cancer cells to treatments with both nanoformulations of curcumin compared with free curcumin. Our results indicated that the nanogel formulation of curcumin was 70-85% more effective in killing cancer cells, even at concentrations lower than IC_{50} of free curcumin. This was also confirmed morphologically by modified acridine orange/ethidium bromide staining assay and fluorescent microscopy. Importantly, nanocarriers alone displayed practically no cytotoxicity. Curcumin is non-toxic to normal tissues; therefore, it can be used in much higher doses than conventional chemotherapeutic drugs. We conclude that nanogel carriers offer an innovative way to encapsulate curcumin and to obtain more effective anticancer therapeutics than curcumin alone with a potential to specific tumor targeting, e.g. using antibodies against surface receptors of breast cancer cells such as IGFR and HER2.

BODY: The following Statement of Work was proposed:

Task 1. **To fabricate the biodegradable curcumin-combretastatin nanocells and evaluate the nanoconstructs. This will be done in collaboration with the National Cancer Institute.**

a. Nanocells will be characterized by the release kinetics using reverse phase HPLC and EC50

b. Characterize nanoconstructs by the efficacy and toxicity studies.

Originally, this task was proposed to be accomplished in collaboration with the National Cancer Institute. We have approached Dr. Robert Blumenthal, Program Director for the CCR Nanobiology Program, NCI-Frederick (Frederick, MD 21702-1201) and Dr. Pradman K. Qasba, Senior Investigator, CCR Nanobiology program with the collaboration proposal. However, due to the conflict of interests, this collaboration was not considered possible. Instead, we initiated collaboration with Dr. Eric Wickstrom (Professor, Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, PA) to fabricate curcumin-loaded single-walled carbon nanotubes, and with Dr. Serguei V. Vinogradov (University of Nebraska Medical Center, Omaha, NE) to synthesize curcumin-loaded colloidal nanogels. We thought it reasonable to explore chemically and structurally different kinds of carriers for drug delivery in order to achieve the maximal effect in killing the cancer cells. In addition, our proposal included using dual-targeted nanoconstructs that would include both the anticancer agent (curcumin), and anti-angiogenic agent combretastatin. However, we had to request the extension on this part of the research that involves testing the nanoconstructs in co-culture of breast cancer cells and HUVEC human endothelial cells due to the unforeseen delay in obtaining combretastatin from OXiGENE, Inc. We had submitted the request for the extension and had received the approval promptly. The final report date has been changed to 30 March 2008. On January 16, before combretastatin was obtained from OXiGENE, the PI of this project, Anna Reeves, was involved in a major car accident (she was hit head-on by a drunk driver), that resulted in her disability: an additional extension had been requested and the final report date had been moved up to 30 September 2008. Due to above-mentioned circumstances the studies involving combretastatin have not been accomplished, and thus not included in the following report.

The synthesis and characterization of curcumin-SWCNT and curcumin-nanogels are described below.

SWCNTs (single-walled carbon nanotubes).

It is well established now that various inorganic nanomaterials, including nanowires, nanoshells, nanocrystals, and nanotubes, exhibit physical properties promising for cancer imaging and therapy (17-19). Carbon nanotubes belong to the family of fullerenes and consist of graphite sheets rolled up into a tubular form. These structures can be obtained either as single- (characterized by the presence of a single graphite sheet) or multi-walled (formed from several concentric graphite sheets) nanotubes (20, 21). Carbon nanotubes can apparently cross the cell membrane as “nanoneedles” without perturbing or disrupting the membrane and localize into cytosol and mitochondria. However, the mechanisms are poorly understood (22, 23). Single-walled carbon nanotubes (SWCNT) have been shown to shuttle various cargoes across cellular membrane

(19). It has been recently shown that NIR (near infrared light) can heat up the bundles of SWCNT (nanobombs) and cause their explosion (24). This is highly important in biomedical applications as it's known that cells are transparent to NIR (700-1100 nm wavelength) light. Thus, targeted SWCNT can be used as cancer therapeutics by themselves or with encapsulated drugs that could be released upon exposure to the NIR light. Our future research plans in collaboration with Prof. Wickstrom involve using the NIR light to release curcumin from the SWCNT.

Preparation of the SWCNT and loading them with curcumin.

1. Preparation of SWCNT solution. 90% pure SWCNTs (Nano-Lab (www.nano-lab.com), Catalog number D1L110-P, Lot number 06091501) were subjected to the acid washing step. Briefly, 300 mg of the SWCNTs were added to 200 mL of acid mixture of sulfuric and nitric acid (3:1 respectively) and mixed in the reflux apparatus at 80˚C with water flow turned on to condense acid vapors for at least eight hours;
2. The mixture was filtered using glass fiber filter and filter housing to separate out the SWCNTs that remained as a solid residue on the filter;
3. Filtered SWCNTs were washed with DI water until the pH of the drained solution reached 4 or higher;
4. Washed SWCNTs were resuspended in 300 mL of the sterile phosphate buffered saline solution (PBS) and agitated for one hour at room temperature in ultrasonicator (Fisher Scientific FS60H) to break the SWCNTs bundles into individual nanotubes. The solution was then microcentrifuged at 20,000 x g for 30 minutes. Sediments containing mostly large conjugations of SWCNTs were discarded and supernatants were collected.

SWCNTs were loaded with curcumin as follows:

5. Pellet was resuspended in 10 mL 70% isopropanol to sterilize, then sedimented 10 min at 20,000 x g. Supernatant was poured off.
6. Pellet was resuspended in 30 mL sterile PBS. 0.3 mL of 1.0 mM curcumin in ethanol was added to a final concentration of 10 μM, then incubated overnight at 37˚C.
7. Suspension was sedimented 5 min at 4,000 rpm, 3,300 x g. Supernatant was poured into autoclaved glass 15 mL centrifuge tubes, then sedimented 10 min at 20,000 x g. Absorbance spectrum vs. control sample without curcumin indicated 10 μM curcumin, 1.5 mg/L SWCNT.

Loaded with curcumin SWCNTs along with the treatment with curcumin alone were used for *in vitro* experiments using breast cancer cell lines.

Nanogels.

Curcumin has undoubtedly demonstrated a considerable promise as a potent and safe natural compound for cancer therapy and prevention. However, widespread clinical application of this agent has been limited due to its poor systemic bioavailability following oral administration. Due to curcumin's nonpolar nature, it is not easily soluble in water and most of consumed curcumin does not reach the bloodstream. In several Phase I clinical trials where patients were administered up to 10-12 g of oral curcumin daily, only trace amounts of the compound and its conjugates were detected in the peripheral circulation. Recently, in Phase II clinical trials oral curcumin was well tolerated and, despite its limited absorption, demonstrated promising biological activity in some patients with advanced pancreatic cancer (25). One way to overcome the problem of curcumin's poor bioavailability is to develop a delivery system that can render this hydrophobic polyphenol soluble in an aqueous phase medium. Recently, two liposomal formulations of curcumin have been reported. Li et al demonstrated that their liposomal formulation of curcumin had comparable to the free curcumin *in vitro* and *in vivo* inhibitory effects on proliferation, apoptosis, cell signaling and angiogenesis (26). In the most recent report (2008) the treatment of two prostate cancer cell lines with liposomal curcumin (5-10 μM) led to 70-80% inhibition of cell proliferation without cell death, whereas the same extent of inhibition required ten times higher dose of free curcumin (27). The advantage the liposomes provided is the delivery in an aqueous phase, which avoided the use of solubilizing agents

that cause side effects including hypersensitivity reactions. Anirban Maitra, a professor of pathology and oncology at Johns Hopkins, and his collaborators in Delhi-including his father, Amarnath Maitra, a professor of chemistry-used polymer-based carriers to encapsulate curcumin (28). The particles about 50 nm in diameter were synthesized by co-polymerization of Nisopropylacrylamide (NIPAAM), with N-vinyl-2-pyrrolidone (VP) and poly(ethyleneglycol)monoacrylate (PEG-A). The hydrophobic interior of the particles holds curcumin and hydrophilic exterior makes the particles soluble. This way, they can pass easily from the gut to the bloodstream. Once in the blood, the curcumin leaks out as the polymers slowly degrade. Using several pancreatic cancer cell lines, the Johns Hopkins team has shown that both free curcumin and nanocurcumin caused inhibition of clonogenicity at 10 and 15 μM dosages and nanocurcumin retained free curcumin's ability to activate key events of cellular apoptosis. However, we believe that larger size of the nanoparticles would improve their accumulation in the tumor due to an enhanced permeation and retention (EPR) effect. This "suction" effect arises from the unique morphology of tumor vessels, tortuous and leaky due to the enhanced and aberrant neovascularization process (29). The size of the gap junctions between endothelial cells of tumor vasculature varies between 100 and 600 nm. Normally, circulating non-modified nanoparticles larger than 150-200 nm are captured by the RES (reticuloendothelial system) such as macrophages of the liver and spleen (30). Consequently, the nanoparticles should be large enough to avoid leakage into the blood capillaries, but small enough to escape capture by RES, i.e. between 100 and 150 nm. Based on this assumption we selected the nanoparticles ranging in size between 100 and 200 nm for loading with curcumin. Curcumin was encapsulated into polymeric-based carrier, the colloidal nanogels, a recently developed by Dr. Vinogradov and his colleagues a new family of carriers for encapsulation and delivery of drugs and biomacromolecules (31-38). Colloidal microgels have recently received attention as environmentally responsive systems and now are increasingly used in applications as carriers for therapeutic drugs and diagnostic agents. These nanogels are made from a cross-linked network of polyionic (e.g. polyethylenimine, polyacrylic acid, poly-L-lysine etc.) and neutral polymer chains (e.g. PEG, Pluronic/Poloxamer, polyvinyl alcohol, etc.). They contain water-filled interior volume and have excellent dispersion stability. Depending on chemical nature of polymers used in the synthesis, nanogels bind and encapsulate drug molecules with opposite charge, via hydrophobic interactions, hydrogen bonding or due to participation of all these forces. When oppositely charged molecules are associated with nanogel, the whole network becomes more compacted forming core-shell nanoparticles with a diameter between 50 and 150 nm. Stabilized by highly hydrated PEG corona surrounding drug-loaded core, these particles form stable aqueous dispersions. Nanogels have been tested as a potential carrier for oligonucleotide delivery to the brain (34). After intravenous injection of ODN-nanogels in mice, no adverse toxic effects were observed and increased brain and decreased liver/spleen accumulations were noted, compared to the free ODN. Importantly, injected doses of drug-loaded nanogels (50 mg/kg) were well tolerated by animals. There are a number of advantages of proposed nanogel formulation of curcumin over other delivery systems, such as PLGA nanoparticles, liposomes and polymer micelles. The curcumin-nanogel formulations are prepared by mixing the drug solution with aqueous nanogel dispersion with simple post-treatment, thus avoiding the complicated procedures of synthesis of nanoparticles in the presence of encapsulating drug or liposome preparation. High drug loading of the curcumin-nanogel formulations can provide an increased drug concentration in the targeted site. The nanoformulation can greatly reduce an exposure of curcumin to biological degradation after systemic administration.

Preparation and characterization of nanogel NG127-curcumin complexes.

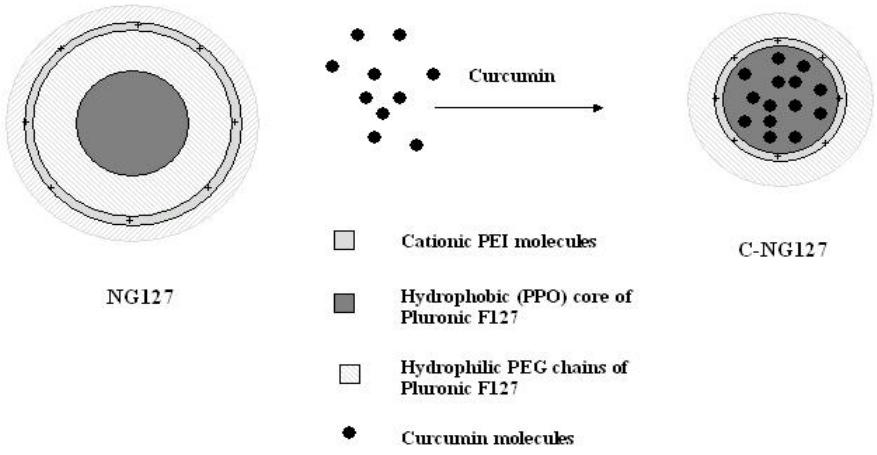
Nanogel synthesis

In the present study we synthesized a biodegradable nanogel network of Pluronic F127 with spermine (NG127) by a stepwise micellar synthesis as described previously with minor modifications (32) (37). In the synthesis we used a biodegradable version of the polycation (SPss) consisted of spermine molecules interconnected by disulfide bridges cleavable in the reducing intracellular environment. The SPss was obtained by cross-linking of spermine molecules with an equimolar amount of dimethyl 3,3'-dithiobispropyonimidate.2HCl (DTBP) (33). An average Mw of the synthesized SPss was equal to ca. 9,000

Da as determined by capillary viscosimetry using samples of PEI with various Mw as standards. The input mass ratio of F127, SPss and PEG (Mw 4,600 Da) components in the synthesis was equal to 3:1:1. NG127 nanogels were isolated from precipitating fraction by centrifugation (3000 rpm, 30 min), dialyzed against diluted HCl for 24 h and lyophilized. ^{1}H-NMR spectrum integrals at δ 4.5 (C$\underline{H_2}$-O of PEG) and δ 2.3-3.5 (C$\underline{H_2}$-N of spermine) were close to the used input PEG/SPss ratio in NG127 nanogels, as well as the total nitrogen content (3.5 μmol/mg) determined by elemental analysis (M-H-W Laboratories, Phoenix, AZ).

Fluorescently labeled NG127 was synthesized by modification of ca. 1% of primary amino groups with rhodamine B isothiocyanate and isolated by gel filtration using NAP-25 columns (GE Amersham, Parsipany, NJ) (34). Fluorescent spectral characteristics of the rhodamine-labeled SP127 (λ_{ex} 549 nm/λ_{em} 577 nm) were measured in phosphate buffered saline, pH 7.4, using a Shimadzu RF5000 spectrofluorimeter. The NG127 contained hydrophobic core and hydrophilic envelope consisted of the biodegradable spermine copolymer cross-linked with the activated ends of F127 and PEG molecules via urethane bond rapidly degrading in aqueous media at pH 4-5. Dynamic light scattering (DLS) of the aqueous solution of NG127 revealed formation of polymeric micelles with an average hydrodynamic diameter of 180 nm. These particles were employed for preparation of complexes with curcumin (Fig.1).

Figure 1



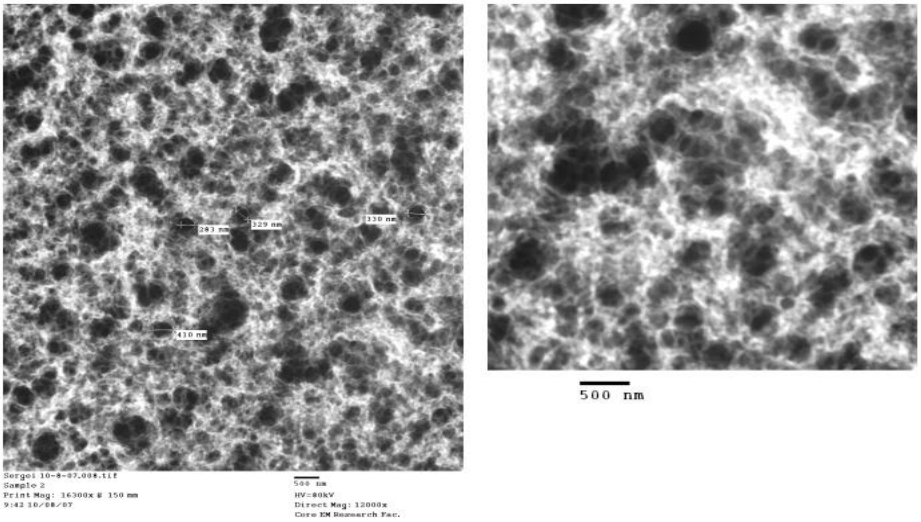
Curcumin encapsulation into nanogels.

A biodegradable nanogel network of Pluronic F127 with spermine (NG127) was used for loading with curcumin. This NG127 contained hydrophobic core and hydrophilic envelope and have an average hydrodynamic diameter of 180 nm. Curcumin loading resulted in nanogel compaction forming particles with the hydrodynamic diameter of 145 $\underline{+}$ 9 nm dispersed in the homogeneous red solution with pH 6.5.

When curcumin solution in DMSO was added to the aqueous solution of SP127, we detected formation of particles with an average diameter of 145 $\underline{+}$ 9 nm dispersed in the homogeneous red solution with pH 6.5, Following formation of the drug-loaded complex, the curcumin-nanogel (C-NG) was isolated by size-exclusion chromatography (SEC) and stored in frozen form. The drug-loaded nanogel was eluted with exclusion volume as dark-red band. Complex formation was accompanied by the observed color change from orange of the curcumin solution in DMSO to dark-red of the aqueous solution of the C-NG complex and the spectral shift of the absorbance maximum from 425 nm to 400 nm. Nanogel-encapsulated curcumin (C-NG), unlike the free curcumin, formed stable aqueous dispersions up to the drug content of 30% wt. Curcumin, a natural hydrophobic polyphenol, contains two phenolic hydroxyl groups (pK_a~5) ionized at physiological conditions. These groups interact with protonated amino groups of the cationic nanogel in the interior volume, additionally stabilizing the encapsulated in hydrophobic core curcumin. Our data show that solubility of the C-NG in water could reach 50 mg/ml or 15 mg/ml by curcumin compared to 25μg/ml for curcumin alone. Physico-chemical characterization of C-NG by transmission electron microscopy is shown

in Figure 2. Drug loading resulted in slight nanogel compaction and formation of particles with the hydrodynamic diameter 50-200 nm and polydispersity 0.24.

Figure 2

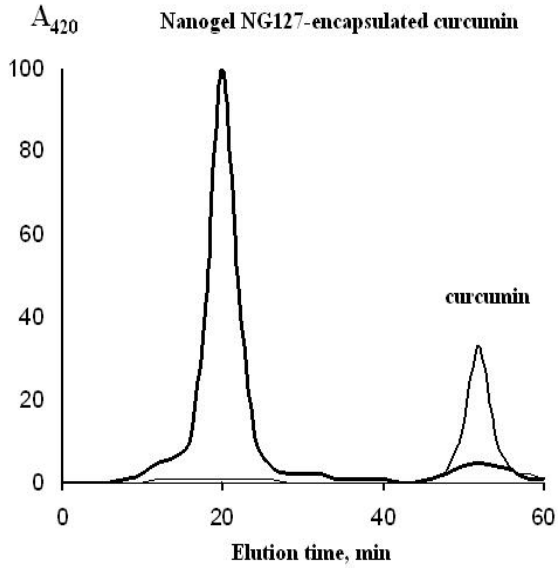


Drug-loaded nanogel particles shown by transmission electron microscopy (TEM).

Curcumin contains two electron-dense phenol rings; after contrasting with vanadate following curcumin encapsulation, the compact particles with electron-dense core were observed with size ranging between 50 and 200 nm.

Compact particles with electron-dense core were observed having size ranging between 50 and 200 nm. Amino groups of empty NG127 could also be covalently modified with rhodamine isothiocyanate (RITC) and used for fluorescent tracing of the nanogel uptake in vitro (λ_{ex} 480/λ_{em} 640 nm). C-NG formulation was characterized by size exclusion chromatography (SEC) on Sepharose S-300 column and showed practically no drug release from the complex with nanogel network (Fig. 3).

Figure 3

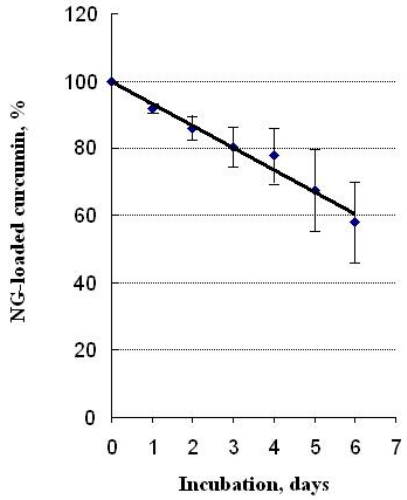


Analysis of one soluble nanogel-curcumin complex formation using size exclusion chromatography (SEC).
Drug-loaded nanogels were isolated by SEC on Sepharose S-300 column. Complex formation was accompanied by the observed color change from orange of the curcumin solution in DMSO to dark red of the aqueous solution of the complex and the spectral shift of the absorbance maximum from 425 nm to 400 nm on Sepharose S-300 column (2 x 70 cm). Running buffer: 0.1 M NaCl, 20% ethanol. Elution: 1 ml/min. UV Detector 420 nm. This complex is stable in physiological conditions (phosphate-buffered saline) and also can be detected by co-migration of the curcumin-absorbing band with a high MW fraction during SEC.

Reverse-phase HPLC was used for the quantitative analysis of curcumin in nanogel formulation injected in methanolic solution using calibration curve for curcumin standard solutions. At high concentration of methanol and pH change from 7.5 to 5, complex stability is dramatically reduced resulting in the release of

free curcumin. By contrast, a sustained drug release (daily 7-10% of loaded curcumin) with the first-order kinetics was observed in PBS at 25°C (Fig. 4).

Figure 4



In vitro curcumin release kinetics.

A sustained drug release (daily 7-10% of loaded curcumin) with the first-order kinetics was observed in PBS at 25°C

CC – curcumin. Drug content in the dialy cell with MWCO 3,400 Da was measured by UV absorbance at 420 nm.

To accomplish Task 1b, the nanoconstructs without curcumin (empty single-walled carbon nanotubes and nanogels) were tested for their toxicity. We have demonstrated that both the empty SWCNTs and nanogels are practically non-toxic to the cells (Fig. 8).

Task 2. To test the effect of nanocells in vitro using breast cancer cell lines alone or in co-culture with endothelial cells.
a.Establish the co-culture of the GFP-positive breast epithelial cell lines MCF-7 and T47D with the human endothelial cell HUVEC. Response of the cells will be measured by TUNEL and flow cytometry.
b. RT-PCR and Western analyses will be performed to analyse the expression of the proapoptotic and prosurvival genes such as NF-kappa B, Bax, Bcl-2, Bcl XL, cytochrome C and caspases.

Three breast cancer cell lines were selected for cytotoxicity studies, the non-metastatic MCF-7 and T47D and highly metastatic MDA-231. The co-culture studies with HUVEC cells could not have been performed for the reasons mentioned above. However, our future plans include both these studies and the analysis of prosurvival and proapoptotic gene expression using RT-PCR and Western analyses as stated in the Statement of Work.

In this present study the cell killing effect of free curcumin and curcumin encapsulated in two types of nanocarriers was evaluated using following assays:

1. intracellular uptake of curcumin formulations by flow cytometry
2. cell viability based on acridine orange/ ethidium bromide staining
3. curcumin formulations cytotoxicity in real-time monitored on microelectronic sensors using a real-time electronic sensing system (RT-CES, ACEA Bioscience Inc, San Diego, CA).

Intracellular uptake of curcumin alone and curcumin-nanogel formulations.

Intracellular uptake of curcumin formulations *in vitro* was assessed using the ImageStream multispectral imaging flow cytometer (Amnis Corp. Seattle, WA). Briefly, the platform produces high-resolution brightfield, darkfield, and fluorescence images of cells prepared in suspension at rates up to 100 cells per second. The IDEAS™ analysis software quantifies over 200 morphometric and photometric parameters for each cell based on its imagery, including parameters that measure sub-cellular location of probes. This technology combines the quantitative power of large sample sizes common to flow cytometry with the high

information content present in microscopic images. MDA-231 cells were treated with free curcumin and with curcumin-nanogel (C-NG) for 24 or 48 hours. The cells were harvested, fixed in 2% paraformaldehide (PFA) and shipped to Amnis. At Amnis, DRAQ5 nuclear dye was added to the samples and imagery acquired on the ImageStream. The MDA-231 cells were 'clumpy' and difficult to run, so the numbers of single cells acquired is about 1000 or less. Results of the analysis of intracellular curcumin in the different cell preparations are shown (Table 1).

Table 1. **Evaluation of intracellular curcumin in MDA-231 cells treated with curcumin alone and curcumin-nanogel (C-NG) formulation.** 67-86% of cells treated with C-NG were positive based on the fluorescence intensity of curcumin, both at 24 and 48 hours after treatment. In contrast, cells treated with free curcumin displayed much lower extent of internalization-almost the background levels at 24 hours, and about 2.5 times higher than untreated cells at 48 hours after treatment. FI-fluorescent intensity.

Cell #	Description	# of Cells (All Cells)	Mean FI of All Cells	% Positive	Mean FI of Positive Cells
1	Untreated	550	4881	3.6	41524
2	Curcumin, 24 h	921	4872	4.7	26546
3	Curcumin, 48 h	757	9254	8.3	28181
4	C-NG, 24 h	846	37835	86.2	42108
5	C-NG, 48 h	890	25393	66.9	31118

The percent of curcumin-positive cells is summarized for the individual groups as is the mean fluorescence intensity (mean FI). The curcumin-nanogel formulation resulted in the highest level of curcumin positive cells in MDA-231 cells.

Additionally, the same preparations of treated and untreated MDA-231 cells were analysed for apoptosis. A number of distinctly characteristic cellular changes occur during the process of apoptosis. Using the ImageStream multispectral imaging cytometer, these changes have been observed to include increased light scatter (dark field imagery), increased intensity modulation of bright field imagery, nuclear condensation and as a consequence, decreased nuclear area and increased nuclear pixel intensity per unit area. These morphometric and photometric features in addition to the classic sub-G0 population observed in traditional cell cycle analysis have been used to assess apoptosis in the cells treated with free curcumin and with both curcumin nanoformulations 24 hours after treatment (Table 2).

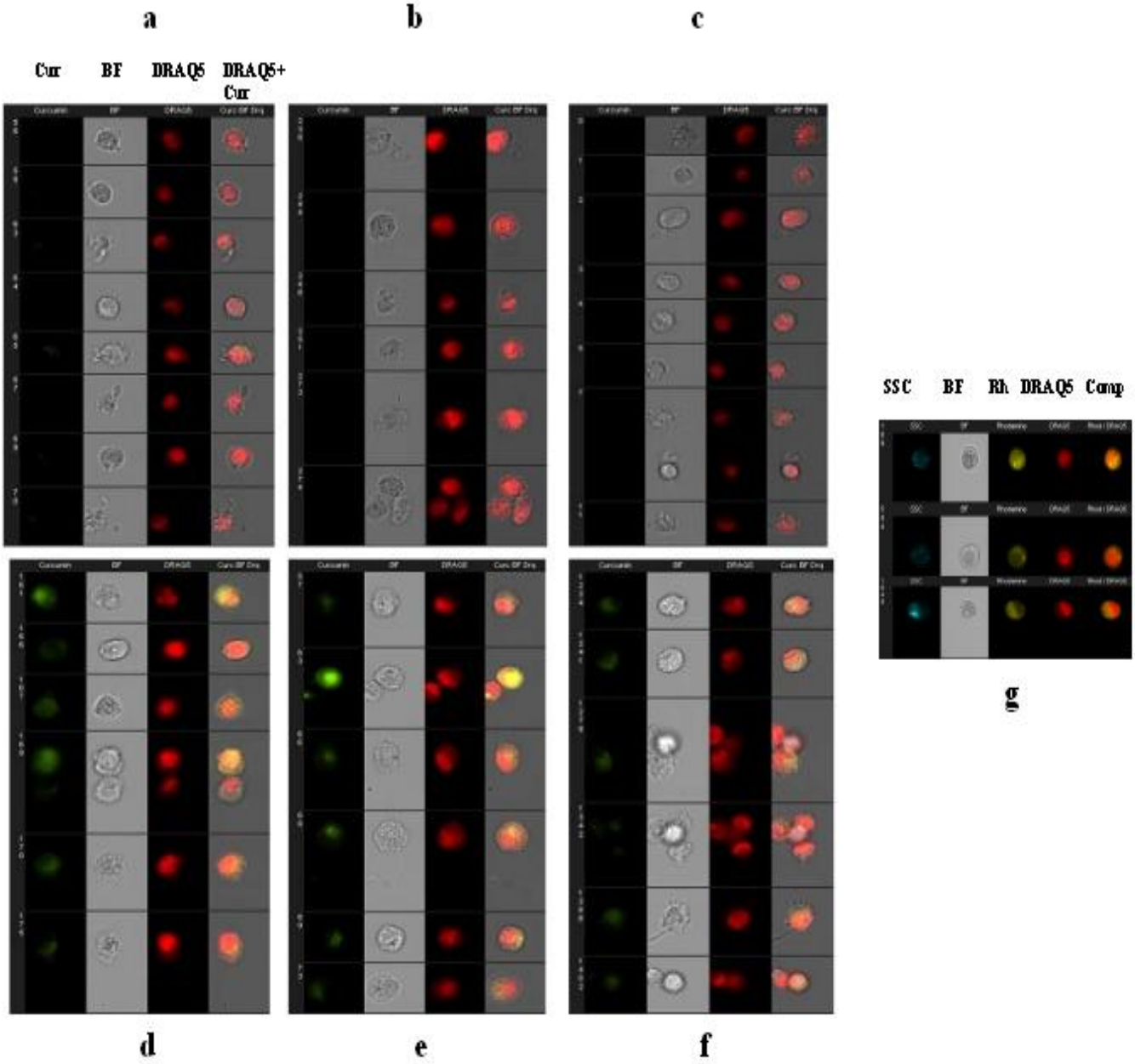
At this time point no significant apoptosis was detected in either treated or untreated cells.

Table 2. **MDA-231 Cells - Apoptosis Values**

	Cell images analyzed	BF Modulation/SSC	Nuclear Max Pixel Area	Nuclear Max Pixel Intensity	Sub G_0
Untreated	7079	4.3%	0.37%	2.35%	1.4%
Curcumin	5225	5.6%	0.1%	0.75%	1.3%
Nanogels	8800	1.8%	0.26%	1.2%	1.2%
C-NG	6659	3.0%	1.2%	2.75%	1.2%
SWNT	8172	3.0%	0.12%	3.0%	3.3%
SWNT Curcumin	10841	2.7%	0.26%	1.7%	1.15%

Cells imagery (Fig. 5)also demonstrates much more efficient internalization of C-NG formulation of curcumin (Fig.5 d-f) compared with free curcumin (Fig.5 b, c) based on both curcumin fluorescence and the rhodamine fluorescence of empty rhodamine-tagged nanogels Fig. 5g).

Figure 5. **Curcumin-positive cells in MDA 231 cells treated with curcumin alone and curcumin-nanogel (C-NG) formulations.** Our results clearly show the efficient internalization of the C-NG into MDA-231 breast cancer cells in contrast to the curcumin alone based on both the curcumin fluorescence (**a-f**) and rhodamine fluorescence (**g**) of the rhodamine-labeled empty nanogels. **a** –Untreated cells; **b** and **c** – free curcumin, 24 and 48 hours after treatment, respectively; **d** and **e**- C-NG, 24 hours, and **f** – C-NG, 48 hours after treatment; **g** – rhodamine-labeled empty nanogels. **Cur** – free curcumin; **BF** – Bright Field; **DRAQ5** – DNA-binding nuclear stain; **SSC** - light scatter (dark field); **Rh** – rhodamine; **Comp** – composite image.



Cell viability based on acridine orange/ ethidium bromide staining

It has been suggested that as a rule, classification of cell death in a given model should always include morphological examination coupled with at least one other assay.

In this study, the cell viability and cytotoxicity of curcumin formulations was analysed based on two assays: the acridine orange /ethidium bromide (AO/EB) staining, and the real-time cell response to treatments which was monitored on microelectronic sensors using a real-time electronic sensing system (RT-CES, ACEA Bioscience Inc, San Diego, CA).

A modified AO/EB staining assay (39) was employed to assess the curcumin formulations-induced cell death. Fluorescence light microscopy with differential uptake of fluorescent DNA binding dyes (such as AO/EB staining) was a method of our choice for its simplicity, rapidity and accuracy. This method doesn't require cell fixation step thus avoiding a number of potential artifacts. Acridine orange (AO) permeates all cells and makes the nuclei appear green. Ethidium bromide (EB) is only taken up by cells when cytoplasmic membrane integrity is lost, and stains the nuclei red. Thus, the live viable cells have a normal green nucleus, and the cells that have died from necrosis or apoptosis will appear orange (Fig. 6A, B). Two breast cancer cell lines, the non-metastatic T47D (A) and highly metastatic MDA-231 (B), were analyzed by this assay. While both cell lines displayed a moderate response to 1 μM curcumin treatment, the C-NG formulation was much more cytotoxic at the dose corresponding 1 μM curcumin, particularly in T47D cell line where the treatment led to a substantial cell death. The observed levels of cell death were similar to those caused by much higher dose of curcumin alone (about 10 μM). Interestingly, the T47D cells seemed to be more sensitive to the C-NG formulation compared to MDA-231 cells. A strong depletion in the cell population was clearly evident at 3 μM of curcumin encapsulated in NG127, while in MDA-231 cells it was effective at 10 μM.

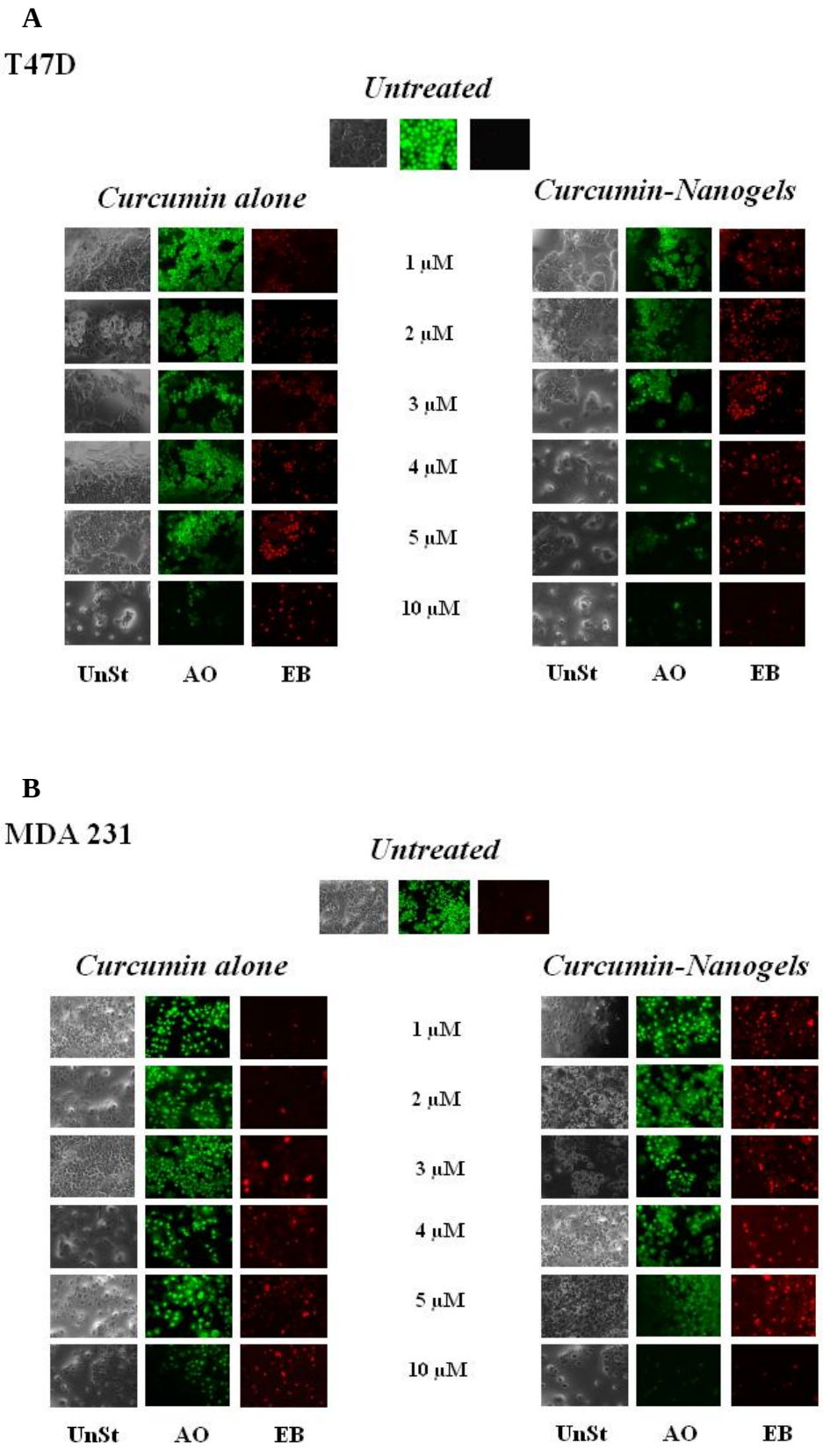


Fig. 6. ***Comparison of curcumin and curcumin-nanogels formulation T47D (A) and MDA-231cells (B) cytotoxicity based on acridine orange/ ethidium bromide staining***
Two breast cancer cell lines were examined using this method T47D (A) and MDA-231 (B). Cells were treated with indicated concentrations of curcumin alone and C-NG and stained 48 hours after treatment. While both of these cell lines displayed a moderate response to curcumin treatment, the nanogel formulation of the curcumin was much more effective in cell killing, particularly in T47D cell line: the dose of the C-NG formulation corresponding to the lowest dose of free curcumin (1 µM) led to a substantial cell death in these cells.

Cytotoxicity of free curcumin and curcumin-nanogel formulations.

A real-time cell electronic sensing (RT-CES) system (ACEA Bioscience Inc, San Diego, CA) was used for label-free, dynamic measurement of cell response to treatments (40). Briefly, cells were grown onto the surfaces of microelectronic sensors, which are comprised of circle-on-line electrode arrays and are integrated into the bottom surfaces of the microtiter plate. Changes in cell status such as cell number, viability, morphology, and adherence were monitored and quantified by detecting sensor electrical impedance. The dynamic responses of the cells to different treatments were continuously monitored by the RT-CES system allowing real-time, continuous monitoring and quantitative recording of the cell's response to the treatments.

The basic principle of the RT-CES system is depicted below (Fig. 6).

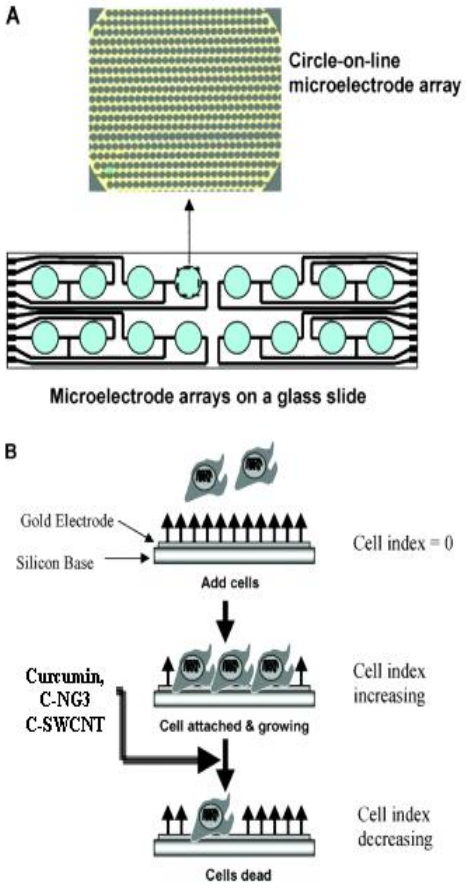


Fig. 6. ***Microelectrode array device and cell electronic sensing used to monitor the real-time curcumin formulations cytotoxicity.***

A. Sixteen x sensor devices with 16 circle-on-line electrode array units are fabricated on glass slides which represent 16 individual detection units with 9 mm as the center to center spacing. An enlarged unit of the circle-on-line electrode array is shown in the top panel.

B. A schematic illustration of the principle of cell detection on the sensor. The presence of cells affects the local ionic environment leading to an increase in electrode impedance. If more cells attach to the electrodes, there will be a large increase in electrode impedance, leading to an increase in cell index (CI). The CI decreases when cells die off, resulted from the exposure to curcumin formulations.

Previously, the pilot experiment was performed to confirm that the CI value obtained on the RT-CES system is quantitatively correlated with the cell growth and to determine the optimal cell number for the following treatment with the drug (Fig. 7, courtesy of Acea Bioscience, Inc).

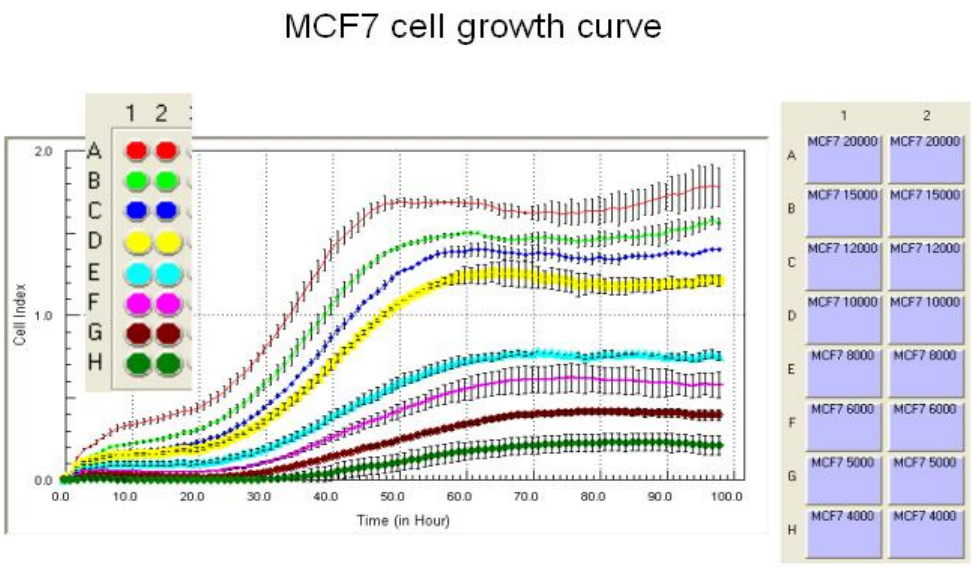


Fig. 7. ***Dynamic monitoring of MCF-7 cell growth on the RT-CES system.*** The growth of the MCF-7 cells on the RT-CES system was dynamically monitored. Cells were seeded in replicates at 4, 5, 6, 8, 10, 12, 15, and $20x10^3$cells/well and proliferation for each cell line was monitored every hour. The growth curves at each cell density (colored wells on the slide, insert) are shown in respective color.

The CI values were found to be linearly correlated with cell growth. Because the sensor device format is compatible to a standard microtiter plate, the starting cell number and cell culture medium volume used for the 16 x sensor device were similar to the cell culture in the standard 96 well microtiter plate. For real-time calculation of CI, the background of impedance for each sensor well was measured in the absence of the cells. To do so, 50 µL of cell culture media was added into each well for the baseline measurement, followed by addition of the cells to the sensor wells. Once the cells were added to the sensor wells, the sensor devices were placed into the incubator and the real-time CI data acquisition was initiated by the RT-CES analyzer under the control of the RT-CES software. To ensure a consistent initial cell condition for cytotoxicity assays, the curcumin formulations were added after CI reached a range between 1.0 and 1.2, dependent on cell types, indicating about 40-50% cell confluence. Following treatment the sensor devices were then mounted back to device stations placed inside a CO2 incubator. The CI was automatically monitored by the RT-CES system. The results of the experiments in all three cell lines treated with nanogel- and SWCNT-encapsulated curcumin are shown in Fig. 8. MDA-231 (A), T47D (B) and MCF-7 (C) cells were seeded into the 16x sensor device, and cell proliferation was then monitored every hour for up to 98 hours.

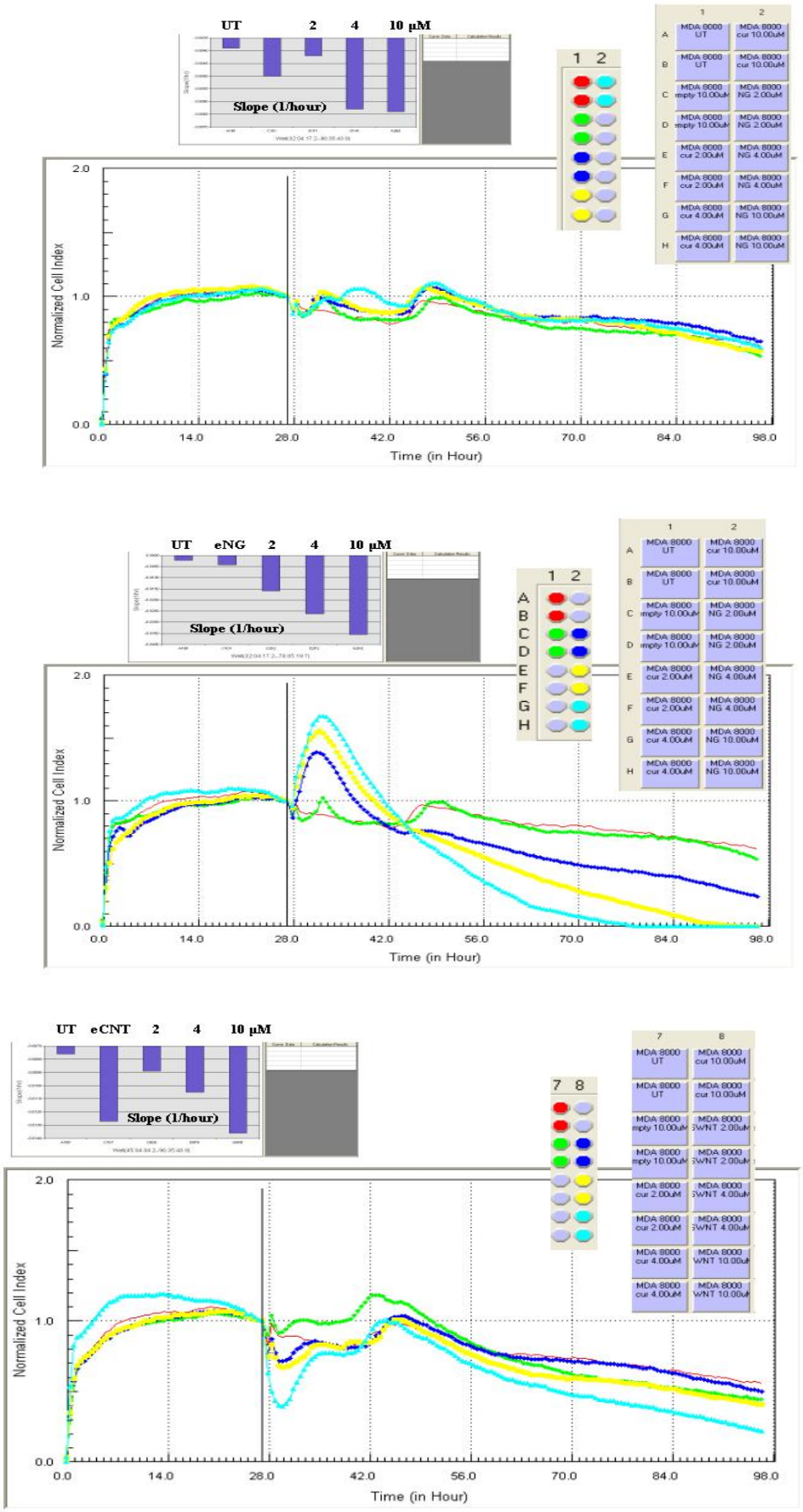
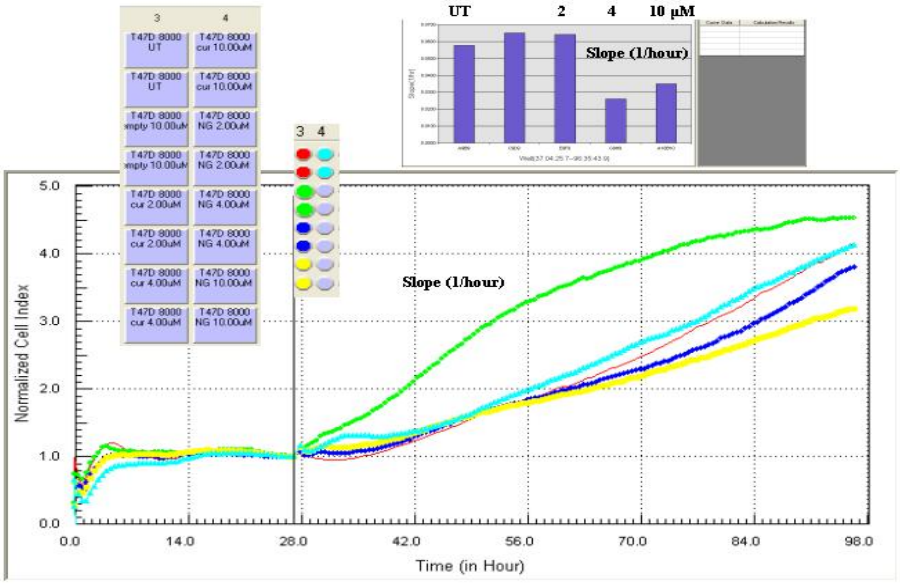


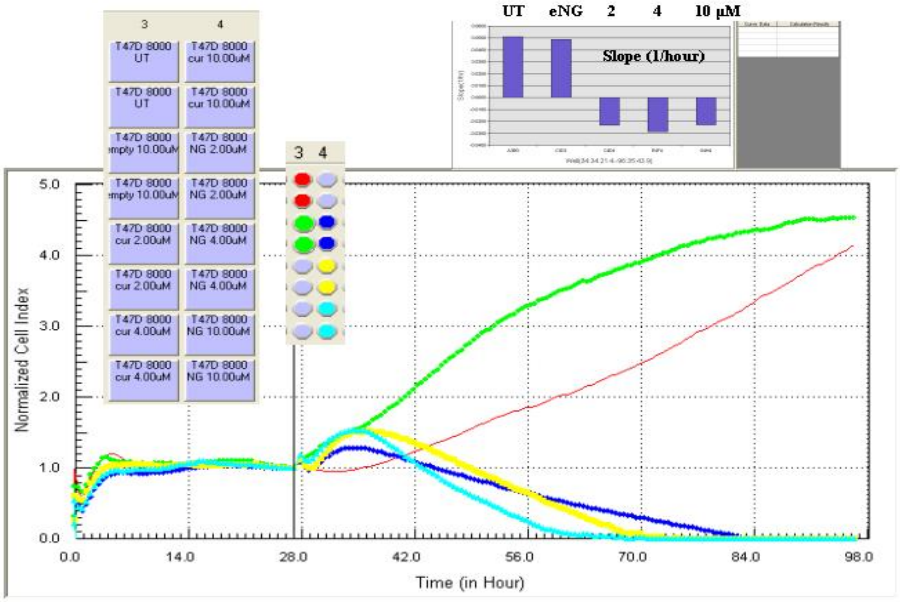
Fig. 8. ***Real-time curcumin formulations cytotoxicity monitored on microelectronic sensors.***

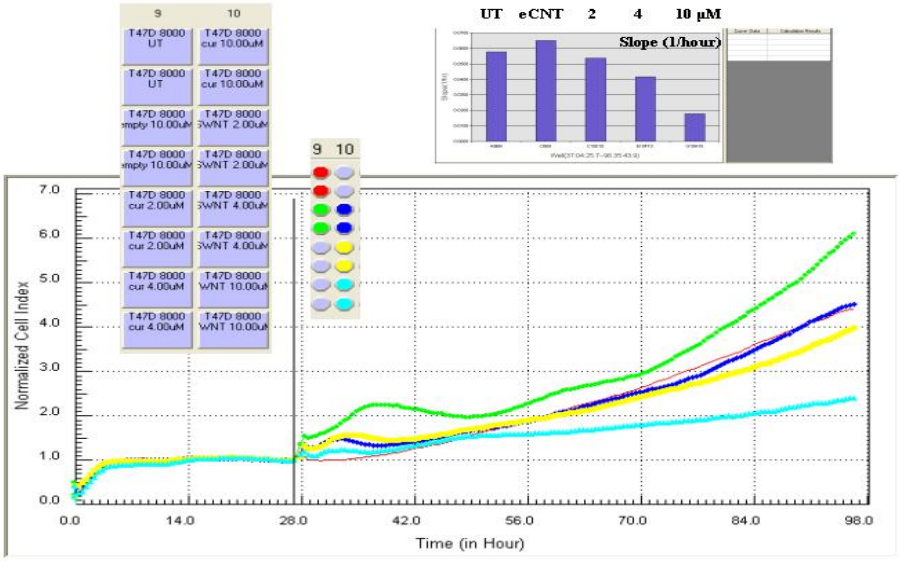
A. MDA-231 cells

Once the cells entered the exponential growth phase, cells were treated with free curcumin (top panel), C-NG (middle panel), or C-SWCNT (bottom panel) at different doses as indicated, specifically, 2, 4, and 10 µM of each drug formulation in duplicates (corresponding wells highlighted in respective colors). UT-untreated; eNG-empty nanogels, eSWCNT-empty SWCNTs. Y-axis-cell index, x-axis-time (hours). For the slope analysis (graphs in inserts) the linear portion of the corresponding curve was used.

Fig. 8B. ***T47D cells***







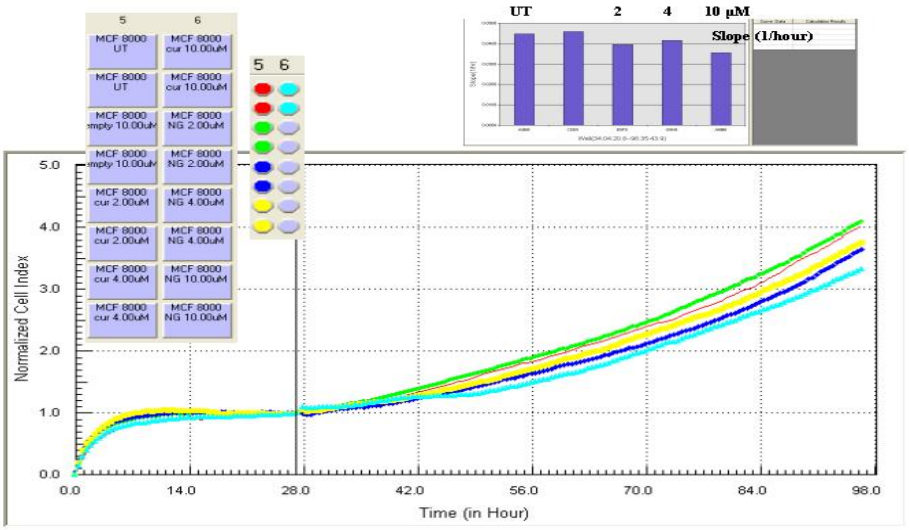
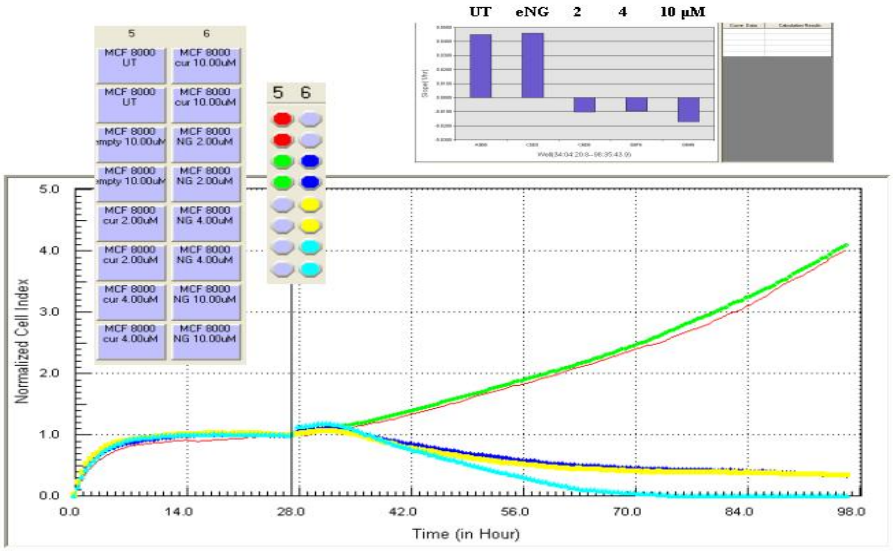
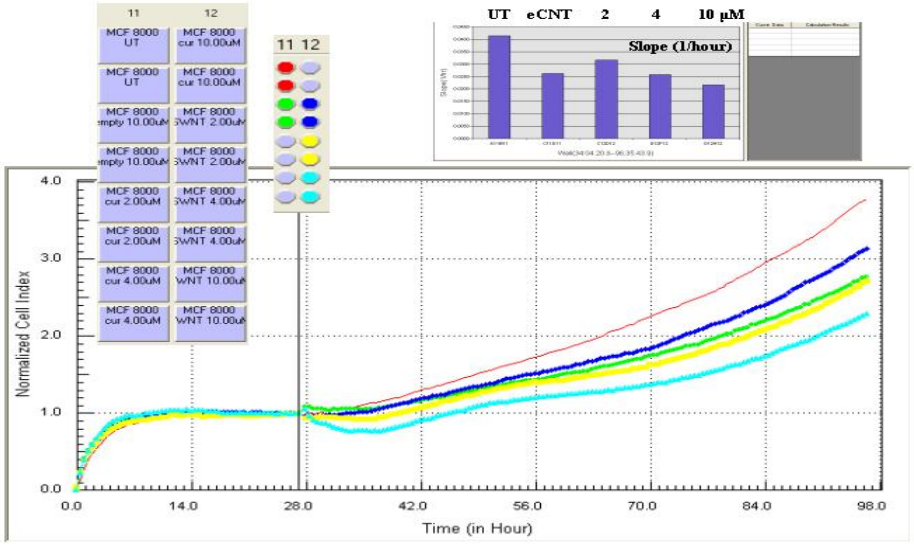


Fig. 8C. ***MCF-7 cells***





The cell index significantly decreased as a result of the cell death after exposure to nanogel formulation of curcumin in contrast to the treatment with free curcumin or C-SWCNT: even the lowest concentration of the

C-NG resulted in immediate cytotoxic response to this curcumin formulation, whereas C-SWCNTs cytotoxicity effect was much less prominent and required higher concentration of the drug. Notably, all three treatments were significantly more cytotoxic in metastatic breast cancer cell line MDA-231 in oppose to the other two non-metastatic cell lines T47D and MCF-7, where only high concentrations of the curcumin formulations caused cytotoxic response. Importanly, empty nanogels and SWCNT did not display any cytotoxicity in all of the three cell lines. Interestingly, free curcumin at even 10 μM concentration did not cause any cell death in all three breast cancer cell lines. This is in agreement with recent report on liposomal formulation of curcumin where only when used in doses higher that 50 μM curcumin alone inhibited proliferation of prostate cancer cells withour affecting their viability [Thangapazham, 2008 #27].

KEY RESEARCH ACCOMPLISHMENTS: The concept proposed here focuses on developing a novel therapeutic nanodevice capable of circumventing the key obstacles in cancer therapy such as toxicity and targeted drug delivery by incorporating a potent anti-cancer natural agent curcumin into biodegradable nanoconstructs.

- Two chemically different nanomaterials, single-walled carbon nanotubes (SWCNT) and colloidal nanogels (NG), were employed as nanocarriers for anticancer phytotherapeutics curcumin.
- Curcumin was successfully loaded into both SWCNT and NG; a high curcumin loading of the nanogels was achieved: the entrapment efficiency reached 30%.
- The widespread clinical application of curcumin has been limited due to its poor systemic bioavailability following oral administration. Due to it's nonpolar nature, curcumin is not easily soluble in water and most of consumed curcumin does not reach the bloodstream. We have developed a delivery system that can render this hydrophobic polyphenol soluble in an aqueous phase medium. Our data show that solubility of the C-NG in water could reach 50 mg/ml or 15 mg/ml by curcumin compared to 25μg/ml for curcumin alone.
- The biodegradable network of nanogel-curcumin complexes was found to be highly stable at neutral pH. However, pH change from 7.5 to 5, the acidic pH typical for tumor microenvironment, results in degradation of nanogels and the release of curcumin.
- The curcumin-nanogel formulation demonstrated significantly higher intracellular uptake than free curcumin (up to 15-fold).
- The findings of our study demonstrate the enhanced anticancer effectiveness of the nanogel-based formulation of curcumin compared with the drug alone while empty nanogels displayed virtually no cell cytotoxicity. SWCNT formulation of curcumin seemed to be less effective in cancer cell killing. Higher loading of curcumin is required to achieve better efficacy of this type of curcumin formulation. In addition, SWCNT must be tagged with fluorescent label in order to track the C-SWCNT trafficking: unlike nanogels, carbon nanotubes might have interfered with curcumin fluorescence as no signal from curcumin was detected.
- Interestingly, it appears that the ER-negative, highly metastatic MDA-231 cell line was more sensitive to the nanogel formulation of the curcumin compared with the other two, non-metastatic cell lines, T47D and MCF-7. It is well known that patients with ER-positive breast cancer often respond to hormonal therapy and have a better prognosis than patients with ER-negative tumors.
- There are a number of advantages of drug-nanogel formulations over other delivery systems, such as degradable PLGA nanoparticles, liposomes and polymeric micelles: 1. the curcumin-nanogel formulations are prepared by simple mixing of the drug solution and the dispersion of cationic nanogels, thus avoiding the damaging procedures of nanoparticle synthesis in the presence of encapsulating drug or complicated liposome preparations; 2. an evident advantage of nanogels is that they can be manufactured in large quantities at low cost and hence are most effective; they are also stable at storage in lyophilized form; 3. curcumin-loaded nanogels could be easily enough decorated with special ligands having high affinity for overexpressed cancer cellular receptors, allowing the curcumin to home in on specific tumors; 4. importantly, the nanogel-encapsulated drugs are capable to cross the cellular and endosomal membrane barriers because of the membranotropic property of

NG127, a property that can be modified by a proper design of nanogels and their polymeric composition; 5. post-synthetic modification of nanogels with cell-specific peptide ligands via PEG linkers provides a way to modulation of their target binding and biodistribution properties.

- We are currently initiating animal studies to determine whether curcumin-nanogel formulation can exert any effect against tumors *in vivo*. The results of this present study clearly demonstrate the superior tumor cell killing capacity of curcumin-nanogel formulation compared to free curcumin. We strongly believe that with this formulation of curcumin, we will overcome a substantial obstacle to widespread clinical use of this potent natural agent and offer the prospect of vastly improving the efficacy of anticancer treatment.

REPORTABLE OUTCOMES: 1. Abstract "Era of Hope" meeting, Baltimore, MD, June 2008 "Curcumin-nanogels and curcumin-carbon nanotubes as more efficient formulation of curcumin, a popular anticancer dietary spice" Anna E. Reeves 1, Serguei Vinogradov2, Eric Wickstrom3, Balaji Panchapakesan4, Mansoor M. Ahmed1.
1Geisinger Clinic (Weis Center for Research), Danville, PA, 2University of Nebraska Medical Center, Omaha, NE, 3Thomas Jefferson University, Philadelphia, PA, 4University of Delaware, Newark, DE.

2. Submitted for publication in Cancer Research: Curcumin-encapsulating nanogels as effective anticancer formulation of curcumin, a popular dietary spice. Anna Reeves[1], Ph.D., Serguei V. Vinogradov[2], M.D., Phil Morrissey[3], Ph.D. and Mansoor M. Ahmed[1*], Ph.D.
[1]Weis Center for Research, Geisinger Clinic, Danville, PA, USA; [2]University of Nebraska Medical Center, Omaha, Nebraska, USA; [3]Amnis Corporation, Seattle, WA, USA.

CONCLUSION: The nanodevices designed to introduce anticancer drugs into cells are expected to increase drug's killing capacity and reduce toxic side effects. The proposed work goes step further. It offers an innovative, cost-effective nanodevices loaded with the anticancer agent, natural compound curcumin that is non-toxic to the normal cells and can be used in much higher doses than conventional chemotherapeutic drugs. Hence, we believe that our proposed strategy represents a promising approach for the treatment of breast cancer with the potential of a greater success than currently used therapy. In future work we are planning to incorporate larger amount of curcumin into the SWCNT-based nanoconstructs to achieve higher cancer cell killing effect. In addition, both the nanogel-curcumin and SWCNT-curcumin complexes will be functionalized with antibodies specific for surface receptors of breast cancer cells such as IGFR and HER2 for more specific cancer cells targeting.

We believe that this work holds a great promise to make an impact on cancer patients in the very near future by providing an innovative and safe anticancer nanodevice. It emphasizes the value of interdepartmental and interdisciplinary collaboration, a trend becoming a hallmark of contemporary cancer research program.

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