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Journal Name

ARTICLE

## Bi-modal Cancer Treatment Utilizing Therapeutic Ultrasound and Engineered Therapeutic Nanobubble

Received 00th January 20xx,  
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Herein we report a bi-modal cancer treatment technique using therapeutic ultrasound in presence of novel sorafenib loaded nanobubble for hepatocellular carcinoma. Therapeutic ultrasound was used for improving fusion-triggered drug release from nanobubbles leading to enhanced cytotoxicity in HepG2 cells by greater than two folds improvement in IC50 values. This strategy could also be made compatible with catheter-based devices to derive a highly localized treatment approach, a concept that might be extendable to metastatic cancers as well.

### Introduction

For many cancers such as hepatic, breast and prostate, thermal therapy is considered an alternative, minimally invasive technique to destroy cancer tissue.<sup>1</sup> Cryo-ablation,<sup>2,3</sup> radio frequency,<sup>4,5</sup> microwave heating,<sup>6,7</sup> and high-intensity focused ultrasound (HIFU)<sup>8-10</sup> are thermal therapy modalities under serious investigation, with several commercial offerings. The use of ultrasound to treat tumors has been investigated in prostate, liver, kidney, and brain<sup>11-20</sup>. The ultrasound mediated destruction of microbubbles helps to increase the deposition rate of therapeutic drugs across the vessels or the cells.<sup>21,22</sup> Ultrasound exposure in conjunction with administration of microbubbles enhances sonoporation of cancer cells.<sup>23-25</sup> It may be possible that the microbubbles were sensitizing the vasculature that lowers damage threshold temperatures and enhances drug uptake. Researchers showed targeted drug delivery combined with focused ultrasound exhibited better treatment outcome when compared to tumors treated with chemotherapeutic agents in mouse pancreatic cancer model.<sup>26</sup>

It is anticipated that similar techniques can be applied successfully for liver cancer treatment. However, to realize the full potential of this technique, the treatment strategy needs

to be improved further. In previous reports, temperature-sensitive liposomes were used to achieve increased drug to flow through the bloodstream by minimizing clearance and non-specific uptake.<sup>27</sup> Upon reaching microvessels within a heated tumor, the loaded drug was released and quickly penetrate as reported in case of ThermoDox<sup>®</sup> (Celsion), demonstrating significant improvements to the drug release rates and drug uptake in heated tumors (~41°C).<sup>27</sup> Preliminary evidence from various studies suggest that combining chemotherapy with localized heating was safe.<sup>28,29</sup> This indicated the treatment success when chemotherapy was combined with thermal energy. Sorafenib (4-[4-[[4-chloro-3-(trifluoromethyl)-phenyl]carbonylamino]-phenoxy]-N-methyl-pyridine-2-carboxamide) has been used as a kinase inhibitor under brand names Nexavar, BAY 43-9006 and sorafenib tosylate to treat advanced cancers of the kidney, liver, or thyroid.<sup>30</sup> However, patients treated with sorafenib alone had limited impact due to very low response rates of 2% and median overall survival (OS) of 10.7 months.<sup>31</sup> Hepatic arterial infusion chemotherapy (HAIC) using an implantable port-catheter system with response rates of 20-48% for advanced hepatocellular carcinoma (HCC),<sup>32</sup> has been a promising regional treatment. However, arterial thrombosis has been a complication associated with the use of VEGF inhibitor, sorafenib and the combination of the VEGF inhibitor with other systemic chemotherapies increasing the complication rate of thrombosis.<sup>33</sup> In such a complicated situation for use with potential sorafenib in HCC, bi-modal therapy using therapeutic ultrasound (US) and sorafenib loaded Nanobubble can lead to better therapeutic strategy.

The ultrasound radiation has been applied effectively to cause cavitation and transfer their energy into the tissue. In traditional sonoporation, dose of ultrasonic frequency can be 20 KHz and the ultrasound intensity may be in the range of 5 W/cm<sup>2</sup> and 55 W/cm<sup>2</sup>. The US probe may have a distance from the tissue in the range of 1 millimeter to 10 millimeters. The

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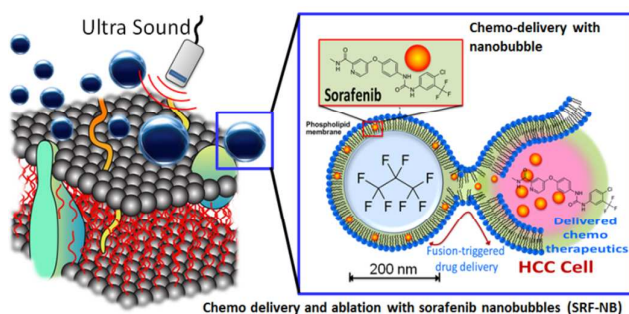
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† Footnotes relating to the title and/or authors should appear here. Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

ultrasound radiation may be continuous or pulsed. The application period may vary in range of 30 seconds to 5 minutes, preferably 1 minute for continuous exposure or about 10 to 20 minutes for pulsed exposure with a 5% duty cycle, respectively.<sup>34</sup> On the other hand, presence of microbubbles varies the dose levels to 1 MHz, 1-2 W/cm<sup>2</sup>, 20% duty cycle, 1-5 min.<sup>35,36</sup> In this work the optimized strategy improves the dose by at least 2 fold to a 500 kHz focused transducer applied to a single 2-cycle US pulse for 2 min using sorafenib-loaded nanobubbles.

Here in we report the delivery of sorafenib encapsulated nanobubble under therapeutic ultrasound exposure *in vitro* extendable to site specific delivery using steerable catheter based ultrasound (CBUS) device for treatment of hepatic tumors. We envision that localized delivery of the chemotherapeutic agent encapsulated in a nanobubble, by virtue of requiring lower administered dose, will reduce systemic toxicity while simultaneously enhancing drug uptake in the targeted lesions facilitated by therapeutic ultrasound (Fig. 1). Such an approach could potentially also be adapted to treat cancer metastasis from various primary tumors.



**Fig. 1** Schematic representation of the cancer treatment utilizing therapeutic ultrasound and synchronous nanodelivery of molecularly targeted therapeutics. Blue box: depicts the fusion-triggered sorafenib delivery from nanobubbles.

## Results and discussion

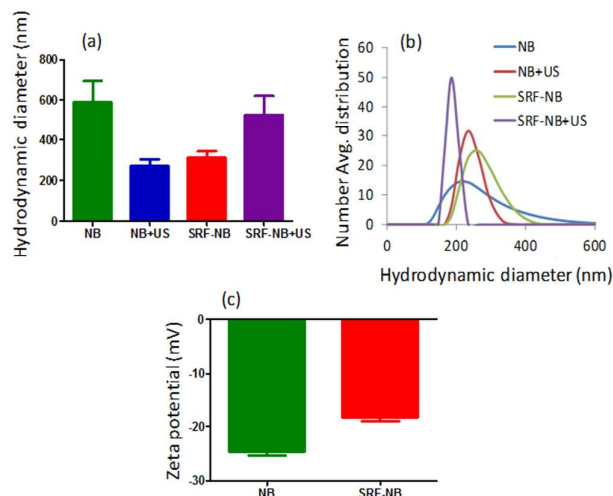
In this work, we utilize nanoencapsulated sorafenib developed to protect and retain the compound in the particle until it is liberated within the target cell. The proposed study will utilize a novel combinatorial delivery approach of bi-modal therapy using therapeutic ultrasound (US) and sorafenib loaded nanobubble for better therapeutic strategy. We envision that localized delivery of the chemotherapeutic agent encapsulated in a nanobubble, by virtue of requiring lower administered dose, will reduce systemic toxicity while simultaneously enhancing drug uptake in the targeted lesions facilitated by therapeutic ultrasound. Such an approach could potentially also be adapted to treat cancer metastasis from various primary tumors.

The bi-modal cancer therapy in HCC, HepG2 cells, was initiated with preparation of sorafenib loaded nanobubbles and optimizing US parameters in specially designed set up for *in vitro* cell culture followed by determining growth regression. In a typical procedure, the nanobubbles (NBs) were prepared

using a thin-film hydration-sonication technique. Briefly, a mixture of phospholipids (180 mg lecithin PC, 10 mg PEG2000-DSPE, and 10 mg cholesterol) was dissolved in 10 mL of chloroform and transferred into a test tube to form a thin phospholipid film by reduced pressure evaporation. The material was then hydrated with 40 mL of hydration liquid, which consisted of 10% glycerol (v/v) and 2 mg/mL Pluronic F-68, at 4 °C overnight followed by probe sonication at 37 °C. The lipid suspension was then transferred to a 50-mL centrifuge tube, and the air above the liquid was replaced with C<sub>3</sub>F<sub>8</sub> gas using a long, fine needle and a 50-mL syringe. Finally, the micro-probe of a Q700, Qsonica Sonicators, Newtown, CT was placed at the air-liquid interface, and the solution was allowed to further sonicate at amplification 4 for 5 minutes to form the nanobubbles of 500±50 nm (Fig. 2a) which reduced to 250±25 nm (Fig. 2b) after centrifugation at 50 g for 5 min. For the incorporation of the drug, surfactant mixture included 2.25 mole% of sorafenib (SRF) (ca. 3000 Nexavar™ molecules/nanobubble) to produce sorafenib-nanobubbles (SRF-NBs) of ~ 250 nm (Fig. 2a) which remained almost of same size with ~225 nm (Fig. 2b). Prepared nanobubbles, with or without the incorporation of drug, were found to be stable and responsive to US exposure for at least 2 weeks during the reproduction of the experiments performed. Generally, the high stability of nanobubbles can be described based on the nature of the entrapped gas in the core, structure of outer boundary, stabilization through phospholipid capping and charge potential of bubble surface. Perfluorocarbons (i.e. C<sub>3</sub>F<sub>8</sub>) possessing a different density than that of air and being poorly soluble in water, have been shown to increase both the echogenicity of the US contrast agent and the stability. Nanobubble dynamic has been found different from micron-sized droplet (e.g. microbubble) studies for targeted imaging and drug delivery, and therefore requires substantially different acoustic parameters and optimization. Nanobubble sonoporation require less administered dose while offering better delivery presumably due to the fact that decrease in size of bubbles increases the internal pressure of entrapped gas significantly, leading much better response toward sonoporation even at lower ultrasonic dose.<sup>37</sup>

Achieving high drug loading, surface area and stability of drug delivery vehicles has always been major concern for biomedical researchers. Decrease in size of delivery vehicles to nanoscale would potentially improve the available surface area for enhanced loading of the drug with increased pressure in core of the bubble. The use of microbubbles for ablation therapy is known before. The respective diameter of microbubbles (MBs) and nanobubbles (NBs) are 10–50 μm and <200 nm. For NBs, the total free energy of the system is supposed to increase along with the formation of NBs unless the surface was extremely rough. However, high Laplace pressure inside NBs would likely cause them to dissolve into solution quickly. Study has revealed that the interface of NBs consists of 'hard' H-bonds similar to those originate in ice and gas hydrates. This may lead to reduced diffusivity of NBs that helps to maintain adequate kinetic balance of NBs against high internal pressure. The particles at the nanoscale offer added

advantages in terms of delivering drugs. At the nanoscale, the acquired surface area is high leading to facile cellular interaction, high payload incorporation and systemic stability. All the constituents of nanobubbles are pharmaceutically acceptable (e.g. phospholipids and C3F8). Since, sorafenib is also FDA approved, the potential for clinical translation of this nanoplatform is remarkable. By enabling the delivery of chemotherapeutics in 'nano' form, the required effective dose can be brought to enhance treatment efficacy as the side effects from chemotherapy will be significantly be reduced as compared to conventional administration of the chemotherapy.<sup>37</sup>

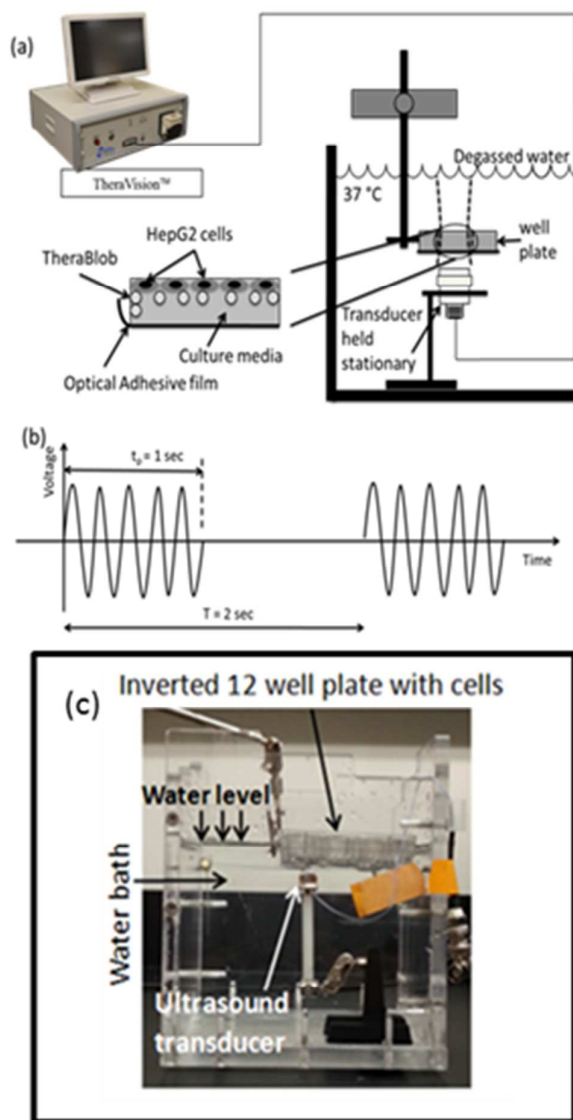


**Fig. 2** Physico-chemical characterization of SRF-NBs. (a) Hydrodynamic diameter distribution in aqueous suspension before and after US exposure without centrifugation (b) after centrifugation and (c) zeta potential distribution of NBs and SRF-NBs.

Added to this, the existence of a monolayer shell presented a significant barrier to gas escape from the core into the aqueous medium. On the other hand, the charge on the surface of the nanobubbles additionally contributed to their stability. Electrophoretic (Zeta) potential measurements showed that the nanobubbles obtained from lipids were  $-26 \pm 3$  mV while SRF-NB was  $-20 \pm 5$  mV (Fig. 2c). The presence of highly negative electrophoretic potential is indicative of the presence of adequate repulsive forces to prevent coalescence and inter-bubble aggregation. To verify the oncolytic effect of SRF-NB, cytotoxicity assays were performed with or without the presence of therapeutic US. As a model system for *in vitro* cancer culture, we chose human hepatocellular carcinoma (HCC) cell line (HepG2) to evaluate the functional therapeutic potential of SRF-NB.

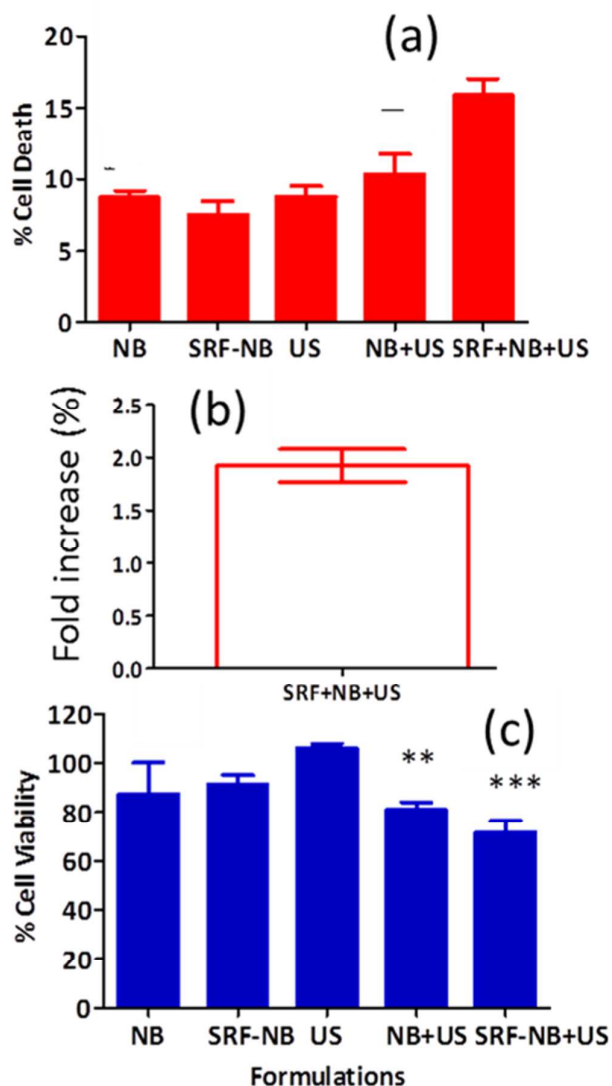
To investigate the potential additive/synergistic effects of ultrasound and chemotherapy on HepG2 cells, various biological experiments were performed in monolayer of cells. Cells were cultured to 80% confluence in a 12 well plate. During the ultrasound experiment the cell plates were filled with EMEM (pH 7.4), covered by a MicroAmp optical adhesive

film (Applied Biosystems, Foster City, CA) to act as an acoustic window and placed inverted in a tank of degassed water (Fig. 3a-c). The optical adhesive film also prevented the solution in the wells from mixing with the water bath. The PBS solution had NB and SRF-NB based on the respective controls and treatment configurations. A rectangular flat ultrasonic transducer with centre frequency of 2.4 MHz was used to expose the cell at the surface of each well for 2 minutes duration at acoustic output power of 10 W and pulse rate of 0.5 Hz. The sonication was delivered using the TheraVision™ (Acoustic MedSystems, Savoy, IL) control unit as shown in Fig. 3a.



**Fig. 3** (a) Schematic of the experimental setup, and (b) pulse sequence used for the ultrasound exposure at 2.4 MHz and pulse rate of 0.5 Hz (c) A digital picture of the experimental set up showing the placement of the 12 well plate and the ultrasonic transducer driven by US ablation system.



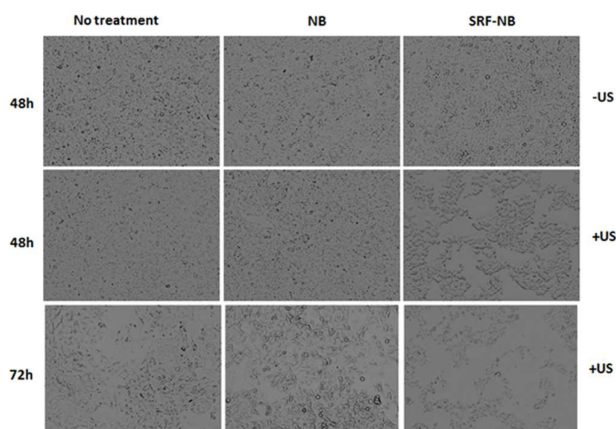


**Fig. 4** Optimization of US parameters. Trypan Blue (24h) (a, b) and MTT assay (c) for HepG2 cells at 48h post treated with nanobubbles (NB) and Sorafenib-encapsulated nanobubbles (SRF-NB) exposed to ultrasound. Biostatistical Analysis was performed Using one way ANOVA with Bonferroni Post Test considering cells only treated with US as negative control.

HepG2 cells were treated with NB and SRF-NB in either the presence or absence of US. *In vitro* studies were conducted to optimize the ultrasound experimental parameters: output acoustic power, exposure durations, pulse rate and duty cycle. Cell viability using Trypan Blue, MTT assay and microscopic images was thoroughly analyzed to finalize the optimized parameters. Cell viability was measured using Trypan Blue (Fig 4a, 24h) and MTT assay at 48h after treatment with 5  $\mu$ M concentration of sorafenib loaded in NBs. Although this

therapy regime is assisted by US exposure, the final outcome of cell growth regression is caused by activity of sorafenib, which depends on time of incubation post-delivery to the cells. Considering this fact the time of incubation post US treatment was varied to higher time points. Trypan blue assay results showed ~2 fold higher % cell death (Fig 4b) on US exposure after SRF-NB treatment while US exposure on NB treated cells do not enhance it significantly (Fig. 4a). Similarly, MTT assay revealed that US exposure on SRF-NB treatment significantly decreased % cell viability by ~30% (Fig. 4c) at optimized US parameters of at 2.4 MHz and pulse rate of 0.5 Hz for 2 min.

Optimized US parameters were used on cells treated with SRF-NBs loaded with sorafenib concentration ranging from 1.8 to 15  $\mu$ M. Cellular assay results were corroborated with bright field microscopy images (Fig. 5). The half maximal inhibitory concentration ( $IC_{50}$ ) was calculated as 12.5 compared to 32  $\mu$ M in case of parent sorafenib molecule<sup>38</sup> when SRF-NB were incubated and post incubated with HepG2 cells (Fig. 6). Biostatistical analysis was performed using one way Analysis of variance (ANOVA) with Bonferroni Post Test considering cells with US as negative control. Cellular entry of small molecule is typically not influenced by US exposure. Since the present work focuses on the effect of US exposure on nanobubble-enabled delivery of SRF, SRF-NB was always used as control formulation without US application.



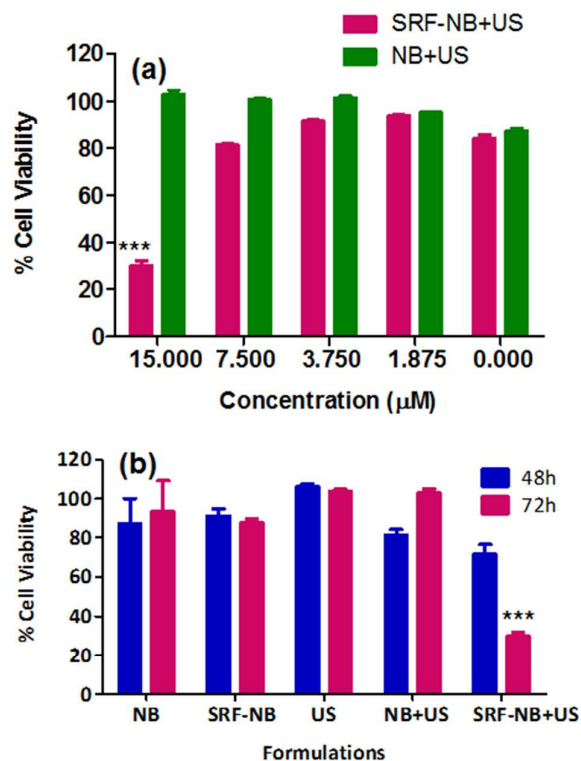
**Fig. 5** Bright field images of the HepG2 cells treated with 5  $\mu$ M of sorafenib loaded in NBs showing morphological variations before and after US exposure at optimized US parameters of at 2.4 MHz and pulse rate of 0.5 Hz for 2 min.

To establish the selective synergistic effect in HCC, combination index (CI) calculations were performed for individual formulations including NB, SRF-NB, US and NB+US, SRF-NB+US. Results were from  $IC_{50}$  calculated for SRF-NB+US, SRF-NB and SRF are 12.5, 60 (not shown) and 32  $\mu$ M, respectively. CI was calculated as 0.59 (considerably less than 1 while just additive ( $\geq 1$ )) indicating the significant synergistic effect from combination of SRF-NB and US combination.

“The CI has been analysed using the formula:

$$CI = (Cax/IC_{50a}) + (Cbx/IC_{50b})$$

where CA,50 and CB,50 are the concentration of factor A and B used in combination to achieve 50 % drug effect. IC<sub>50,A</sub> and IC<sub>50,B</sub> are the concentrations for single agents to achieve the same effect. A CI of less than, equal to, or more than 1 indicates synergistic, additive or antagonistic effect, respectively.<sup>39</sup>



**Fig. 6** Optimization of sorafenib concentration and time dependent variability in % cell viability. (a) MTT assay for HepG2 cells at 48h post treated with NB and SRF-NB (1.875-15 μM of sorafenib in SRF-NB and same equivalent for NB) and exposed to ultrasound and (b) decreasing % cell viability with increased time of incubation post US exposure while being treated at 15 μM concentration of SRF in SRF-NB and same equivalent of NB. Biostatistical Analysis was performed using one way ANOVA with Bonferroni Post Test considering cells only treated with US as negative control.

## Experimental

### Materials and Methods

HepG2 cells were procured from ATCC. Fetal bovine serum was purchased from GIBCO®, Life technologies. The probe sonication was performed by QSONICA Sonicators. MeOH was purchased from fisher chemicals. EMEM was purchased from ATCC® 30-2003, Pen-strep from Sigma, Trypsin from gibco® by lifetechnologies™, lecithin as COATSOME from NOF corporation, Sorafenib from Selleckchem.com, C3F8 from

Specialty Gases of America (OH), Trypan blue from sigma, MTT (Thiazolyl blue tetrazolium bromide) from sigma and DMSO from MACRON fine chemicals™.

### Physico-chemical characterization of NB and SRF-NB.

#### Dynamic light scattering

Average hydrodynamic diameter distributions for NB and SRF-NB formulations before and after exposure to optimized ultrasonication (NB+US and SRF-NB+US) were determined using Malvern Zetasizer ZS90 particle size analyzer while scattered light was collected at a fixed angle of 90°. A photomultiplier aperture of 400 mm was used, with the incident laser power was adjusted to obtain a photon counting rate between 200 and 300 kcps. Measurements for which the measured and calculated baselines of the intensity autocorrelation function agreed to within +0.1% were used to calculate hydrodynamic diameter values. All determinations were made in multiples of 3 consecutive measurements with 10 runs each.

#### Zeta potential determination

Zeta potential ( $\zeta$ ) values for the NB and SRF-NB formulations before and after exposure to optimized ultrasonication (NB+US and SRF-NB+US) were determined with a nano-series Malvern Zetasizer zeta potential analyzer. Measurements were made following dialysis (MWCO 20 kDa dialysis tubing, Spectrum Laboratories, Rancho Dominguez, CA) of nanoparticle suspensions into water. Data were acquired in the phase analysis light scattering (PALS) mode following solution equilibration at 25 °C. Calculation of  $\zeta$  from the measured nanoparticle electrophoretic mobility ( $\mu$ ) employed the Smoluchowski equation:  $\mu = \epsilon\zeta/\eta$ , where  $\epsilon$  and  $\eta$  are the dielectric constant and the absolute viscosity of the medium, respectively. Measurements of  $\zeta$  were reproducible to within  $\pm 4$  mV of the mean value given by 3 determinations of 10 data accumulations.

#### US set up and optimization

TheraVision™ Ultrasound Ablation system (Acoustic MedSystems, IL) was used to drive the flat square ultrasonic transducer. The transducer was held positioned such that the acoustic beam is centered at each well in the 12-well plate. A pulse rate of 0.5 Hz was used for all the experiment. The transducer was held stationary and the plate was moved to expose each separate well containing cells for the respective configurations in the plate. The size of the transducer was 18 mm diagonally (Acoustic MedSystems, IL), which was slightly smaller than the diameter of each well (22 mm) such that each well is exposed by the collimated acoustic beam. Degassed water at 37 °C was used for all the experiments. The RF generator in the TheraVision™ system uses a sophisticated circuit design that minimizes changes in pressure amplitude

and frequency settings. Each well was exposed for 2 minutes. During the ultrasound experiment the plates covered by a MicroAmp optical adhesive film (Applied Biosystems, Foster City, CA) to act as an acoustic window and placed inverted in a tank of degassed water. The optical adhesive film prevented the solution in the wells from mixing with the water bath and acted as acoustic window. The plate was inverted so that the TheraBlob would float and be near the cell surface. Typically the four farthest corner wells in a 12-well plate were used for the ultrasound experiment to avoid any ultrasound exposure to the adjacent wells.

### Trypan blue and MTT Assay

The cell viability of various formulations were investigated by Trypan blue and MTT assay using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay in presence of 10% FBS in antibiotic free media. Experiment was performed in 12 well plates (Cellstar®; Germany) growing 200,000 HepG2 cells per well 24 h before treatments. Experiments were performed for various concentrations of NB and SRF-NB ranging from 15 to 0.9375  $\mu\text{M}$  of before and after exposure to US. Cells were incubated for 24h before performing either trypan blue or MTT assay after 48 and 72h. Collected cells after incubation time were used for trypan blue assay. In a viable cell trypan blue is not absorbed; however, it traverses the membrane in a dead cell which distinguishes the dead cells in blue colour under a microscope while live cells are excluded from staining. This staining method is also described as a dye exclusion method. After incubation period, cells were treated with MTT (20  $\mu\text{L}$ , 5mg/mL) per well and further incubated for 4 h. The yellow tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is cleaved by dehydrogenases to its purple formazan derivative (MTT-formazan) with maximum absorbance at 560-595 nm.<sup>40</sup> The intensity of purple formazans indirectly reveals the mammalian cell survival and proliferation.<sup>41,42</sup> At the end of the incubation entire medium was removed from the wells and 1000  $\mu\text{L}$  DMSO was added to dissolve blue colored formazan crystals and segregated in 5 wells for each treatment of 96 well plates. The percentage cell viability was obtained from plate reader and was calculated using the formula % Viability =  $\frac{\{[A630(\text{treated cells}) - (\text{background})]\}}{\{[A630(\text{untreated cells}) - \text{background}]\}} \times 100$ .

### Statistical Analysis

Data were analyzed using analysis of variance (graph Pad Prism 5.0) ( $p < 0.05$ ). Data are presented as the mean  $\pm$  standard error of the mean unless otherwise stated where ever applicable.

### Conclusions

In conclusion, we have successfully prepared novel sorafenib loaded nanobubbles, SRF-NB for the treatment of hepatocellular carcinoma under exposure of therapeutic US.

Sorafenib is a FDA approved drug for first line treatment of liver cancer. The nano-enabled delivery of sorafenib using bubbles has not been explored. Our results indicated that the combination and synergistic use of sorafenib-entrapped nanobubble and US mediated therapy might be a novel approach of treating liver cancer. US ablation techniques were used to facilitate the drug delivery and improved ablation of HCC. We were able to achieve  $\sim 70\%$  growth inhibition of HepG2 cells after treatment with 15  $\mu\text{M}$  sorafenib loaded NBs post incubated for 48h while US parameters with frequency of 2.4 MHz and pulse rate of 0.5 Hz for 2 min were exposed to HepG2 monolayer after 24h of plating cells. Bi-modal therapy could achieve  $\sim 2$  fold improvement in  $\text{IC}_{50}$  of free sorafenib. Although, further studies are needed to substantiate the promise that such an approach holds for effective treatment of HCC and secondary (metastatic) liver cancers, combination of US exposure with US responsive therapeutic NBs could improve the therapy regime for HCC *in vitro*.

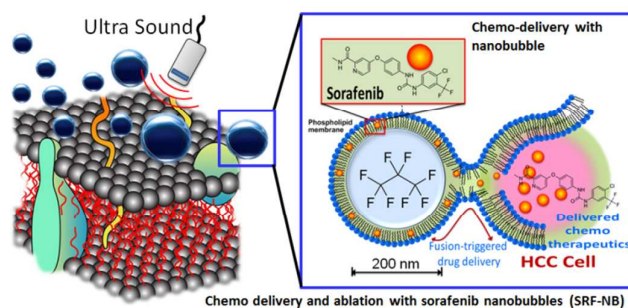
### Acknowledgements

Physico-chemical measurements were carried out in part in the Frederick Seitz Materials Research Laboratory Central Research Facilities, University of Illinois. We thank University of Illinois at Urbana-Champaign for financial support.

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We developed a bi-modal cancer therapy comprising sorafenib loaded ultra-sonic responsive nanobubble (SRF-NB) for ultrasonic assisted delivery in hepatocellular carcinoma.