Nanomaterial-Assisted Acoustic Neural Stimulation

Chapter X Nanomaterial-Assisted Acoustic Neural Stimulation

Attilio Marino[1,*], Giada Graziana Genchi[1], Marietta Pisano[2], Paolo Massobrio[2], Maria Teresa Tedesco[2], Sergio Martinoia[2,3], Roberto Raiteri[2,3], Gianni Ciofani[1,4,*]

[1]Istituto Italiano di Tecnologia, Smart Bio-Interfaces, Viale Rinaldo Piaggio 34, Pontedera (Pisa) 56025, Italy

[2]Università di Genova, Department of Informatics, Bioengineering, Robotics and System Engineering, Via Opera Pia 13, Genova 16145, Italy

[3]CNR, Institute of Biophysics, Via De Marini 6, Genova 16149, Italy

[4]Politecnico di Torino, Department of Aerospace & Mechanical Engineering, Corso Duca degli Abruzzi 24, Torino 10129, Italy

*attilio.marino@iit.it; gianni.ciofani@iit.it

Abstract

Wireless and non-invasive stimulation of neural system, especially at the central level, is a considered a critical issue not only for the treatment of a variety of pathological conditions, such as, epilepsy, chronic pain and obsessive-compulsive disorders, but also for reducing the debilitating motor symptoms of movement disorders such as Parkinson's disease, dystonia, and essential tremor. In this chapter, the potential of piezoelectric nanostructured materials for remote non-invasive neural stimulation is presented.

X.1 Introduction

The word "piezoelectricity" derives from the ancient Greek, and it literally means "electricity resulting from pressure". Piezoelectric materials, indeed, generate an electric potential when subjected to a mechanical strain (direct piezoelectric effect); conversely, the application of an electric field to a piezo-material induces its own deformation (reverse piezoelectric effect). Since the discovery of piezoelectricity in the late nineteenth century, piezoelectric materials have been exploited in several different devices, for applications ranging from biomedical (e.g., ultrasonic imaging systems) to automotive fields (e.g., air-bag sensors) (Marino et al. 2017).

The rapid development of nanotechnology has reshaped our knowledge in the fields of physics, chemistry, material science and biology. Nanomedicine refers to the biomedical application of nanomaterials and is one of the branches of nanotechnology, which has been mostly influenced in the course of this scientific/technological revolution (Zhang et al. 2008). Researchers working in nanomedicine recently developed a variety of non-invasive and biocompatible tools capable of remotely delivering specific physical and chemical stimuli in the deep tissue, at single cell level or even with subcellular resolution (Genchi et al. 2018)

In this context, piezoelectric nanomaterials represent a class of nanotransducers, both organic and inorganic, that can be exploited not only for the remote excitation of the neural cells, but, more in general, for the stimulation of the electrically excitable cells, such as cardiomyocytes, osteoblasts and skeletal myotubes (Marino et al. 2017). Specifically, piezoelectric nanomaterials can be activated with different mechanical energy sources, such as vibrations and acoustic pressure waves in the audible (sound) or non-audible (ultrasound, US) frequencies (Royo-Gascon et al. 2013; Inaoka et al. 2011; Wang et al. 2007).

Concerning US, these pressure waves deeply and safely penetrate soft biological tissues, and are clinically exploited for diagnostic purposes (e.g., sonography). Efficient piezoelectric nanotransducers, such as the ones characterized by barium titanate, are able to generate electric potentials in the order of millivolt and remotely activate neural cells when exposed to US intensities similar to the ones used for sonography (Marino et al. 2015). Moreover, US waves can be focused into deep tissues through hyperlenses in order to maximize the US intensity in a specific region of the tissue (Zhang et al. 2009). For these reasons, US represents an ideal and safe source of mechanical energy that can be efficiently transduced into biologically relevant electrical cues.

In addition to US, the piezoelectric neural stimulation mediated by acoustic waves in the audible frequencies has been extensively investigated; such studies have been carried out in order to develop a new generation of single-component cochlear implants able to transduce the mechanical waves of sound into electrical signals for the stimulation of the spiral ganglion neurons. These piezoelectric devices have been designed for substituting the functions of the cochlear sensory epithelium, which are compromised in certain types of deafness (Inaoka et al. 2011).

Piezoelectric materials can be exploited not only as actuators for indirect electric stimulation, but, taking advantage of the reverse piezoelectric effect, also as sensors to detect, measure and accumulate the biomechanical energy developed by single cells and tissues (Nguyen et al. 2012). In this technological framework, piezoelectric nanostructured devices have been incorporated in artificial pacemakers to obtain self-powered battery-free cardiac stimulation systems (Hwang et al. 2015).

In this chapter, the piezoelectric nanomaterials that have been adopted in the biomedical field will be described and the biological effects of the acute and chronic nanoparticle-assisted piezo-stimulation on neural cells will be reported.

Moreover, we will provide a chronological overview of the discovery of the neural activation with this indirect electric stimulation approach, starting from the first experimental evidences to the recent electrophysiological proofs obtained on primary neurons. Finally, *in vivo* exploitation of the piezo-stimulation approach for activation of the neural system will be presented in the last section.

X.2 Piezoelectric nanostructured materials applied to nanomedicine

The generation of small electric charges upon the application of mechanical stimuli to piezoelectric nanomaterials is a unique phenomenon in the context of remote stimulation of cells and tissues. Electrical cues are known to foster specific biological responses, and piezoelectric nanomaterials own the ability to act as real "nanotransducers", thus allowing obtaining "wireless" and remote electric stimulation thanks to non-invasive excitation through mechanical sources (usually US or vibrations).

Inorganic piezoelectric nanomaterials can be ceramic or polymeric, with piezoelectric nanoceramics usually showing higher piezoelectric features than polymers. Perovskites (like barium titanate and lead zirconium titanate) and wurzites (like zinc oxide and zinc sulphide) are among the mostly investigated piezoelectric nanoparticles.

Concerning piezoelectric polymers, poly(vinylidene difluoride) (PVDF) and its copolymers show the best piezoelectric features and have been widely investigated to promote cell stimulation, for example on rat spinal cord neurons (Royo-Gascon et al. 2013) and on human adipose tissue derived stem cells (Ribeiro et al. 2015).

Boron nitride nanotubes (BNNTs), inorganic nanomaterials with structural affinity to carbon nanotubes, have been tested, among others, by our group, and showed beneficial effects as nanotransducers on PC12 neuron-like cells (Ciofani et al. 2013) and on pre-osteoblast human cells (Genchi et al. 2018). In the latter example, they have been used as nanofillers in P(VDF-TrFE)-based scaffolds stimulated with ultrasounds.

Other studies of ours also provided the first direct evidences of piezoelectric stimulation of cell cultures mediated by barium titanate nanoparticles (BTNPs): in particular, experiments have been performed on SH-SY5Y human neuroblastoma cells in the presence of nanoparticles and stimulated with US (Marino et al. 2015). In this work, BTNPs owing tetragonal crystalline structure, and thus piezoelectric, were tested to demonstrate neuronal stimulation, whereas nanoparticles with cubic crystalline structure (and thus non-piezoelectric) were used as a negative control. A physical model has also been developed to corroborate obtained findings.

Barium titanate nanoparticles, analogously to BNNTs, have been further exploited as fillers to improve piezoelectric properties of scaffolds, giving

interesting results on both neuron-like cells (Genchi et al. 2016) and on human pre-osteoblasts (Marino et al. 2015).

Finally, it is worth to mention the potentialities of piezoelectric stimulation of cancer cells. It is in fact well-known, as low-intensity electric stimulation represents an alternative treatment able to affect cancer cells without the use of any drugs/chemicals, and to significantly enhance the effects of chemotherapy by reducing multidrug resistance with the impairment of the plasma membrane translocation of P-glycoprotein (P-gp), encoded by the MDR1 gene, the overexpression of which is associated with chemotherapy resistance. Recently, our group provided the first evidences of the efficacy of this antitumor approach mediated by piezoelectric BTNPs and US, respectively on breast cancer (Marino et al. 2018) and on glioblastoma multiforme cells (Marino, Almici et al. 2019) (Fig. X.1).

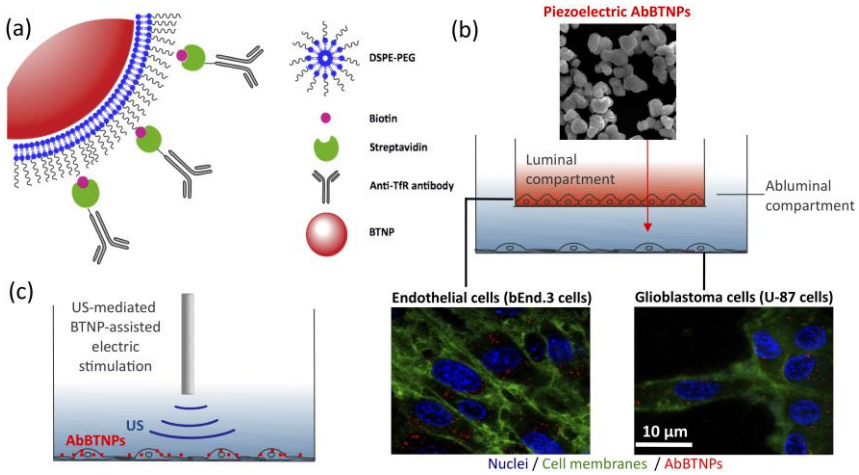

Fig. X.1 Example of piezoelectric stimulation of glioblastoma cancer cells (U87). BTNPs have been functionalized (a) in order to promote the crossing of an in vitro blood-brain barrier model (b). Synergic effects of a chemotherapy drug (temozolomide) and indirect electric stimulation promoted apoptosis and reduced proliferation (c). Reproduced with permission from Marino et al. (2019); Copyright Elsevier.

X.3 Nanoparticle-assisted piezoelectric stimulation of neural cells

X.3.1 Wireless nanoparticle-assisted modulation of electrophysiological activity

Different strategies have been proposed and implemented not only to regulate gene expression and drive cell differentiation, but also to induce short term effects, in particular to modulate neuronal electrical activity both at the single cell and network level. A neuronal network can be considered as a complex, highly interconnected circuit where signaling is based on the collective effects of electric charges, neurotransmitters, and action potentials.

Stimulation of excitable cells can be provided using electrodes both *in vitro* and *in vivo*, and deep electric stimulation plays an important role in many medical treatments for different pathological conditions (e.g., Parkinson's disease, cardiac arrhythmia, chronic pain) (Lonzano et al. 2019; Luan et al. 2014; Keifer et al. 2014). Nanomaterial-based coatings of electrodes and nanostructured electrode surfaces have been shown to improve electrode/cell coupling (lower impedance, higher charge injection capability) and, consequently better stimulation performances in terms of smaller applied voltages, lower power losses and, consequently, less tissue perturbation/damage.

Non-invasive *in vivo* neural stimulation represents the most effective solution for restoring lost neural functions and correcting neurological disorders in several diseases. Moreover, *in vitro* neuromodulation allows investigating a wide range of complex phenomena, from neural development to synaptic plasticity. A wireless, spatially resolved and "steerable" stimulation technology would represent a major advancement for *in vitro* experiments as well (Wang and Guo 2016).

Electromagnetic fields and acoustic waves have both been shown to elicit neuronal responses *in vivo* and *in vitro*, however they both allow poor spatial resolution when targeting regions deep in the brain. In this context, piezoelectric nanoparticles can be used as a localized transducer that is remotely driven by either an acoustic control signal and turn it into a suitable neuronal stimulus with sub-cellular spatial resolution and response time in the millisecond range (Rojas et al. 2018). Cell-type specificity can also be achieved because nanomaterials can be surface-modified and bio-conjugated (Marino et al. 2019).

X.3.2 Ultrasonic fields for neuromodulation

US can propagate as compression/rarefaction longitudinal waves in gases, liquids, and biological soft tissues. Soft biological tissues, which contain a large

amount of water, behave as liquids, from the point of view of sound propagation; harder tissues, such as bones, allow propagation of shear wave. While propagating, US interacts with matter either by reflection, absorption, or scattering, as electromagnetic waves such as light do, even if at a much lower rate. Such interactions attenuate the US intensity and release energy either as heat that, if not dissipated fast enough can increase locally the temperature and generate mechanical forces (acoustic radiation forces) that can, in turn, generate a stream of fluid, or cause the fast expansion/shrinking of gaseous microbubbles in the fluid (acoustic cavitation). The amount and the type of interaction depend on wave parameters (frequency, wavelength, speed of propagation, and intensity) as well as properties of the liquid or tissue (density and elastic modulus).

Effects of US propagation on biological tissues are at the basis of US medical diagnostic imaging; more in general, effects of US pressure waves on biological tissues, have been studied for safety (US dosimetry (O'Brien 2007)), therapeutic (tissue ablation (Hesley et al. 2013)), local drug delivery (Carpentier et al. 2016), thrombolysis (Bader et al. 2016), as well as imaging purposes (e.g., US localization microscopy). An extensive description of such reciprocal interactions can be found in (Dalecki 2004), while Maresca and colleagues provided a recent review of US biophysics at cellular and molecular levels (Maresca et al. 2018).

For neuromodulation, short pulses at low amplitudes are used in order to minimize thermal effects (i.e., heating) and provide mechanical actuation through radiation force avoiding cavitation. Several *in vivo* and *in vitro* studies showed either excitatory or inhibitory neuronal responses to directly applied US. Such results have been recently reviewed (Naor et al. 2016, Blackmore et al. 2019).

Although many models describing the mechanism at the basis of neural response have been proposed, a comprehensive understanding based on experimental evidences requires further investigation of US biophysics at the single cell and single channel level. Such understanding would allow exploiting US-protein interactions in order to increase sensitivity and selectivity to US stimulation by genetically modifying selected neurons to over-express mechanoreceptors. Such interesting approach, for which the proposing investigators coined the term sonogenetics, has been demonstrated for invertebrates (Kubanek et al. 2018); however, a proof of principle employing mechanoreceptors suitable for mammalians is still to be provided.

Coupling nanomaterials to transduce the US primary stimulus has the potential advantage of a better defined and, consequently, better controlled stimulation mechanism, which renders the stimulation more selective.

X.3.3 Indirect proofs and first demonstration of neural activation upon piezoelectric nanoparticle-assisted stimulation

Demonstration of the nanoparticle-assisted piezoelectric stimulation was primarily complicated by the vibrations of the electrophysiological electrodes used for monitoring the neural activity during the exposure to acoustic waves. All the traditional approaches of electrophysiological recording, such as, intracellular, extracellular and patch clamp whole cells suffer from this technical issue.

For this reason, the first proofs of neural stimulation with this approach were fundamentally indirect. Specifically, our group firstly investigated the *in vitro* development of a neural network during chronic piezoelectric stimulation (Ciofani et al. 2013). In this pioneering work, PC-12 neural-like cells were incubated with BNNTs, and the axonal outgrowth of these cells was monitored in concomitance with the chronic US stimulation ("BNNTs+US"). Results were compared with those obtained from cells incubated with BNNTs but not exposed to US ("BNNTs"), from cells exposed to US without BNNTs ("US"), and, finally, from negative control cultures ("Control"). Interestingly, the combined piezoelectric "BNNTs+US" treatment was able to remarkably promote the development of the neural network with respect to the other experimental classes ("BNNTs", "US", "Control"), both in terms of increased percentage of differentiated cells (+ 15-20%) and of enhanced number and length of β3 tubulin-positive neurites. No significant biological effects were found between the different control conditions ("BNNTs", "US", "Control"). Interestingly, the stimulation tests performed in the presence of non-specific blockers of Ca^{2+} channels (i.e., lanthanum ions ($LaCl_3$)) indicated as the enhanced neural differentiation induced by the chronic "BNNTs+US" stimulation was mediated by the Ca^{2+} influx; this result enforced the hypothesis of an effective indirect electrical stimulation since the intracellular Ca^{2+} elevations are required for the development of PC12 neurites during electric stimulation (Manivannan and Terakawa 1993). An increased axonal outgrowth was also observed in our work (Ciofani et al. 2013), when piezoelectrically stimulating ("BNNTs+US") SH-SY5Y cells, therefore highlighting a good versatility of this nanotechnology-based approach.

Other indirect experimental evidences of the efficacy of the piezoelectric stimulation on neural cells were subsequently collected by other independent groups by using piezoelectric membranes, films, microfibers and nanofibers (Lee and Arinzeh 2012; Genchi et al. 2016). As an example, the group of William Craelius developed piezoelectric PVDF films able to transduce mechanical vibration (50 Hz frequency) into oscillating electrical fields; thanks to this indirect electric stimulation, Craelius's group was able to promote the neurite outgrowth in rat spinal cord neurons (Royo-Gascon et al. 2013).

The first direct demonstration of the neural cell activation in response to nanomaterial-assisted piezo-stimulation was subsequently obtained by our group in BTNP-treated SH-SY5Y-derived neurons thanks to Ca^{2+} imaging investigations (Marino et al. 2015). At 24 h of BTNP incubation, nanoparticles resulted mostly

associated to the plasma membranes of these neurons, both at the level of cell bodies and of the neurites. After an acute 5 s exposure to US stimulation, high-amplitude Ca^{2+} waves were evoked only in SH-SY5Y-derived neurons that were previously incubated with piezoelectric BTNPs (tetragonal crystal); no high-amplitude Ca^{2+} waves were observed in US-stimulated cells that were not pre-incubated with BTNPs or that were pre-incubated with non-piezoelectric BTNPs (cubic crystal). This experimental evidence indicated that the cell activation was mediated by the piezoelectricity of the material and not by other non-specific phenomena (e.g., mechanical and thermal). Coherently, the stimulation in the presence of gentamicin, a blocker of mechano-sensitive cation channels, was not able to affect cell activation. The evoked high-amplitude Ca^{2+} waves resulted both tetrodotoxin (TTX) and cadmium (Cd^{2+}) sensitive, therefore indicating as the opening of voltage-gated Na^{+} and Ca^{2+} membrane channels was involved, respectively. Finally, experimental evidences were further corroborated by an electroelastic model of the voltage generated by BTNPs when exposed to different US intensities; the generated voltages are in the order of millivolt, values compatibles to the ones required for the activation of voltage-sensitive channels.

X.3.4 Electrophysiological recording of primary cultures: our results

Despite the difficulties related to electrophysiological recording using tip electrodes, planar multielectrode arrays (MEA) did not result significantly affected during US exposure. In this framework, it was possible to reversibly induce an excitatory response on *in vitro* networks of hippocampal or cortical neurons with low intensity ultrasonic pulses in the presence of piezoelectric BTNPs (Rojas, Tedesco et al. 2018). The sketch in Fig. X.2 describes the working principle of the method: in the presence of piezoelectric nanoparticles (NPs) adsorbed onto the cell membrane, neurons irradiated with an US pulse modify their spontaneous electrical activity. Without NPs or with non-piezoelectric NPs, the same US stimulus does not affect the electrophysiology of the neurons. In the following, we describe the developed procedures for the electrophysiological recording during piezo-stimulation and the main obtained results.

Neurons were extracted and dissociated from the cortex and hippocampus of rat embryos (E18), plated onto a commercial MEA (Multi Channel Systems MCS GmbH), and kept in incubator for 20-30 days in order to allow the development and maturation of an interconnected and spontaneously active neuronal network. With the MEA device the correlated electrophysiological activity was easily recorded. Sample preparation, set-up description and the experimental procedure were previously described (Rojas et al. 2018). Briefly, BTNPs were dispersed in culture medium 12 h before the experiment; BTNPs unspecifically bound the plasma membrane of the cell bodies and of the neurites, as observed by confocal

microscopy. In control experiments, primary cultures on MEA were incubated with BTNPs characterized by a cubic crystal structure, thus not showing piezoelectric behavior. Each circular electrode (30 μm in diameter) of the array records the extracellular field potential generated by the cells sitting on top or near it (typically one to three cells). In order to verify the capability of the network to respond to external stimuli and maintain such capability after US exposure, a standard electrical stimulation protocol was performed before and after the US stimulation experiments.

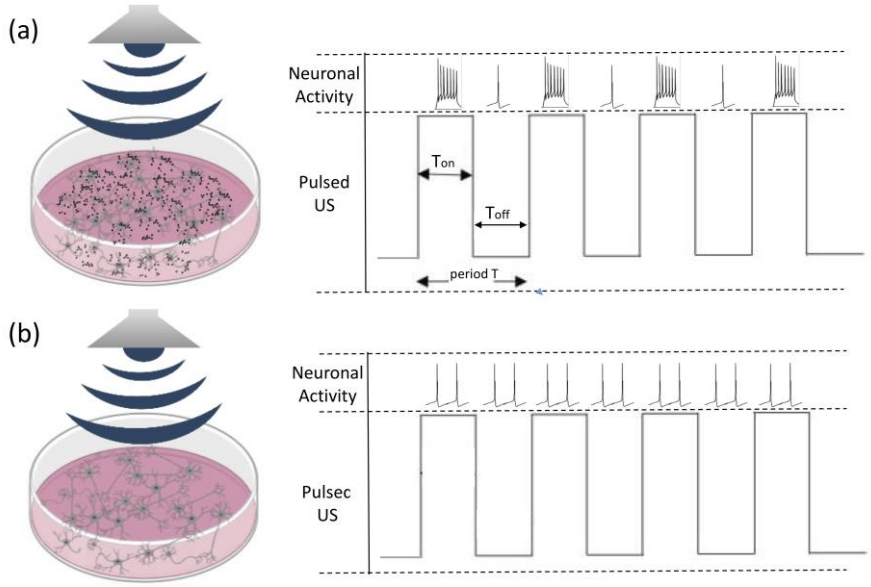

Fig. X.2 Piezoelectric BTNP-mediated ultrasound stimulation. (a) In the presence of piezoelectric BTNPs and low intensity US, electrical activity of primary neurons is significantly higher with respect to the spontaneous one. (b) The US stimulation has no effect on the electrical activity of neurons cultured without BTNPs.

US at 1 MHz frequency and relatively low intensity (~1 W/cm^2) was generated using a KTAC-4000 system (Sonopore) and transmitted to the MEA chamber through a thin thermoplastic film coated with a layer of acoustic gel. The pressure field generated by the piezoelectric transducer was experimentally characterized using a chamber with a miniaturize hydrophone (Teledyne RESON, model TC4038). US stimulation consisted of a sequence of US pulses of the same duration as schematically depicted in Fig. X.2; such periodic stimulation pattern is usually defined by its period and its duty cycle.

Fig. X.3 reports the result of a typical US stimulation experiment in the presence of piezoelectric BTNPs. Raw MEA recordings were processed by using a spike detection algorithm in order to extract the neuronal firing activity. Raster-plot representing with single points each detected spike as a function of the

recording electrode (numbered from 1 to 60, in the vertical axis) and of time (horizontal axis) is shown in Fig. X.3a. Fig. X.3b shows a magnification of the raster plot during piezo-stimulation. The red lines indicate, the switching on and off of the US generator. The black trace in Fig. X.3b represents the trend over time of the instantaneous firing rate, averaged over the 60 electrodes. The raster plot also shows spontaneous activity before and after the US stimulation. It is easy to recognize an increase in the firing activity over all the recording area during the stimulation, and, in particular, when the US is switched on (black trace in Fig. X.3b).

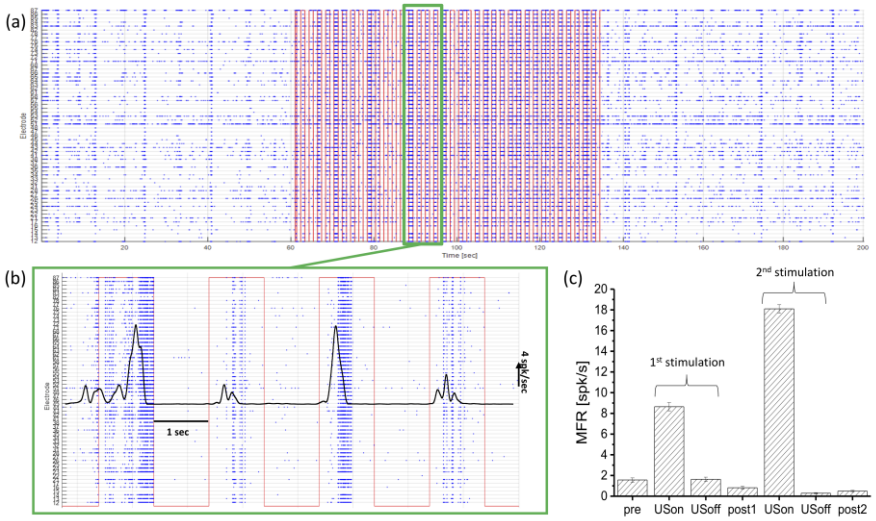

Fig. X.3 MEA recording of the electrical activity in primary neurons during BTNP-assisted and US-driven piezoelectric stimulation. (a) Raster plot recorded from 60 microelectrodes before, during, and after US stimulation. The red line indicates when the US is switched on and off (single pulse duration = 1 s, pulse frequency = 0.5 Hz). (b) Raster plot during the train of US stimulations; the superimposed black trace represents the trend over time of the instantaneous firing rate. (c) Mean firing rate (MFR) calculated before, during and after two successive trains of US stimulations.

In order to quantify the increase in electrical activity, we calculated the average firing rate (expressed as spikes per second) of the 60 electrodes. Specifically, we calculated both the instantaneous firing rate, defined as the reciprocal of the interval between successive spikes, and the mean firing rate (MFR), defined as the number of spikes in a certain time interval divided by the duration of the interval. In order to calculate the MRF values reported in Fig. X.3c, we considered 90 s before and after the stimulation ("pre" and "post" intervals) and over 90 consecutive 1-second intervals (total duration = 90 s) during which the US was switched on ("USon" interval) or off ("USoff" interval). Plot in Fig. X.3c reports the MFR values of two series of piezo-stimulations interspersed with 5 min of

interval. The MFR remarkably increases during the US pulses; such increase is both repeatable and reversible.

Moreover, we investigated the MFR ratio, defined as the difference between the MFR during "USon" and "Usoff" intervals, divided by the sum of the MFR values during a specific period of time (Fig. X.4). MFR ratio can vary between -1 (spiking activity only during the "USoff" intervals) and +1 (spiking activity only during the "USon" intervals). Fig. X.4a shows the MFR ratio measured by the recording electrodes of the array in response to different pulse repetitions. The average of the MFR ratios in Fig. X.4a is 0.78; this remarkably high MFR ratio is a clear indication of the increased activity when the US stimulation is applied in primary cultures pre-incubated with piezoelectric BTNPs. We also observed in a previous work of our group that the induced increase in firing activity during piezo-stimulation depends on the intensity of the generated US (Rojas et al. 2018). The graph in Fig. X.4b reports the average MFR ratio values obtained by stimulating the primary neurons pre-incubated with piezoelectric BTNPs, pre-incubated with non-piezoelectric BTNPs, or non-incubated with BTNPs. The corresponding values, close to zero, indicate that the US stimulation alone, in our experimental conditions and with the adopted low intensity levels, does not elicit any relevant response of electrical activity in the neural network. Moreover, since the cubic crystal non-piezoelectric BTNPs are not able to induce any significant increase of the MFR under US exposure, we can affirm that the piezoelectricity of the nanomaterial is required for the transduction of the US pressure stimulus into a biologically relevant excitation cues.

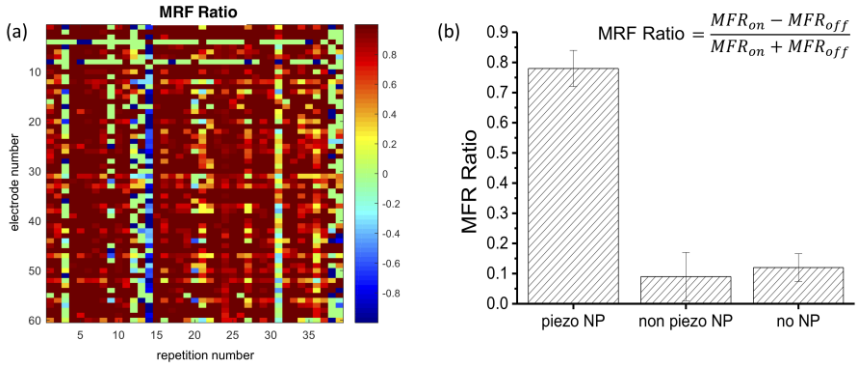

Fig. X.4 Analysis of the MFR ratio in response to piezoelectric stimulation. a) MFR ratio measured by the recording electrodes of the array in response to different US pulse repetitions in primary neurons pre-incubated with piezoelectric BTNPs. b) Average MFR ratio values obtained by stimulating the primary neurons pre-incubated with piezoelectric BTNPs, pre-incubated with non-piezoelectric BTNPs, or non-incubated with BTNPs.

In addition to the spiking activity, dissociated cortical or hippocampal cultures display peculiar patterns of electrophysiological activity, named bursts. A network burst is defined as a fast sequence of spikes (at least 5 spikes) and indicates fast re-

depolarization at single cell level and the almost synchronous activation at network level. Burst activity can be characterized by several parameters, such as the bursting rate (BR), the burst duration (BD), and the number of spikes in each burst (SpkxBurst). All these parameters can be averaged over all the electrodes of the array and over a certain interval of time. We calculated the mean values of these parameters before, during, and after US stimulation in the presence of piezoelectric BTNPs. Fig. X.5 reports the values of the above mentioned parameters after normalization to their respective maximum values; normalization has been carried out in order to plot them on the same dimensionless scale.

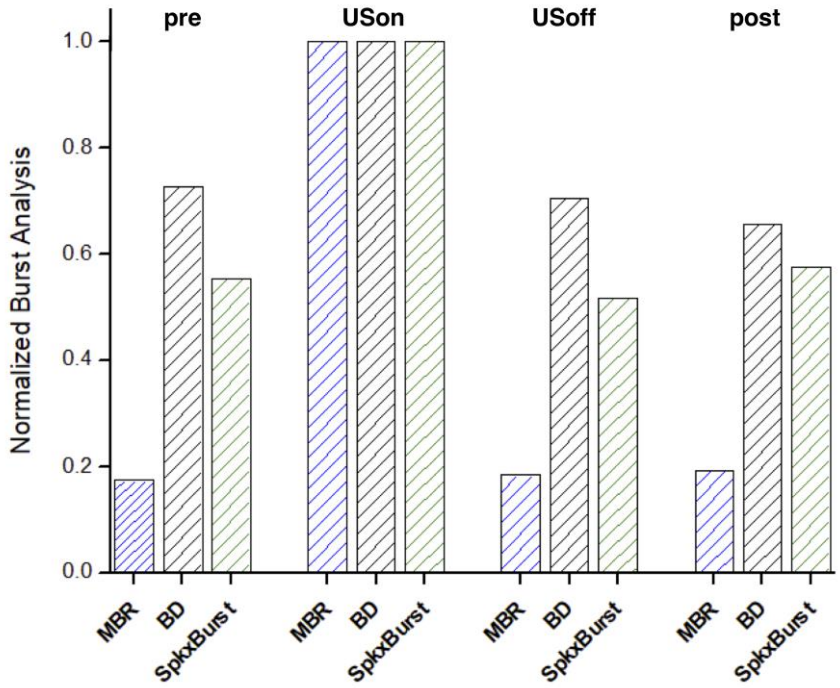

Fig. X.5 Network burst activity before, during, and after US stimulation for a representative experiment with piezoelectric NPs. Different parameters are plotted for each interval: mean burst rate (MBR), burst duration (BD), inter burst interval (IBI), and number of spikes per burst (SpkxBurst). Mean values are normalized with respect to the maximum during the experiment.

The first relevant observation is that US stimulation not only increases spiking activity (MFR) of cultures incubated with piezoelectric BTNPs, but also their bursting activity: during the pulses delivery, the MBR increases about 80% (up to 23.11 ± 0.8 bursts/min). Nonetheless, the MBR increase is limited to the USon intervals. When the US is off, MBR tends to the values measured before (*i.e.*, pre interval) and after (post interval) the stimulation (i.e., pre and post interval, respectively). Consistently with the MBR, also BD and SpkXBursts increase

during the “USon” intervals, indicating as the evoked bursts are longer and with a higher number of spikes than the spontaneous ones. To summarize, the analysis of the bursting activity suggests two main observations: (i) the effect of the US stimulation is temporally confined and does not evoke any plastic change at the network level, and (ii) US stimulation models the bursting activity both in terms of number of evoked bursts as well as their shape (i.e., duration). An increased evoked bursting activity (longer bursts with more spikes than the spontaneous ones) can be interpreted as an increased transmission of information throughout the network which is related to the stimulation.

The most intuitive interpretation of the working mechanism of US stimulation mediated by piezoelectric nanoparticles is that the US pressure field deforms the NPs. As a consequence, the NPs, which are interfaced to the cell membrane, generate electric potential differences. A simple analytical model of the mechano-electric transduction mechanism of piezoelectric BTNPs subjected to pressure field has been previously provided (Marino et al. 2015). The estimated voltage amplitudes generated by the US intensity range used in these cited works and in the experiments presented here (0.8 - 1 W/cm^2) correspond to about 0.2 mV. Since the NPs are interfaced to the cell membrane, such local voltage sources can increase the probability of activation of voltage-gated membrane channels, hence statistically increasing the membrane depolarization and, eventually, the probability that an action potential is generated.

Although preliminary, such results offer an intriguing opportunity to exploit US as an external signal for a highly selective neuromodulation technique based on nanotechnology.

X.4 Piezoelectric devices for *in vivo* neural stimulation and regeneration

In vivo neural stimulation has been shown as fundamental for tissue integrity regeneration and maintenance after trauma, and has promising implications concerning tissue function development and recovery in the case of other pathological conditions (such as genetically-derived sensorineural hearing loss, SNHL), including iatrogenic ones (for instance drug-derived SNHL). Traditionally, it is attained by electrodes which can either externally or internally be applied, but its principal drawbacks consist of electric field attenuation through tissues when external electrodes are used, the high invasiveness of the surgical interventions required for internal electrode positioning/substitution, as well as power management and resupply (Cogan 2008). Wireless stimulation is therefore highly desirable to circumvent these issues, and different approaches have to date been developed, including direct stimulation with ultrasonic waves (Menz et al. 2013; Hertzberg et al. 2009) and indirect stimulation with acoustic/ultrasonic waves mediated by piezoelectric materials (Hwang et al. 2015). The application of

mechanical stimulation to piezoelectric materials intimately interacting with biological environments indeed enables the treatment of deep tissues with high time resolution, though it requires further studies and technological advances in particular in order to improve spatial resolution (Inaoka et al. 2011). In the following, the most important examples of applications of piezoelectric materials to *in vivo* neural stimulation will be presented, and future directions on the topic will be suggested based on the current technological and nanotechnological opportunities.

Pioneering work on piezoelectric material application to *in vivo* regeneration of nerves after trauma was conducted by Aebischer and co-workers since late 1980s. In their work, they demonstrated that poled piezoelectric PVDF channels designed for nerve guidance supported transected sciatic nerve regeneration to a higher extent than unpoled (non-piezoelectric) channels in mice. Nerves regenerated in poled channels indeed featured a higher number of myelinated axons than those in unpoled channels, both at early and late stages of regeneration after implantation (Aebischer et al. 1987). In a following study on a rat transected sciatic nerve model, a higher number of myelinated axons were also achieved using positively charged piezoelectric poly(vinylidenefluoride-trifluoroethylene) (PVDF-TrFE) channels compared to unpoled channels. To a lower extent than positively charges one, also negatively charged channels supported better axonal regeneration, thus demonstrating the influence of polarity on neuronal regeneration (Fine et al. 1991).

Over the latest decades, PVDF and its copolymer with trifluoroethylene have also been used for different applications, like sensorineural hearing loss treatment. SNHL is a condition of impaired neural stimulation of the cochlear nerves due to altered or missing cilia on the cochlear epithelium (as an inheritable or acquired disorder). In this concern, Inaoka and co-workers developed and characterized a PVDF membrane based-device equipped with an interdigitated aluminum electrode array in view of its utilization as a self-powered cochlear prosthesis (Fig. X.6). The multilayered device was designed as a sensor with acoustic/electric signal conversion capability in the absence of battery. Although over-dimensioned compared to cochlear anatomy, the piezoelectric device showed a tonotopic response (in the 6.6-19.8 kHz range in air, and in the 1.4-4.9 kHz in silicone oil). It also generated maximum electrical response from an electrode positioned at the site of maximum vibration amplitude. The device was also scaled down for application in deafened guinea pigs (as rodent models for SNHL), and the PVDF membrane was demonstrated to induce auditory brain-stem responses upon sound stimulation and amplification of the electrical output. In this case, metal electrodes were implanted in the cochlea and the membranes were used externally. When implanted in the *scala tympani* of the basal turn of the cochlea, the device however did not develop an electric output sufficient to activation of auditory primary neurons, which was partially ascribed to suboptimal anatomical positioning (Inaoka et al. 2011). Another work from Tona and co-workers aimed at optimizing neural stimulation based on PVDF-TrFE films as electrodes inserted into the *modiolus* of guinea pigs. This enabled a significant decrease in the

thresholds of electrically evoked auditory brainstem responses compared with those of electrodes placed in the *scala tympani*. Due to the modest histological alterations detected with long term analyses, this study represented a further step in the application of piezoelectric films to neural stimulation in cochlear prosthetics (Tona et al. 2015).

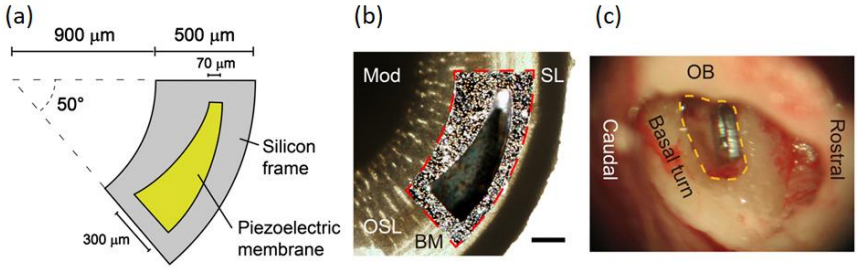

Fig. X.6 Design of a piezoelectric device based on a PVDF film (a) for treatment of sensorineural hearing loss in a rodent model (guinea pig). Superposition of the image of an implantable piezoelectric film and the basal turn of the guinea pig cochlea (b), where BM stands for basilar membrane, Mod for *modiolus*, OSL for *osseous lamina*, and SL for spiral ligament. A microscopic view of an implanted device in the basal turn of the guinea pig cochlea (c). The yellow dotted line highlights an opening in the basal turn of the cochlea (OB stands for *otic bulla*). Reproduced with permission from Inaoka et al. (2011); Copyright National Academy of Sciences of the Unites States of America.

Other studies also aimed at applying different piezoelectric materials to cochlear nerve stimulation: for instance, lead-based composites (lead magnesium niobate–lead titanate crystal with saturation polarization, an epoxy composite with saturated polarization, an epoxy composite with unsaturated polarization, and lead zirconate titanate crystal with saturated polarization) were implanted in the *scala tympani* of a feline model for SNHL by Guo and co-workers (Guo et al. 2011). By measuring the maximum decline of hearing thresholds, this investigation demonstrated that composite II supported better hearing ability recovery than the other materials, and supported evaluation of piezoelectric material performances relying on hydrostatic piezoelectric constants d_h and g_h (Guo et al. 2011).

As another application field of piezoelectric materials, deep brain stimulation (DBS) was demonstrated through a ternary, lead-based composite thin film (lead indium niobate–lead magnesium niobate–lead titanate, also termed PIMT) deposited on a flexible plastic substrate and connected to the primary motor (M1) cortex of a murine model. Upon moderate linear bending, the PIMT film indeed could generate a high current (far above the threshold for real-time DBS of the cortex) and a high voltage that enabled significant forearm movements (1.5-2.3 mm) in anesthetized mice (Hwang et al. 2015).

X.5 Conclusions

This overview of the available literature demonstrates that further investigations are necessary for a realistic application of piezoelectric materials *in vivo*, as clear indications on neuronal survival and function on mid- and long-terms are still missing. Future studies will have to consider carefully long-term interaction of the materials with the host, and that high piezoelectric performances are ensured along with high safety and proper anatomical site targeting. In particular, lead-based materials of high neurotoxicity (Bressler and Goldstein 1991) should be replaced by more biocompatible compounds (for instance: barium titanate), and targeting should be addressed from different perspectives by implementation of nanotechnology tools, such as nanoparticles, nanotubes, etc. in order to guarantee effective spatial resolution of stimulation from the cellular down to the subcellular level (Genchi et al. 2016; Salim et al. 2018). Recent literature has clearly shown that piezoelectric nanomaterials and nanocomposites can in particular operate a mechanoelectric signal transduction suitable to (1) opening voltage-gated ion channels on cell membranes (in particular, Ca^{2+} channels), and (2) triggering intracellular signal transduction cascades (Ciofani et al. 2013; Genchi et al. 2016; Wang and Guo 2016). Since the modulation of these events is involved in neuronal cell communication and survival (Wojda et al. 2008), the role of nanomaterials and nanocomposites (which often show completely different properties compared to their bulk counterparts) in responding to environmental stimulation but also in taking advantage from body motion (heart beating, respiration etc.) —yet still largely unexplored— will increasingly be determinant for proper addressing of neural stimulation and regeneration.

References

Aebischer, P., R.F. Valentini et al. (1987) Piezoelectric guidance channels enhance regeneration in the mouse sciatic nerve after axotomy. Brain Research **436**(1):165-168

Bader, K. B., G. Bouchoux et al. (2016). Sonothrombolysis. Advances in Experimental Medicine and Biology **880**: 339-62.

Blackmore, J., S. Shrivastava et al. (2019). Ultrasound Neuromodulation: A Review of Results, Mechanisms and Safety. Ultrasound in Medicine and Biology **45**(7): 1509-1536.

Bressler, J.P., G.W. Goldstein et al. (1991) Mechanisms of lead neurotoxicity. Biochemical Pharmacology **41**(4):479-84

Carpentier, A., M. Canney et al. (2016). Clinical trial of blood-brain barrier disruption by pulsed ultrasound. Science Translational Medicine **8**(343): 343re2.

Ciofani, G., S. Danti et al. (2013). Enhancement of neurite outgrowth in neuronal-like cells following boron nitride nanotube-mediated stimulation. ACS Nano **4**(10): 6267-77.

Cogan, S.F. (2011) Neural Stimulation and Recording Electrodes. Annual Review of Biomedical Engineering **10**:275-309

Dalecki, D. (2004). Mechanical bioeffects of ultrasound. Annual Review of Biomedical Engineering **6**: 229-48.

Fine, E.G., R.F. Valentini et al (1991) Improved nerve regeneration through piezoelectric vinylidenefluoride-trifluoroethylene copolymer guidance channels. Biomaterials **12**(8):775-780

Genchi, G.G., L. Ceseracciu et al. (2016) P(VDF-TrFE)/BaTiO3 nanoparticle composite films mediate piezoelectric stimulation and promote differentiation of SH-SY5Y neuroblastoma cells. Advanced Healthcare Materials **5**(14): 1808-1820.

Genchi , G.G., A. Marino et al. (2016) Barium titanate nanoparticles: promising multitasking vectors in nanomedicine. Nanotechnology **27**:232001

Genchi, G. G., A. Marino et al. (2017). Remote Control of Cellular Functions: The Role of Smart Nanomaterials in the Medicine of the Future. Advanced Healthcare Materials **6**(9).

Genchi, G. G., E. Sinibaldi E. et al. (2018). Ultrasound-activated piezoelectric P(VDF-TrFE) / boron nitride nanotube composite films promote differentiation of human SaOS-2 osteoblast-like cells. Nanomedicine: Nanotechnology, Biology and Medicine **14**(7): 2421-2432.

Guo, S., X. Dong et al. (2011) Properties Evaluation of Piezoelectric Materials in Application of Cochlear Implant. Ferroelectrics **413**(1):272-278

Hertzberg, Y., A. Volovick (2010) Ultrasound focusing using magnetic resonance acoustic radiation force imaging: Application to ultrasound transcranial therapy. Medical Physics **37**(6):2934-2942

Hesley, G. K., K. R., Gorny et al. (2013). MR-guided focused ultrasound for the treatment of uterine fibroids. CardioVascular and Interventional Radiology **36**(1): 5-13.

Hwang, G.-T., Byun M. et al. (2015). Flexible Piezoelectric Thin-Film Energy Harvesters and Nanosensors for Biomedical Applications. Advanced Healthcare Materials **4**(5): 646–658.

Hwang, G., Y. Kim et al. (2015) Self-powered Deep Brain Stimulation via a Flexible PIMNT Energy Harvester. Energy and Environmental Science **8**:2677-2684

Inaoka, T., H. Shintaku et al. (2011). Piezoelectric materials mimic the function of the cochlear sensory epithelium. Proceedings of the National Academy of Sciences of the United States of America **108**(45): 18390–1839.

Keifer, O. P., J. P. Riley et al. (2014). Deep Brain Stimulation for Chronic Pain. Neurosurgery Clinics of North America **25**(4): 671–692.

Kubanek, J., P. Shukla et al. (2018). Ultrasound Elicits Behavioral Responses through Mechanical Effects on Neurons and Ion Channels in a Simple Nervous System. The Journal of Neuroscience **38**(12):3081-3091.

Lee, Y. S. and T. L. Arinzeh (2012). The influence of piezoelectric scaffolds on neural differentiation of human neural stem/progenitor cells. Tissue Engineering Part A. **18**(19-20):2063-72.

Lin, F,, S. E. Shelton et al. (2017). 3-D ultrasound localization microscopy for identifying microvascular morphology features of tumor angiogenesis at a resolution beyond the diffraction limit of conventional ultrasound. Theranostics. **7**: 196–204.

Lonzano, A. M., N. Lipsman et al. (2019). Deep brain stimulation: current challenges and future directions. Nature Reviews Neurology **15**(3): 148–160.

Luan, S., I. Williams, et al. (2014). Neuromodulation: present and emerging methods. Frontiers in Neuroengineering **7**(27).

Maresca, D., A. Lakshmanan et al. (2018). Biomolecular Ultrasound and Sonogenetics. Annual Review of Chemical and Biomolecular Engineering **9**: 229-252.

Manivannan, S. and S. Terakawa S. (1993) Rapid filopodial sprouting induced by electrical stimulation in nerve terminals. The Japanese Journal of Physiology **1**: S217-20.

Marino, A., E. Almici er al. (2019). Piezoelectric Barium Titanate Nanostimulators for the Treatment of Glioblastoma Multiforme. Journal of Colloid and Interface Science **538**: 449-461.

Marino, A., S. Arai et al. (2015). Piezoelectric Nanoparticle-Assisted Wireless Neuronal Stimulation. ACS Nano **9** (7): 7678–7689.

Marino, A., J. Barsotti et al. (2015). Two-photon lithography of 3D nanocomposite piezoelectric scaffolds for cell stimulation. ACS Applied Materials and Interfaces **7**(46): 25574-25579.

Marino, A., M. Battaglini M., et al. (2018). Ultrasound-Activated Piezoelectric Nanoparticles Inhibit Proliferation of Breast Cancer Cells. Scientific Reports **8**(1): 6257.

Marino, A., G. G. Genchi et al. (2017). Piezoelectric Effects of Materials on Bio-Interfaces. ACS Applied Materials and Interfaces **9**(21): 17663-17680.

Menz, M.D., Ö. Oralkan et al. (2013) Journal of Neuroscience **33**(10): 4550-4560

Naor, O., S. Krupa et al. (2016). Ultrasonic neuromodulation. Journal of Neural Engineering **13**(3): 031003.

Nguyen, T. D., N. Deshmukh, et al. (2012). Piezoelectric nanoribbons for monitoring cellular deformations. Nature Nanotechnology **7**(9): 587.

O'Brien, W. D. Jr. (2007) .Ultrasound-biophysics mechanisms. Molecular Biology **93**(1-3): 212-55.

Ribeiro, C., J. Pärssinen et al. (2015). Dynamic piezoelectric stimulation enhances osteogenic differentiation of human adipose stem cells. Journal of Biomedical Materials Research Part A **103**(6):2172-5.

Rojas, C., M. Tedesco et al. (2018) Acoustic stimulation can induce a selective neural network response mediated by piezoelectric nanoparticles. Journal of Neural Engineering **15**(3): 036016.

Royo-Gascon, N., M. Wininger et al. (2013). Piezoelectric Substrates Promote Neurite Growth in Rat Spinal Cord Neurons. Annals of Biomedical Engineering **41**(1): 112–122.

Salim, M., D. Salim et al. (2018) Review of nano piezoelectric devices in biomedicine applications. Journal of Intelligent Material Systems and Structures **29**(10):2105–2121

Tona, Y., T. Inaoka et al. (2015) Development of an electrode for the artificial cochlear sensory epithelium. Hearing Research **330**(2015) 106e112

Wang, Y. and L. Guo (2016). Nanomaterial-Enabled Neural Stimulation. Frontiers in Neurosciences **10**: 69.

Wang, X., J. Liu, et al. (2007). Integrated Nanogenerators in Biofluid. Nano Letters **7** :2475–2479.

Wojda, U., E. Salinska et al. (2008) Calcium ions in neuronal degeneration. International Union of Biochemistry and Molecular Biology- Life **60**(9):575-590

Zhang, L., F.X. Gu et al. (2008). Nanoparticles in medicine: Therapeutic applications and developments. Clinical Pharmacology and Therapeutics **83**(5): 761-769.

Zhang, S., L. Yin et al. (2009). Focusing ultrasound with an acoustic metamaterial network. Physical Review Letters **102**(19): 194301.