Optimization of electrical stimulation parameters for enhanced cell proliferation on biomaterial surfaces

AQ2 **Ashutosh Kumar Dubey, Shourya Dutta Gupta, Bikramjit Basu**

Laboratory for Biomaterials, Department of Materials Science and Engineering, Indian Institute of Technology, Kanpur 208016, Uttar Pradesh, India

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Abstract: From the point of view of biocompatibility of bone analog materials, cell–material interaction is of fundamental importance. In this article, we report the effect of pulse electric field stimulation on cell–material interaction by analyzing cellular functionality and viability. An in-house fabricated pulse electric field setup was used for the application of electric field during cell culture experiments. To optimize voltage/electric field, the first set of exploratory experiments was conducted with varying field strength at fixed frequency, and subsequently, the frequency of the electrical stimulation was varied to study its influence on the proliferation of L929 mouse fibroblast cells on gelatin-coated control disc. Subsequently, L929 cells were cultured on hydroxyapatite (HA) and HA-40 wt % $BaTiO_3$ composite. Cell-cultured samples were analyzed qualitatively as well as quantitatively using fluorescence microscope and scanning electron microscope. It has been demonstrated that due to the application of electric field during the cell culture experiment, the cell proliferation and the cell spreading on the surface of the biomaterials were enhanced within a narrow window of voltage/frequency of electrical stimulation. At lower field intensities, the energy density is quite low and increases parabolically with field strength. There is no significant increase in the temperature ($\Delta T \sim 10^{-5}$ K) of the medium due to the application of short duration pulse electric field. This led us to believe that electric field with appropriate strength and duration can enhance the cell–material interaction. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B:000–000, 2011.

Key Words: pulsed electric field, calcium phosphate(s), cell–material interactions, $BaTiO_3$

INTRODUCTION

It is known that continuous movement of charged ions across cell membrane leads to net charges around a living cell, and therefore, the interaction of cells with the external electric field is anticipated. Recently, our research group reported the deterministic analytical model to evaluate the interaction of external electric field with a single living cell.[1] In a follow-up study, we implemented stochastic model to incorporate the concept of fluctuations in interactions of cellular organelles with electric field.[1] Although a number of theoretical attempts have been made in the analysis of cell–electric field interactions,[1–4] the experimental confirmation to assess whether electric field can substantially influence cell–material interaction is rather limited.[5,6] In a work carried out by Bourguignon et al.,[7] it has been reported that the rate of protein and DNA synthesis of human fibroblasts could be enhanced for the specific combination of stimulation parameters.

A recent study by Ercan et al.[8] showed that the application of DC pulse electric stimulation each day for 1 h at a voltage of 15 V and frequency of 20 Hz on anodized titanium enhanced the osteoblast proliferation by 72% after 5 days of culture. However, no systematic investigation was carried out to probe into the influence of electrical stimulation parameters such as field strength, frequency, and time duration. A hypothesis was put forward by Vodovnik et al.[9] supporting that electrical currents enhance the healing rate of chronic wounds and retard the growth of tumor.

A given combination of electrical stimulation parameters has been found to have different response on various cell types from same tissue. Under an applied potential difference, the osteoblast-like cells migrated toward the negative electrode, whereas the osteoclasts migrated in opposite direction (toward positive electrode).[10] These results have important cellular basis for hypothesizing the clinical application of electrical stimulation for bone healing.[11] Field-induced cell shape changes, directional alignment, and field polarity-dependent cell migration have been reported in number of studies.[10,12–14] The threshold for field-induced shape changes and perpendicular alignment in embryonic quail fibroblasts was measured at field strength ~1 V/cm.[4] Currently, there is a considerable interest to enhance the physical interaction, for example, adhesion, proliferation, and differentiation of living cells with various biomaterial surfaces. It is expected that successful experimental exploitation of this concept can lead to *ex vivo* tissue formation, and this will facilitate the faster osteointegration of synthetic implant material.

AQ9 **Correspondence to:** B. Basu; e-mail: bikram@iitk.ac.in
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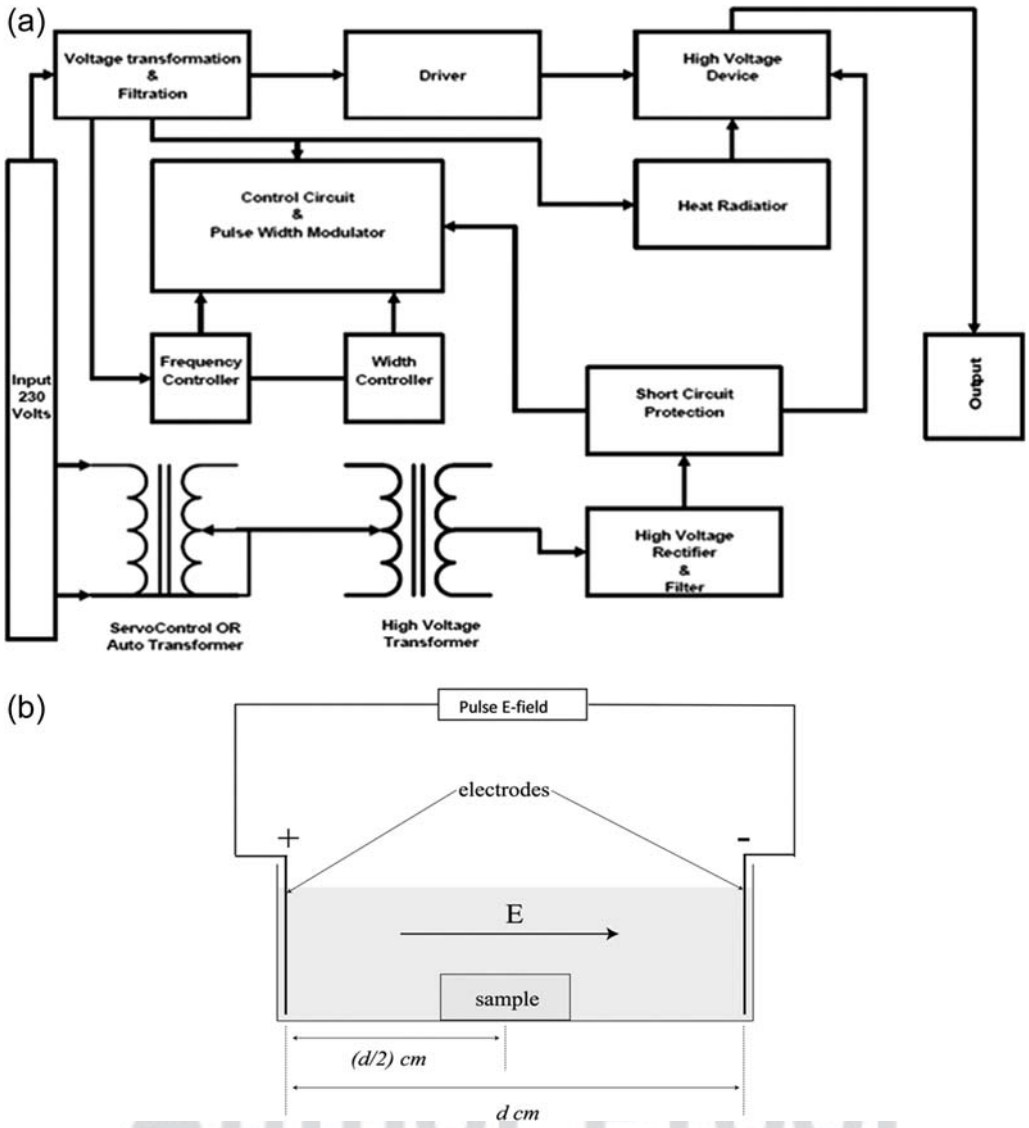

FIGURE 1. (a) Block diagram of the entire electrical setup assembly. (b) Schematic diagram showing the position of electrodes and direction of E-field with respect to the sample in one of the eight-well culture plate. [Color figure can be viewed in the online issue, which is available at AQ8 wileyonlinelibrary.com.]

It is well known that a layer of adsorbed proteins mediates cell adhesion to biomaterial surfaces.[15] The type, quantity, and expression of these adsorbed proteins are dependent on substrate properties and more importantly on chemistry and hydrophobicity.[16] Because of electrically charged nature of proteins, the external electric field can also influence protein adhesion on the material surface. Because of the dielectric nature of cell membrane, the effect of external electric field is expected to depend on frequency and field strength. The aim of this study is to optimize the external stimulation parameters such as voltage, time duration, and frequency for enhancing the growth and proliferation of cells in contact with biomaterial surfaces. ~~For the first time~~ we have shown that the optimal electrical stimulation parameters are dependent on the substrate used in the *in vitro* cell culture experiments. The results of the experiments have been analyzed both qualitatively as well as quantitatively.

MATERIALS AND METHODS

Electrical setup

An in-house electric field setup is developed for applying the pulse electric field during *in vitro* cell culture studies. Figure 1(a) shows the block diagram of electric field F1 application system. The voltage from main power source is filtered and applied to all other major units in the circuit, which include function generator (FG) for pulse generation, a switching circuit (SC), and high voltage variable power supply (PS). Both the frequency and the width of the applied pulses are modulated with the FG, which can generate pulses in the frequency range of 0.3 Hz to 1 MHz. Metal–oxide–semiconductor field-effect transistor (MOSFET)

switch is used to modify the frequency and voltage of the applied pulses as desired for the experiment. The SC unit is responsible for the application of pulses of desired frequency and voltage to cells. The pulses from FG are fed to the SC through a high power MOSFET, controlled by a driver circuit. An opto-coupler isolates the FG from the PS, thus, protecting its low current signal circuitry from high power transients, generated in the PS. The PS can deliver a variable DC output voltage in the range of 1–600 V within an error margin of 0.3 V. The recommended operating temperature range for both the PS and FG is 0–50°C, and therefore, the above described setup is suitable for cell culture experiments, which are conducted at 37°C. A short circuit protection unit protects all the major components in the setup. To facilitate uniform parallel electric field across the electrodes and minimize fringing effects, rectangular-shaped culture well plates ($40 \times 30 \times 12$ mm^3; NUNC) were used. The duty cycle of the pulse waveform is given by the equation, % duty cycle $= (t_p{*}f){*}100$; where, t_p = pulse width and f = frequency of the wave.

Synthesis of model biomaterials

In the present study, two model biomaterials, that is, hydroxyapatite (HA) and HA-40 wt % $BaTiO_3$ (HA-40BT) were selected. Pure HA is widely recognized for its excellent biocompatibility. However, pure HA is an electrical insulator. In reality, natural bone is a piezoelectric composite,[17] and therefore, 40 wt % $BaTiO_3$ added HA composite is developed to enhance the piezoelectric property. Preliminary experimental measurements reveal HA-40BT composition enhances the dielectric constant (measured at 1 MHz) from 11.5 (HA) to 20.4 in the composite as well as increases piezoelectric strain coefficient from 0 pC/N (HA) to 0.5 pC/N in the composite. The electrical properties of HA-40BT are fairly comparable with that of natural bone.

The detailed synthesis procedure for HA has been discussed in our earlier report.[18] For the synthesis of $BaTiO_3$, stoichiometric amounts of $BaCO_3$ and TiO_2 were weighed and mixed in a ball mill using agate as grinding media and acetone as a milling medium for 6 h in agate jar. The mixed powders were dried overnight in oven. The dried powders were calcined at 1000°C for 6 h in a platinum crucible.

To synthesize the biocomposites, appropriate amount of in-house synthesized HA and $BaTiO_3$ were ball milled for 16 h in a planetary ball mill (agate jar and balls) with acetone as milling medium. The powder was pressed uniaxially to form pellets of 12 mm diameter and 2–3 mm thick. These green pellets were sintered via conventional sintering route in air. Monolithic HA sample was sintered at 1200°C for 2 h, whereas HA-40BT was sintered at 1300°C for 3 h. The heating and cooling rates were maintained at 5°C/min. The sintering temperature has been optimized on the basis of sintered bulk density of the sample.

Material characterization

XRD analysis was carried out using Cu Kα radiation (λ = 1.54184 Å) to identify and to characterize the different phases present in the sintered samples. Scanning electron microscope (SEM) analysis was undertaken to study the grain structure of the sintered samples. To observe the microstructure, the samples were first polished using diamond paste, and they were thermally etched for 15 min in air at a temperature of 100°C lower than their respective sintering temperatures.

In vitro experiments

Mouse fibroblast (L929) cell lines (ATCC, USA) were used for cell culture experiment. The culture media used for these cells was Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum (Sigma Aldrich) and 1% penicillin/streptomycin solution (Sigma Aldrich). The cells were seeded on the sterilized samples at a density of 3×10^5 cells/mL in the rectangular eight-well culture plates. The samples were sterilized in an autoclave at 121°C temperature and 15 pound pressure for 20 min.

Electric field stimulation. The equipment, designed for the application of pulse electric fields during culture experiments, can be operated in the voltage range of 0–500 V and frequency range of up to 1 MHz. Because of the high tunability of the parameters, a wide range of cell types as well as materials with different properties (biocompatibility, electrical properties, etc.) can be electrically stimulated during culture experiments. Figure 1(b) shows the schematic diagram of the culture well with the sample and electrodes. The electric field is applied by parallel electrodes, and the direction of electric field is parallel to the sample surface.

In all cell culture experiments, unless otherwise mentioned, initially the cells on the control sample/biomaterial surface were incubated for 6 h under field-free conditions, and then the electric field was applied for 5 min, and the same process is repeated once more, before the entire culture system was incubated for 12 h. Also, the distance between the positive and negative electrodes during the experiment was 3.0 cm (unless otherwise mentioned). In all our experiments, stainless steel plates were used as electrodes. To compare the results, same set of samples were kept inside the incubator, and no electric field was applied to the culture solution. The electrical stimulation was carried out using the earlier described pulse electric field setup outside the CO_2 incubator, and immediately after stimulation, the culture solution is placed inside the incubator. This kind of electric field stimulation sequence was adopted because of the concept that fibroblast-like cells adhere moderately within first few hours and the first electrical stimulation of 5 min was given after first 6 h to ensure the application of electric field mostly on the adhered cells.

The first set of experiments was conducted at varying voltages of 1, 2, 5, 10, 15, 20, 25 V and a frequency of 100 Hz (duty cycle 4%) on gelatin-coated control glass discs [see Figure 2(a)]. The pulse width during this experiment was 400 μs. F2

Subsequently, we studied the effect of frequency of the electric stimulation on the cell adhesion characteristics on the gelatin-coated control discs [see Figure 2(b)]. To study the variation of cell proliferation with frequency of the

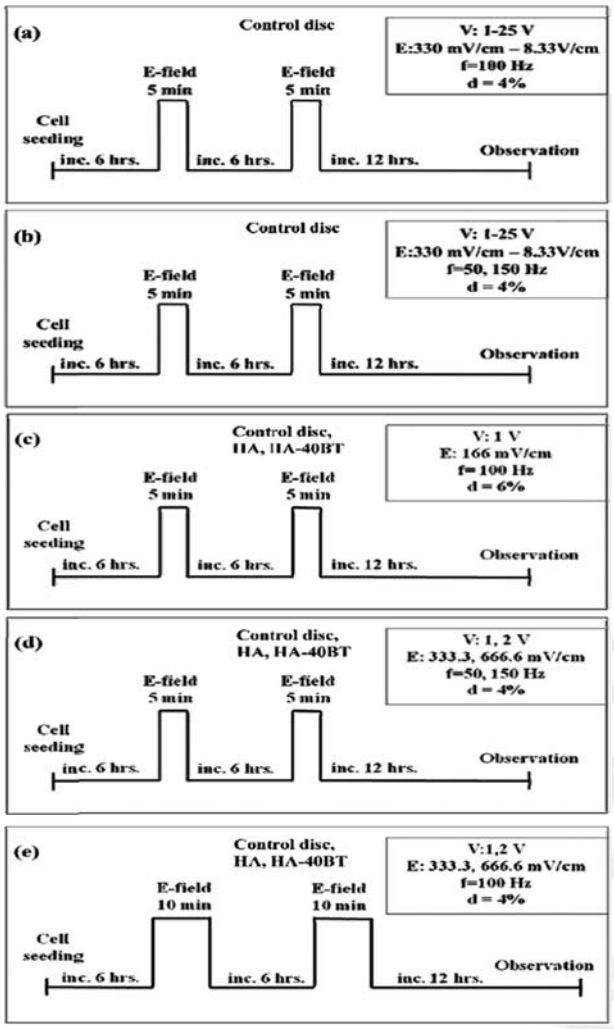

FIGURE 2. Schematic of sequence of the electrical stimulation (a) first set of experiments, (b) second set of experiments, (c) third set of experiments, (d) fourth set of experiments, and (e) fifth set of experiments. (Here, inc. refers for incubation in field free condition).

electrical stimulation, we performed the second set of experiments at voltages of 1, 2, 5, 10, 15, 2, and 25 V with two other frequencies of 50 and 150 Hz.

Based on the initial results performed on gelatin coated control disc, third set of cell culture experiments [see Figure 2(c)] was carried out at 1 V, 100 Hz, and with a duty cycle of 6% on HA and HA-40BT. In this experiment, the distance between the positive and negative electrodes was kept fixed at 6 cm. After the entire incubation period, the cultured samples were washed twice with PBS and then fixed with 1.5% glutaraldehyde in PBS. A series of ethanol solutions (30, 50, 70, 90, and 100%), each for 10 min twice, was used for dehydration. Finally, the samples were dried using hexamethyl disilazane (Himedia). The samples were then sputter coated with gold, and SEM (Philips, Quanta) was used to study the cell adhesion behavior.

The fourth set of experiments was carried out at a voltage of 1V and 2V and a frequency of 100 Hz (4% duty cycle) on pure HA and HA-40 BT composite [see Figure 2(d)]. The pulse width during this experiment was 400 μs. Fluorescence microscopy was used to study the cell morphology and the cell density on the cultured samples after the necessary staining procedure, as elaborated in Fluorescence Microscopy section.

The next set of experiments was carried out to study how cell proliferation was affected by a change in the duration of the electric field application. This experiment was performed at exactly the same voltage and frequency as the previous one [i.e., voltage of 1 and 2 V and a frequency of 100 Hz (4% duty cycle)] with the exception that the total duration of the electric field was increased from 5 to 10 min [see Figure 2(e)]. Fluorescence microscopy was again used to study the cell morphology and the cell density on the cultured samples after the necessary staining procedure, as given in Fluorescence Microscopy section.

At this juncture, it is important to mention the selection of the above mentioned range of potential and frequency. It is known that the cell response to E-field is dependent on cell type, and to access such response for fibroblast cells, our preliminary experiments with a wide voltage range (0–25 V) reveal that the cell density drastically decreases on application of 10 V or larger potential field. At potential of larger than 10 V, the turbidity and pH of culture medium also showed significant change. In view of this, experiments were performed in the window of 0–10 V. As far as the frequency is concerned, it is known that the natural bone has a resonance frequency of around 300 Hz,[19,20] and therefore, lower frequency range of 50–150 Hz was chosen.

Fluorescence microscopy. After the required incubation time, the samples were washed twice with 1x PBS and then fixed for 30 min using 3.7% paraformaldehyde (PFA) in PBS. The samples were again washed with 1x PBS, and then the adhered cells on the sample were permeabilized using 1x Triton 100. In the next step, the samples were washed again using 1x PBS, and then the cells were blocked using 1% bovine serum albumin for 2 h. The washing of the samples was subsequently carried out using 1x PBS, and then Alexa Fluora 488 Phalloidin (Invitrogen) fluorescent dye was added for 1 h to stain the cytoskeleton of the cells adhered on the samples. This was followed by another step of washing, and finally Hoechst fluorescent dye (Invitrogen) was added for 30 s for the staining of the nucleus. The samples were washed again with 1x PBS. The prepared samples were then observed under fluorescent microscope (Nikon LV100D) to study the cell adhesion and proliferation behavior on the various substrates.

Cell density analysis. Fluorescent/optical transmission microscope images of four nonoverlapping regions on each of the samples were taken to calculate the average cell density on each of the samples. The cell density was subsequently determined for each experiment by counting the number of cells from at least 20 images. The commercial SPSS-13 software was used to analyze the difference in statistical significance of the results. The univariate analysis of variance (ANOVA) method has been adopted for statistical

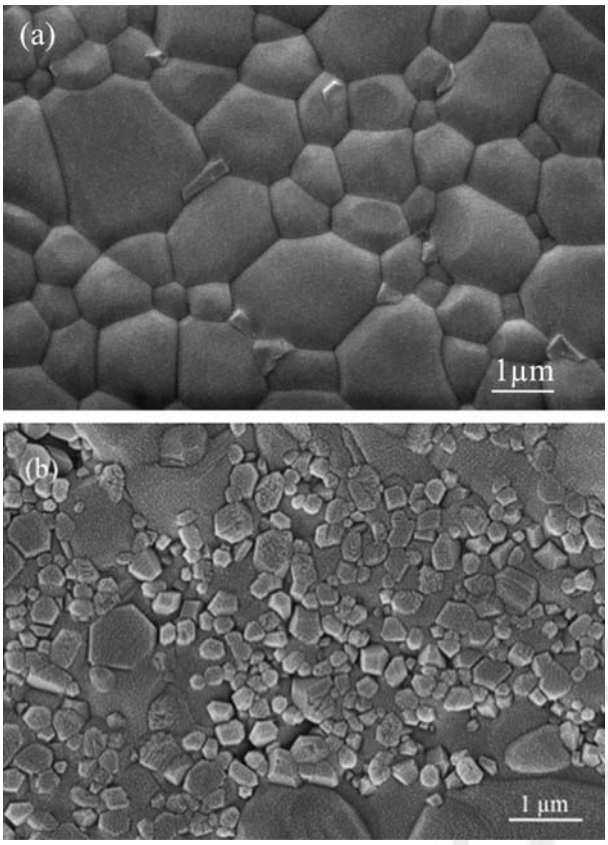

FIGURE 3. SEM micrographs of (a) HAp and (b) HAp-40 wt % $BaTiO_3$ surfaces after thermal etching. Finer grains in (b) are of $BaTiO_3$ as identified by EDS analysis.

analysis, particularly the *post hoc* multiple comparisons of the mean of independent groups using Tukey test (for homogeneous data) at statistical significant value, $p < 0.05$.

RESULTS

Microstructure and phase assemblage

XRD analysis indicated the dominant presence of HA and ferroelectric $BaTiO_3$ phase in sintered microstructures (not shown). The important observation that can be made from the XRD spectra is that no reaction occurred between HA and $BaTiO_3$ during the sintering process.

F3 In Figure 3(a,b), representative SEM images of sintered thermally etched HA and HA-40BT are shown. Although HA grain sizes around 1–2 μm in the monolith, finer HA grains of 1 μm size are observed in the composite (identified by EDS analysis). The difference in sintering conditions can explain such differences in grain size. Also, equaixed $BaTiO_3$ grains with size 200–400 nm are found to distribute homogenously in HA matrix [Figure 3(b)].

Cell adhesion behavior and cell density analysis

A general observation is that the cells have adhered and proliferated uniformly on the entire surface. In our experiments, circular samples were used, and the samples were not fixed on the culture well plate. Therefore, it was difficult to ascertain any specific difference in cell density on samples surfaces regions depending on its closeness to either cathode or anode side. Also, any discussion on the electric field distribution in the cellular medium and its influence on difference in adhered cell density is beyond the scope of present work.

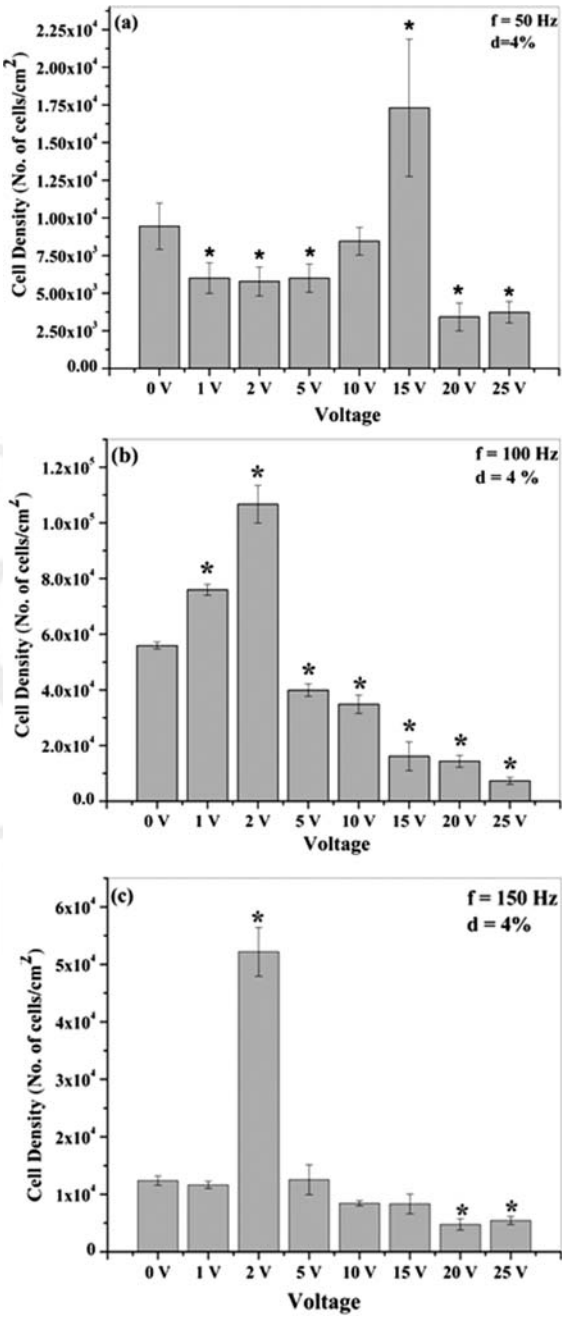

FIGURE 4. The cell density evaluation of L929 fibroblast cells, cultured for 24 h on control plastic disc samples treated with different voltages at (a) 50 Hz, (b) 100 Hz, and (c) 150 Hz. The distance between the electrodes was kept fixed at 3 cm. The cell-seeded samples were first incubated for 6 h, then the electric field was applied for 5 min, and the same process is repeated once more before the entire culture system was incubated for 12 h. For comparison, the untreated samples with cells are incubated for 24 h. Asterisk mark (*) represents significant difference among the samples with respect to control sample at 0 V at $p < 0.05$ and error bars correspond to ± 1.00 SE.

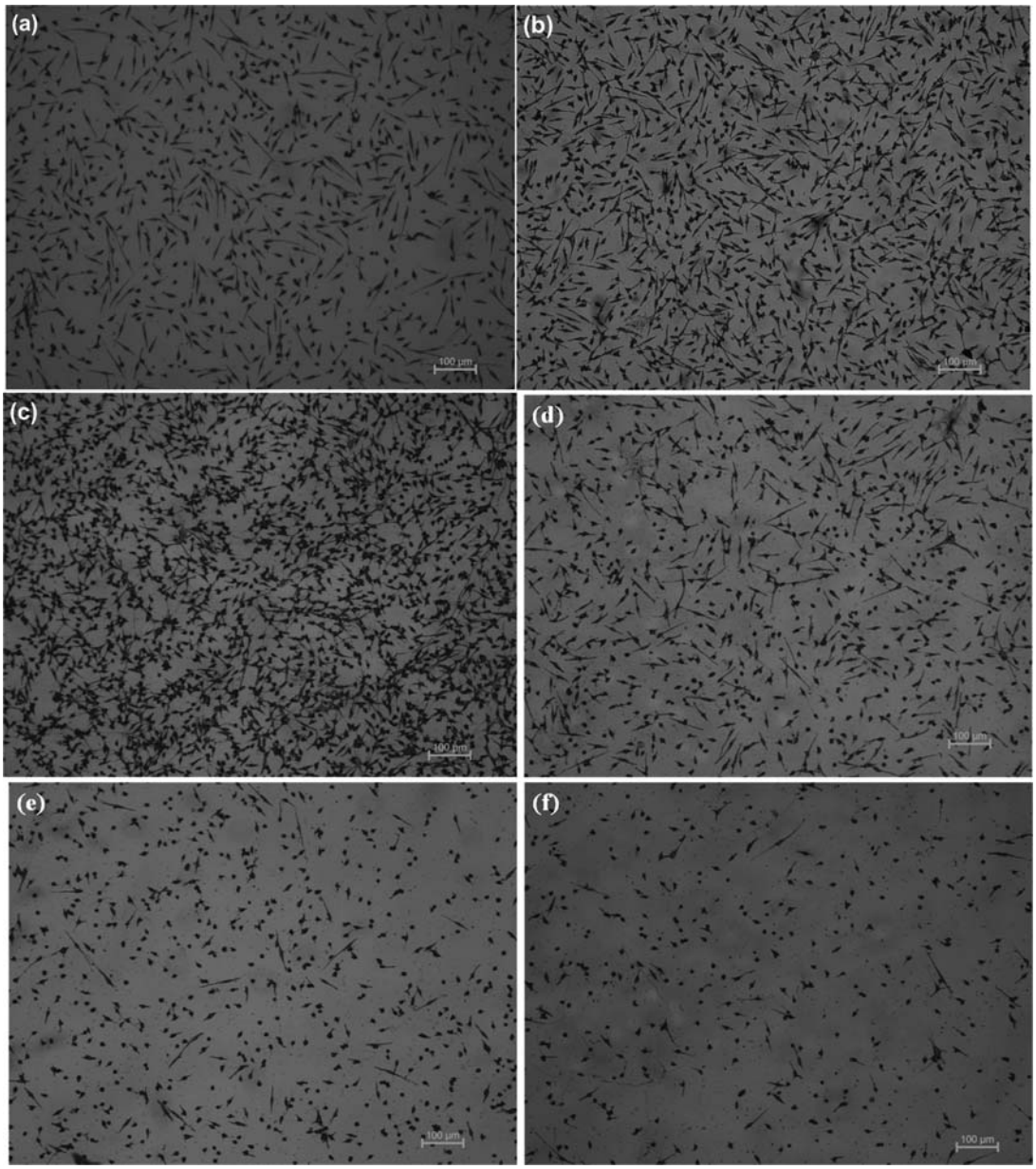

FIGURE 5. Transmission optical microscope images of L929 cells adhered on control disc: (a) Cultured without electric field, (b) 1 V, (c) 2V, (d) 10 V, (e) 20 V, and (f) 25 V. The distance between the electrodes was kept fixed at 3 cm. The cell-seeded samples were first incubated for 6 h and then the electric field (100 Hz, duty cycle 4%) was applied for 5 min and the same process is repeated once more before the entire culture system was incubated for 12 h. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

F4 The result of initial set of experiments [Figure 4(a)] showed that the cell proliferation is significantly affected with the exposure to the external electrical stimulation and in particular with field strength. The cell proliferation increases [Figure 4(a)] with increase in applied voltage (electric field) up to 2 V (666.7 mV/cm). Further increase in voltage has deteriorating effect on proliferation in comparison with unexposed cells. To provide evidence, representative optical transmission microscope images are shown F5 in Figure 5, which shows more pronounced cell adhesion at 2 V [Figure 5(c)] when compared with the untreated sample [Figure 5(a)]. At 25 V (8.33 V/cm), majority of the cells seems to be fragmented [Figure 5(f)]. Another observation is that at higher voltages (>10 V), the color of the media was changed from red to yellowish orange near the positive electrode. This may be due to the change of pH of the media, resulting in the diminishing of the number of cells by death from acidity.

In second set of experiments [Figure 2(b)], two independent experiments at different frequencies (50 and 150 Hz) were performed, while keeping the similar range of applied voltage as above (1–25 V). It is observed that the optimal voltage for enhanced cell proliferation varies with the variation in frequency of the applied electrical stimulus.

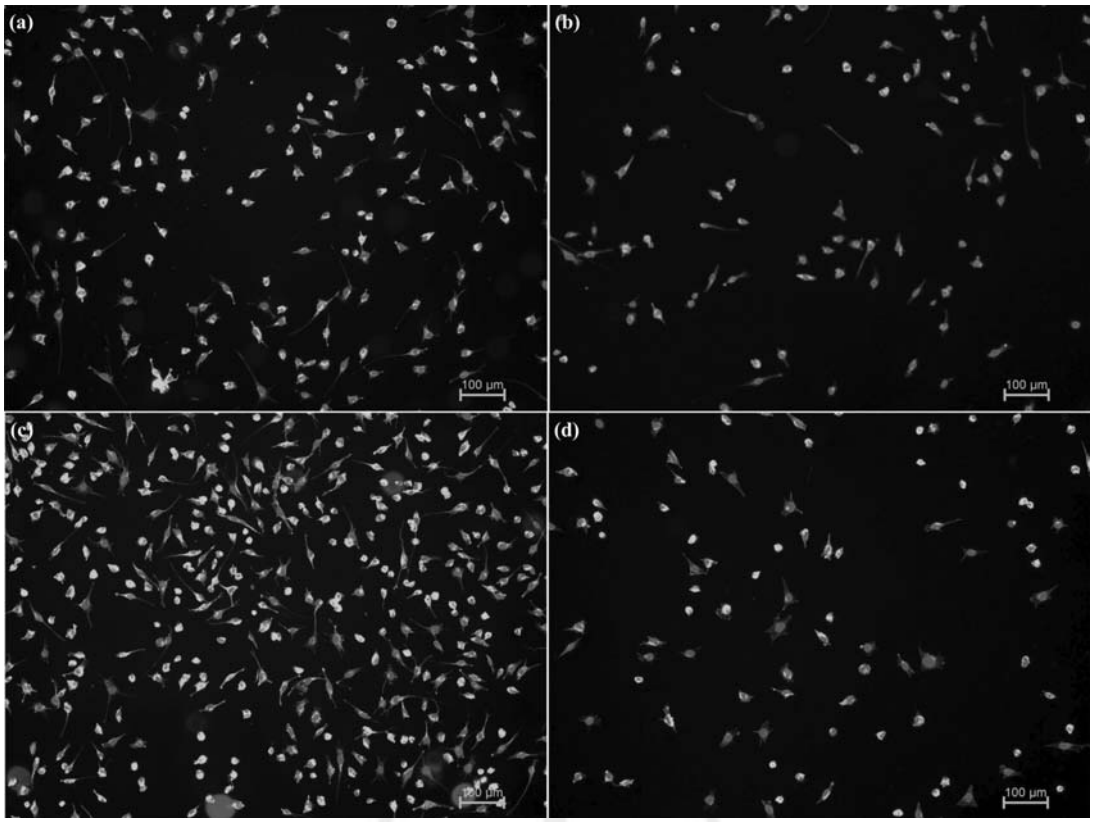

FIGURE 6. Fluorescent microscope images of L929 cells adhered on control disc: (a) Cultured without electric field, (b) 1 V, (c) 15 V, and (d) 25 V. The cell-seeded samples were first incubated for 6 h and then the electric field (50 Hz, duty cycle 4%) was applied for 5 min and the same process is repeated once more before the entire culture system was incubated for 12 h. The distance between the electrodes was kept fixed at 3 cm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

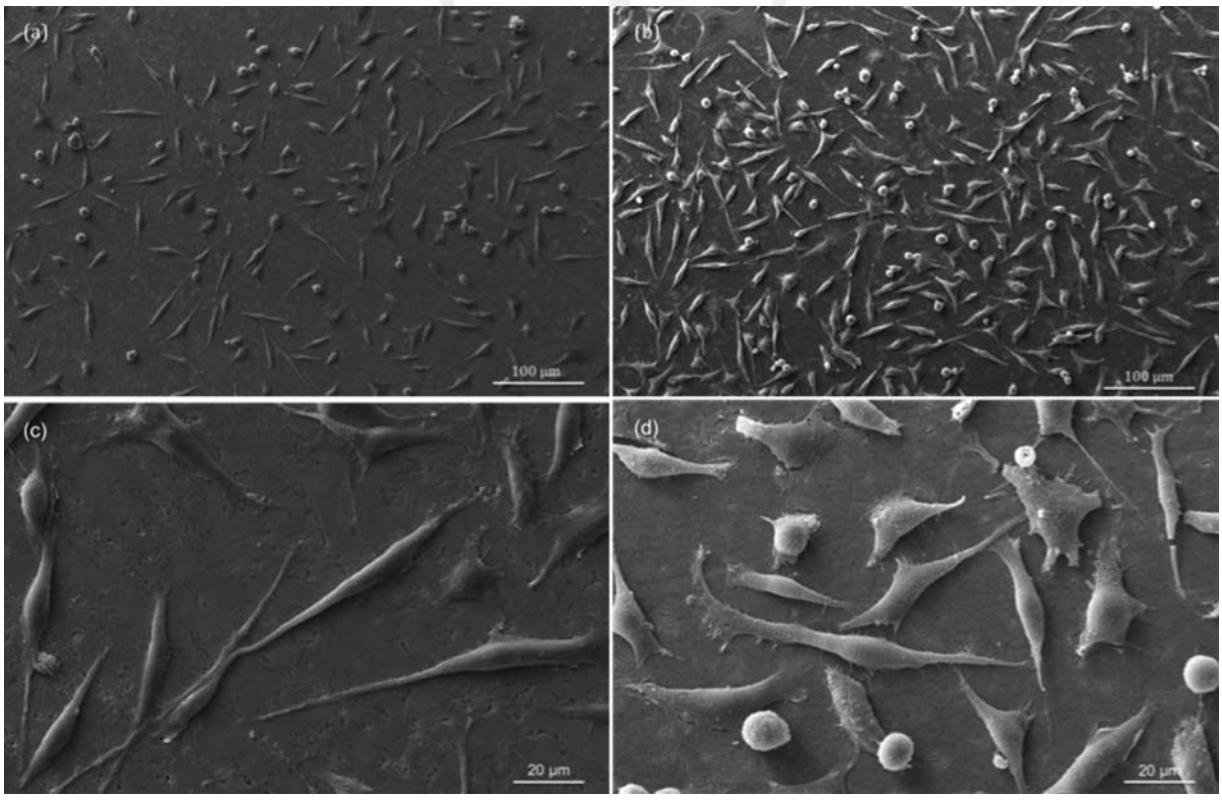

FIGURE 7. SEM images of L929 cells adhered on the HAp-40% $BaTiO_3$: (a) and (c) Cultured without electric field, (b) and (d) Cultured with electric field. The cell-seeded samples were first incubated for 6 h and then the electric field (1 V, 6% duty cycle, and 100 Hz) was applied for 5 min and the same process is repeated once more before the entire culture system was incubated for 12 h. The distance between the electrodes was kept fixed at 3 cm. For comparison, the untreated samples with cells are incubated for 24 h, before SEM analysis.

At 50-Hz frequency, the optimal voltage is 15 V (5 V/cm) [Figure 4(b)], and at frequency 150 Hz, the optimal field strength for enhanced cell proliferation is 2 V (666.7 mV/cm) [Figure 4(c)]. This leads to the conclusion that the optimal voltage for enhanced cell proliferation is shifted toward lower values with increase in frequency of applied electrical stimulation and vice versa [Compare Figure 4(a–c)]. These results are in direct agreement (qualitative) with the results reported by Bourguignon et al.,[9] where they showed that the optimal voltage for enhanced DNA and protein synthesis shifted to higher values at lower frequencies. Figure 6 shows the fluorescent images of the cells adhered on the control disc cultured with and without external stimulation of 1, 15, and 25 V at 50 Hz frequency. The cells treated at a voltage of 15 V [Figure 6(c)] clearly show the enhanced cell density and lamellepodium extensions when compared with the untreated samples. Majority of the cells attain a globular shape, when treated at 25 V.

The next sets of experiments [Figure 2(c)] were carried out at 1 V and 100 Hz frequency. In this case, the duty cycle was 6%, and the distance between the positive and negative electrode was 6 cm, and therefore, the electric field was 166.7 mV/cm. SEM images [Figure 7] revealed the enhanced cell fate processes for the E-field treated cells adhered on the HA [Figure 7(b)] and HA-40BT composites [Figure 7(d)] when compared with the untreated HA [Figure 7(a)] and HA-40BT composites [Figure 7(c)]. The cells adhered on the substrate with elongated morphology (unidirection) [Figure 7(a,c)]. A clearly distinct morphology was seen when the cells were treated with E-field [Figure 7(b,d)].

From Figure 8(a), it is clear that the irrespective of sample composition, the cell proliferation increases in all the treated samples when compared with the untreated samples. In this case, the applied voltage was 1 V and the distance between the electrodes was 6 cm. Therefore, the electric field will be 166.7 mV/cm. However, the material dependent proliferation behavior is observed when exposed to the higher field strength [Figure 8(b)]. In case of control and HA-40BT, the cell proliferation increases with the strength of electric field from 333.3 mV/cm (1 V) to 666.7 V/cm (2 V). On the other hand, higher E-field deteriorates the proliferation on pure HA samples [Figure 8(b)].

Next set of experiments was carried out in low voltage region (1 V, 2 V) and at a frequency of 100 Hz (duty cycle of 4%) on biomaterial surfaces. Figure 9 shows the fluorescent images of the L929 cells cultured on HA and HA-40BT composite materials, in the absence [Figure 9(a,c)] and presence of external electric field [Figures 9(b,d)]. For HA, it was observed that the application of voltage of 1 V (333.3 mV/cm) at 100 Hz, the cell density decreases. This result in conjunction with the results of third set of experiments clearly shows that the optimal voltage for enhancing the cell proliferation lies somewhere between 166.7 and 333.3 mV/cm, which is lower than the optimal voltage evaluated for control disc (666.7 mV/cm). On HA-40BT composite, it is seen that the application of 1 V (333.3 mV/cm) does not show any significant difference from the nontreated composite, but the application of external stimulus of 2 V

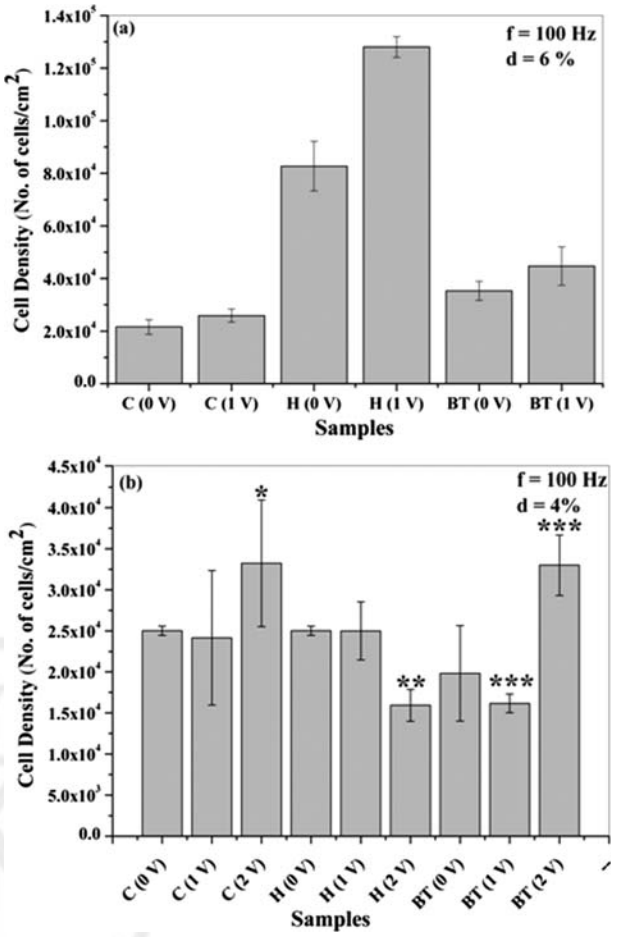

FIGURE 8. The cell density evaluation of L929 fibroblast cells, cultured for 24 h on control plastic disc (C), pure HA (H), and HA-40BT (BT) samples treated with and without electric field (a) 1 V (f =100 Hz, d = 6%) and distance between electrodes was kept fixed at 6 cm (b) 1, 2 V (f = 100 Hz, d = 4%) and the distance between the electrodes was kept fixed at 3 cm. The cell-seeded samples were first incubated for 6 h and then the electric field was applied for 5 min and the same process is repeated once more before the entire culture system was incubated for 12 h. Asterisk mark (*) represents significant difference among the control disc at $p < 0.05$ and error bars correspond to ±1.00 SE. Asterisk mark (**) represents significant difference among the HA samples at $p < 0.05$ and error bars correspond to ±1.00 SE. Asterisk mark (***) represents significant difference among the HA-40BT at $p < 0.05$ and error bars correspond to ±1.00 SE.

(666.7 mV/cm) leads to an increase in the cell density. This clearly shows that the optimal voltage for maximizing the cell proliferation on HA-40BT is probably equal or greater than 2 V (666.7 mV/cm).

In the final set of experiments [Figure 2(e)], the total duration of the electrical stimulation at each step was increased from 5 to 10 min. In case of gelatin-coated control discs, it is seen that a peak occurs at 1 V (Figure 10) when compared with the occurrence of the peak at 2 V, when the exposure time was 5 min. Comparing this with Figure 4, it is observed that the optimal voltage for enhancing the cell proliferation shifts toward lower voltages with an increase in the exposure time. For pure HA, it is seen that the application of 1 V for 10 min duration does not cause any

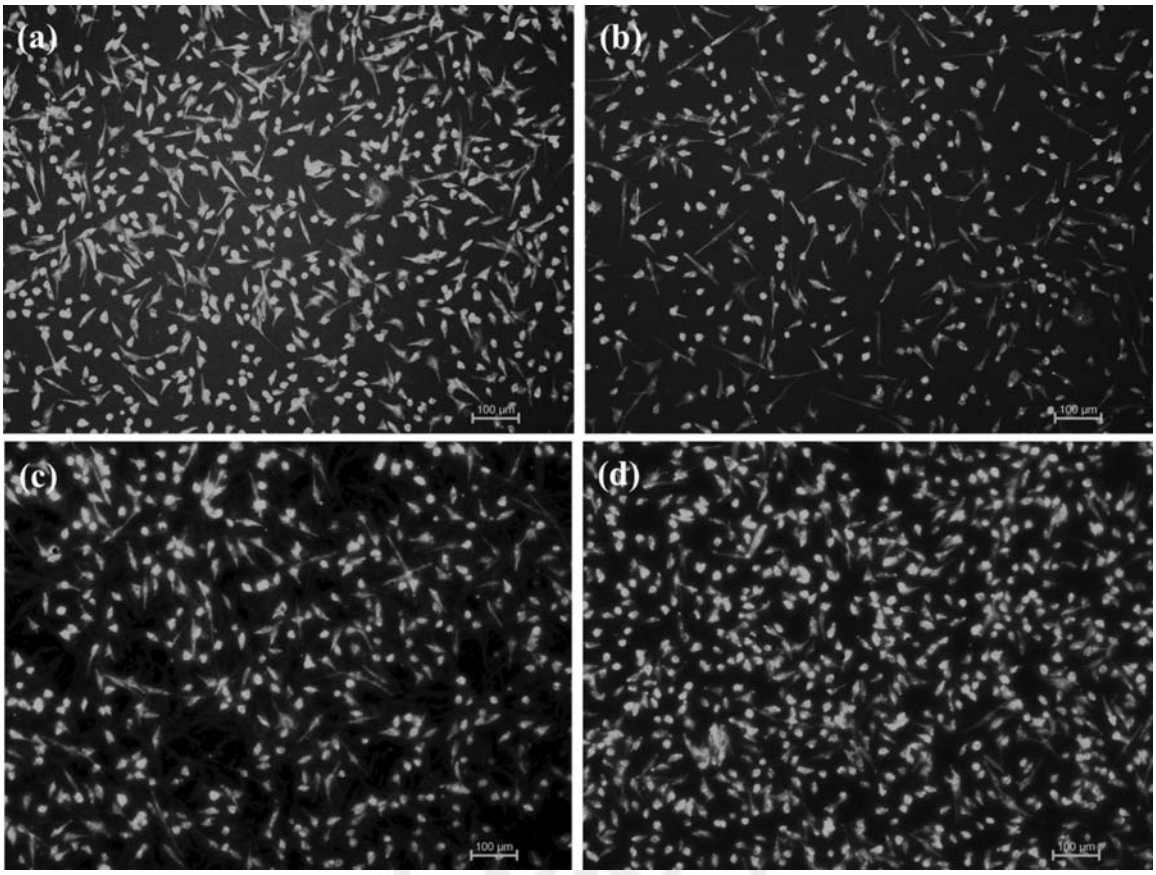

FIGURE 9. Fluorescence optical microscope images of L929 cells (stained with Alexa Fluor® 488 Phalloidin dye) adhered on (a) HA cultured without electric field, (b) HA cultured with electric field 1 V, (c) HA-40BT cultured without electric field, and (d) HA-40BT cultured with electric field 2V. The cell-seeded samples were first incubated for 6 h and then the electric field (100 Hz, duty cycle 4%) was applied for 5 min and the same process is repeated once more before the entire culture system was incubated for 12 h. The distance between the electrodes was kept fixed at 3 cm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significant variation of the cell density with respect to control sample. However, on the application of 2 V for 10 min duration, a significant difference in cell density was observed with respect to untreated sample. Considering cellular functionality on HA-40BT sample, it is seen that the application of the electrical stimulation for 10 min leads to a detrimental effect on cell density, irrespective of the voltage (1 or 2 V). This leads us to believe that the optimal voltage shifts to lower voltages in case of HA-40BT, if the total time of exposure at each stage is increased.

DISCUSSION

The experimental results, as presented in the previous section, clearly demonstrate that E-field stimulation parameters, that is, voltage, frequency, and time duration can individually influence the cellular proliferation of transformed cells (e.g., fibroblasts) on control disc. Additionally, the experiments on a limited number of materials also suggest that the presence of piezoelectric $BaTiO_3$ phase in HA matrix shifts the optimal E-field strength to obtain highest cell density toward higher values, compared with that in case of pure HA.

In particular, we noticed that the external electric field affects the cell–biomaterial interaction in terms of the morphological differences, actin cytoplasmic extension and importantly, cell viability. Even though the cells were found to adhere on both the treated and the nontreated substrates, it is observed that the treated samples boasted of more number of cells as well as increased cell spreading on the surface. The relevant cell fate processes that must be considered in the present context include cell adhesion, cell growth, and proliferation.

The adhesion of cell to the substrate (the biomaterial) is preceded by the formation of an adsorbed layer of adhesion proteins[21] on the substrate. The adhesion is primarily guided by the physical interaction of these adhesion proteins with the protein receptors in the cell membrane. The cell adhesion proteins may originate from the physiological fluid or the cell itself may secrete these proteins in their absence from the physiological fluid.[6,21] The two important substrate properties influencing cell adhesion are substrate roughness[22,23] and substrate surface charge.[24] For our cell culture experiments, the substrate surfaces of both the samples (HA, HA-40BT) were smoothly polished to same extent so as to obtain similar surface roughness values. Thus, any

difference in the cell adhesion is likely not due to the roughness of the surface ($R_a \approx 0.2$–0.5 μm). Also, as no surface treatment technique is used on the substrate prior to the cell culture, no surface charge is generated on the surface. There is a possibility that the formation of the adsorbed protein layer on the substrate may get affected due to the applied electric field. Kotwal et al.[25] reported that fibronectin adhesion on a biomaterial substrate generally reaches equilibrium in about 2 h, and the application of electric field after 2 h does not show any significant effect on the adsorbed protein layer. As in all our experiments, the electric field was applied to the samples after 6 h of incubation, it is highly probable that the applied electric field did not have a major effect on the adsorbed protein layer.

Cell proliferation and cell growth are both cell fate processes, dependent on protein and DNA synthesis within the cell. It has been shown by Bourguignon et al.[9] that protein and DNA synthesis are enhanced in the presence of an optimized electric field. Currently, the mechanism of protein synthesis in the presence of an electric field is not well understood, but a few hypotheses can be put forward: (a) At higher voltage, more current will pass through the culture medium. From the measurements of electrical conductivity of culture medium (2.03 Sm^{-1} at 37°C), we found that high current of 1.03 A flows between the electrodes at high voltage of 25 V. b) the observed color changes from (red to orange/yellow) of the medium at high voltages are reflection of the change in pH from near neutral to acidic regime. As the protein expression is highly sensitive to local changes in pH and knowing that cellular functionality on substrate depends on interaction between cell surface receptors and adsorbed legends on the substrate, it is consistent that cellular proliferation/survival will decrease at higher voltages.

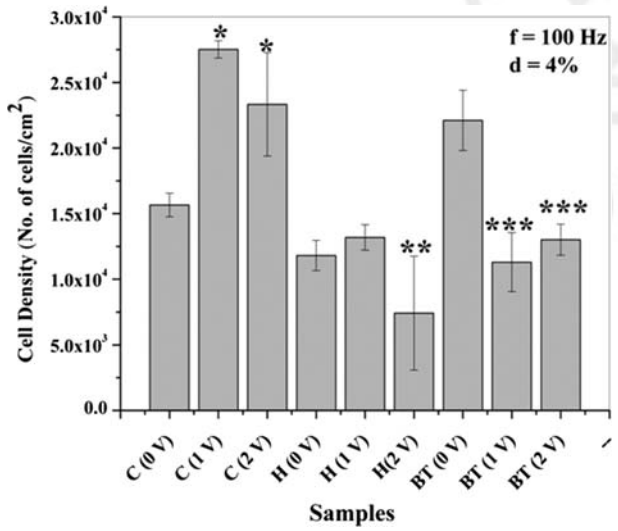

FIGURE 10. The cell density evaluation of L929 fibroblast cells, cultured for 24 h on control plastic disc (C), pure HA (H) and HA-40BT (BT) samples treated with and without electric field. The cell-seeded samples were first incubated for 6 h and then the electric field (1 V, 2 V (distance between electrodes = 3 cm), 100 Hz, 4% duty cycle) was applied for 10 min and the same process is repeated once more before the entire culture system was incubated for 12 h. Asterisk mark (*) represents significant difference among the control disc at $p < 0.05$ and error bars correspond to ±1.00 SE. Asterisk mark (**) represents significant difference among the HA samples at $p < 0.05$ and error bars correspond to ±1.00 SE. Asterisk mark (***) represents significant difference among the HA-40BT at $p < 0.05$ and error bars correspond to ±1.00 SE.

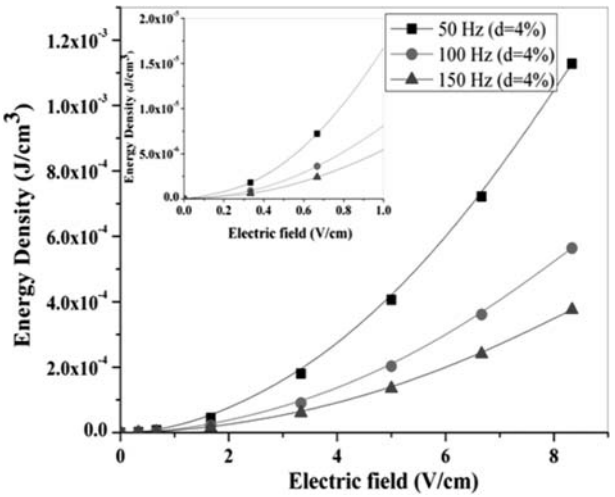

FIGURE 11. Variation of energy density as a function of electric field strength for different frequencies (50, 100, and 150 Hz) at duty cycle of 4%. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

To find out the origin of cell apoptosis at higher field strength, the possibility of an increase in temperature caused by the energy supplied to the medium due to the external electric field has been analyzed. Energy densities at different voltages have been evaluated using the formula used by Kolb et al.[26] From Figure 11, it is clearly evident F11
that at low voltages, the energy density is quite low and increases parabolically with voltage. An increase in temperature caused by the energy supplied to the medium due to the external electric field has been analyzed. Our calculations further reveal that there is no significant increase in the temperature ($\Delta T \sim 10^{-5}$ K) of the medium due to the application of short duration pulse electric field. In the absence of any significant heating effect, an alternative molecular level hypothesis can be considered to explain the effect of tailored E-field stimulation on cellular functionality.

It is possible to hypothesize that the enhancement of the protein and DNA synthesis at low voltages is due to the effect of electric field on internal function of the cell. In a recent study by Schminnelpfeng et al.,[27] it was shown that low frequency electrical stimulation enhanced the cellular proliferation via secondary messenger dependent processes. As far as the molecular mechanism of the cell proliferation is concerned, the electrical signal is expected to activate extracellular signal molecules during field application of 5/10 min after first incubation stage. The extracellular signal molecules can be bound to cell surface receptor proteins and signals can be further transferred via molecular switch mechanisms to intracellular signaling proteins.[28] In a cell specific manner, the intracellular signaling pathway enables

the electric field induced signals to reach the target proteins. Such molecular level mechanism appears to more effective on application of low voltages (1–2 V) for fibroblasts adhered on control glass samples, HA or HA-$BaTiO_3$ composite. However, it is not clear why such signaling mechanism has an inhibition effect at higher voltage, particularly on control sample. Nevertheless, our study confirms that the application of electric field pulse using new stimulation sequences can enhance cell proliferation on control substrate or investigated bioceramic substrate, when applied within a narrow window of E-field parameters.

We believe that the signaling mechanism followed by enhanced protein synthesis or actin polymerization is probably the mechanism through which cellular proliferation is enhanced at low voltages/E-field strengths in the present study. However, the exact molecular level mechanism of how an electric field enhances DNA and protein synthesis with the cell needs further investigation, but it is clear that due to application of an appropriate field, both the processes can be enhanced.

CONCLUSIONS

(a) Using the culture experiments on control disc, it has been demonstrated that the combination of electrical voltage, time duration, and electrical pulse sequence has significant effect on cell fate processes. It is shown that there exists an optimal voltage for achieving maximum cell density, which is dependent on the frequency, duty cycle, and time duration of the electrical stimulation. In case of control discs treated at a frequency of 100 Hz, the optimal voltage was around 2 V (666.7 mV/cm). Larger voltages of more than 2 V were found to have an inhibitory effect on the cellular proliferation.

(b) The optimal voltage for enhanced cell proliferation varies with the frequency of applied electrical stimulation. On increasing the frequency from 50 to 150 Hz, it was observed that the optimal voltage shifted from 15 to 2 V in case of control discs.

(c) The presence of a piezoelectric phase (like $BaTiO_3$) shifts the optimal voltage to higher values in HA-40 wt % $BaTiO_3$ composites when compared with pure HA. In case of pure HA, the optimal E-field strength at a frequency of 100 Hz was found to be around 166.7 mV/cm. In contrast, in case of HA-40 wt % $BaTiO_3$, it was found to be equal to or greater than 666.7 mV/cm.

(d) An increase in the time duration of the electrical stimulation from 5 to 10 min at each stage of electrical stimulation shifted the optimal E-field strength toward lower values in case of both control disc and HA-40 wt % $BaTiO_3$.

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