

BIOIMPEDANCE PLATFORM TO ASSESS DRUGS AGAINST PHOTO-OXIDATIVE CELL BEHAVIOR USING HDFn CELLS

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Abstract

Light radiation plays an important role in skin diseases. In this study, we have provided a simple, high-throughput and real-time bio-analytical method to measure the radiation effect on the human dermal fibroblast neonatal (HDFn) cells by electric cell substrate impedance sensing (ECIS) system. This bio-analytical system was utilized for radiation effects on cells that exposed to various bio-active compounds to treat damage cells. The ECIS physical response is well correlated with standard qualitative results. Prolonged light-exposure caused time dependent growth drop in HDFn cells and these resulted in photo-oxidative damage due to deregulation of intra and extra cellular biochemical activities followed by cell cycle arrest. ECIS measurements show < 50mM concentrations were found to be well tolerated non-toxic safer and its show good protection against light radiation toxicity. The proposed system guides the effective in-vitro model, and would be of wide interest in the field of cosmetics and therapeutics.

Key words: Electric cell-substrate impedance sensing (ECIS), photo-protective drugs, Light radiation induced damage, Cell behaviour, In-vitro light setup

1. INTRODUCTION

Over recent years, the external radiation incidence of morbidity and mortality of human skin damage has continued to increase. The environmental radiation is a one of the potential initiator for the skin cancers (Bowden 2004, Kielbassa 1997, Markovic 2007). The light radiation incident on human skin is depending on the energy and intensity penetration, and it causes cellular dysfunction and severe damage. These might be the photodynamic production of free radicals from the toxic combination of light and low level of oxygen (Boulton 2001, Ruffolo 1984). It suggested that radiation-induced photo-damage of skin cells induced through photo-oxidative mechanisms. Early stages of the diseases can be treated by the correct treatments, and improve appearance of the skin from the radiation toxicity. For this purpose the effective intervention of solar radiation toxicity remains challenging in dermatological field.

On the hand, only few therapeutic drugs are available for the superior activity of radiation toxicity and their related diseases. Also, the potential effect of administration of antioxidants and other active agents are very less because of their instability, irradiation effects, oxidation properties effects, and interact with body fluids (Wondrak 2007). Because of these limitations, topical delivery with carotenoids nano-formulation can play a beneficial effect in skin protection. The bioactive molecules such as beta carotene are of great interest for various biomedical applications such as cosmetics, pigments and therapeutics because of their abundant antioxidant properties which are low side effects and low cost. These are converted in the body to vitamin A, which is necessary for healthy eyes and skin. Beta-carotene is well-characterized antioxidant carotenoids (Paiva 1999). These antioxidants neutralize the free radicals by donating one of their own electrons and prevent the extending their chain reaction. Antioxidant themselves do not become free radicals by donating an electrons because they are stable in either form. Several studies have been performed to increase the efficacy of the treatments through various administrations; the suitable administration route for the radiation toxicity treatment is topical administration, because the skin is a target for radiation induced related diseases (Nichols 2010). Very limited studies have been tried so far been developed for drug loaded nanoparticle with biodegradable polymers and to see the effectiveness in protection. However, to our understanding no study had been involved to formulate

permeation enhanced nano-encapsulation of beta-carotene based on poly(D,L-lactide-co-glycolide) (PLGA) with lipid coated using oleic acid and to study the protective effect against light radiation on human dermal fibroblast (HDFn) cells.

Another controversial point is to obtain qualitative results with continuous monitoring of light radiation induced skin cell behaviour is very difficult with existing methods such as toxicity, cell viability, cell proliferation and apoptosis analysis methods are frequently used in high-throughput screening (Beatty 2000, Coombs 2006, Hao 2002, Joly 2006, Sang 2011, Suzuki 2009, Van Bergen 2009, Wood 2007), though are not suitable for continuous or real-time analysis. To realizing that, the more sensible real-time instrument is needed to fulfil the aboveproblems. Herein, we suggest that the electric cell-substrate impedance sensing (ECIS) as an idyllic system for continuous monitoring (Giaever 1984, Giaever 1993). With bio-impedance system, Bennet et al. have established a model for real-time continuous measurements of light radiation induced damage (Bennet 2013a, Bennet 2013b), and this system has utilized for continuous light radiation toxicity and protective effect of drug formulations in HDFn cells. For that present research developed a permeation enhanced nanoparticle formulations, then were examined for physico-chemical characteristics, cellular uptake and bio-activity in vitro on HDFn cells, and to study the light radiation induced response (attachments, proliferation and mitosis during the cell culture) against different concentration of drug loaded nanoparticle by real-time ECIS analytical tool.

2. MATERIALS AND METHODS

2.1 HDFn Cell Culture on Microelectrode System

The new analytical ECIS applications can be used to study HDFn cell in vitro environmental toxicity activity. The ECIS system allows quantifying the changes in cell morphology and obtaining relevant physical information by measuring the electrical impedance developed by cells, which are cultured in vitro on a microelectrode (Giaever 1993). For ECIS, 48 hours subcultured subconfluent HDFn cells were used, the cells were cultured in standard growth medium (CB-HDF-GM; without phenol red) mixed with require supplements and antibiotic solution at 37°C under a humidified atmosphere of 5% CO₂. Cell amount was calculated using hemocytometer-based trypan blue dye exclusion technique and used.

2.2 HDFn Cell Impedance Measurements

In this experiment, 8-well, 10-electrode (8W10E) array chip has used which can measure more than 2000 cells can be analyzed by this chip. Each detecting electrode has been micropatterned with tiny gold electrodes (250 µm in size). This has been connected with counter electrode, and all electrodes linked to the edge of the array, and were connected to the lock-in amplifier. The final cell concentration in each well was adjusted to 5×10^5 cells in 400 µl of fresh medium. The entire cell culture chip electrode was stabilized with 10 mM cysteine, and then rinsed with fresh culture media before starting the investigations (Giaever 1993). The cell impedance was monitored every second at various frequencies, and selected the best frequency to monitor the HDFn responses.

2.3 Real-time HDFn Cells Measurement under Light Setup

For the light setup, we used custom made light-emitting diode (LED) white light which have been described previously in eye cell studies (Bennet 2013a). The working principle and LED strip light setup in the ECIS system is dramatically shown in figure 1. In brief, the eight rounds LED white lights build in a single strip and was equipped with on/off switches, which was inverted above 2 cm of the cultured chip surface. Each electrode was exposed with same intensity light and the light was ventilated by a fan. All the controllers were controlled from external.

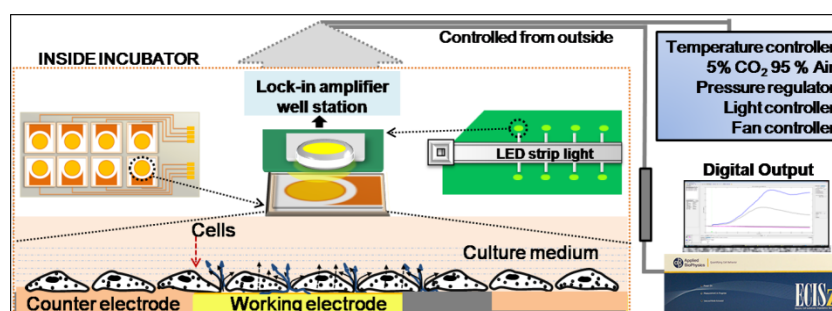


Figure 1. Schematic diagram represented the cell monitoring system under light stimulation with the controlled environment.

2.4 In-vitro Cell Behavioural and Light-induced Toxicity Study by ECIS

For the light-induced toxicity impedance measurement, one well was maintained as a cell-free electrode, another one well maintain as a positive (without light treatment) control; the well was covered with black cap, and the test wells were light exposed continuously. The cell activity changes resulting from a dark environment to light radiation effect were reflected as impedance signals, which registered by the ECIS system. The cellular physical information was examined, and compared. The cell impedance was monitored every second at various frequencies, and data collection and processing were carried out using the software, impedance monitored until the end of the experiments. The software, electrode arrays, and amplifier were obtained from an Applied Biophysics company (Troy, NY, USA).

2.5 Cell Viability and ROS Measurement

In the same time, the cell viability study has been performed using MTT assay and Live/Dead Staining dye kit (following the manufacturer's instructions) for compare with real-time data. The attached cells were exposed to light for 10-hrs, and the viability was quantified every two hrs. Also the each time point the stained cultured electrode chip was examined under fluorescence microscope. Simultaneously, cells were involved to measure the production of ROS using cell-permeable fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Degli Esposti 2002). In order to measure the ROS production we followed the standard protocol. For that we used black walled culture plate with the same environment, as previously described. Finally the fluorescence intensity was determined by spectrofluorometer (λ = excitation 485 nm, λ = emission 530 nm).

2.6 Preparation of β -carotene Loaded Nanoparticle

This was synthesized using emulsion solvent evaporation method with slight alterations (Bennet 2012, Bennet 2013c). Figure 2 describes a nanoparticle preparation method. In brief, the β -carotene (5mg) vortex with PLGA 50:50 / PLGA 85:15 (0.25 g) contains acetone (3ml) for 3 min, then added drop by drop into aqueous solution (30 mL) containing Tween-20 (as emulsifier, 0.02 % w/v) under vigorous stirring at 3000 rpm to get a crude PLGA-drug nanoemulsion. This was coated with lipid; permeation enhancer (oleic acid 4 % w/v) by adding in to the crude emulsion, then immediately ultra probe sonicated for 15 minutes at 40 % amplitude, 20 kHz frequency and on/off cycle (VCX750; Sonics and Materials Inc, Danbury, CT). Colors of the solution changed to milk-white yellow, and then makeup to 50 mL using distilled water. Finally acetone was evaporated using rotary evaporator then the nanoparticle was collected by centrifugation, washed, freeze-dried, and stored at 4°C for future use.

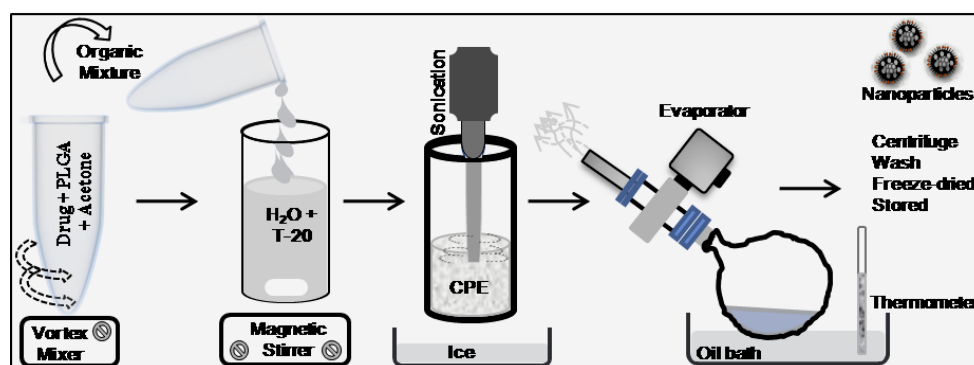


Figure 2. Schematic diagram represent the nanoparticle preparation method by the emulsion solvent evaporation technique.

2.7 Nanoparticle Characterizations

The nanoparticles were characterized using field emission scanning electron microscope (FE-SEM; JEOL-ISM-7500F), photon correlation spectroscopy (DLS; Malvern Zetasizer Nano ZS series) and atomic force microscopy (AFM), they were employed to study of morphology, size distribution, zeta potential and polydispersity index (PI) and topography of the samples. Ultraviolet-visible (UV-Vis) absorption spectroscopy studies were conducted to calculate the drug loading and entrapment efficacy of the nanoparticles using the standard curve by utilizing the standard equations.

2.8 Cytotoxicity Effect and Drugs Screening by ECIS

The high encapsulated β -carotene loaded nanoparticle preparations were involved to ECIS for high throughput screening, in order to achieve the effective and safer treatment, to select the safest nanoparticle concentration. For the drug screening, we followed the standard protocol. Suitable quantities of HDFn cells were seeded in the each electrode well. After the cell attachment, cells were exposed with various dosages (1 to 500 μ M) of 50:50 PLGA ratio samples (which show the uniform high encapsulated particles) and the cellular behavior was monitored. Then the well tolerated concentrations were selected for further studies. Simultaneously, 50% inhibition concentration was determined from the ECIS signal ($ECIS_{50}$) based on standard equation (Bennet 2013b).

2.9 Photo-protective Effects in Real-Time

The HDFn protective effects under light, an appropriate quantity of cells were seeded in each wells, the culture media was adjusted to 400 μ L. After cell attachment, the sub confluent cells were exposed with safest selected different concentration of nano preparation and raw drug by replacing the initial cultured media, then 4-5 hours later the nanoparticle internalized HDFn cells was exposed to light. Similar conditions for positive and negative controls; without any treatment and light treatment respectively. The cell protective effect was examined, and compared. Same time the free radical scavenging activities of β -carotene loaded nanoparticle and raw drugs were measured using DPPH assay and were compared with ECIS data. At the end of ECIS experiment about 10 hours after drug treatments, all wells containing drug treatments cells were involved to access the DPPH free radical scavenging activity which was followed by the standard procedure. Then the scavenging activity was calculated by the standard equation. Above all experiments were performed in triplicate.

3. RESULTS AND DISCUSSIONS

3.1 ECIS Measurements and Light Stimulation Characteristic Responses

To monitor the light-induced changes in cell behaviour by ECIS require a one suitable frequency for each cells. We selected the suitable frequency from 4 to 64 Hz frequencies range obtained from the cell-covered electrodes. The one particular frequency was enough to monitoring the cellular behaviour on exposure to light radiation. Figure 3a shows the typical experiment for select the suitable high responses frequency from whole range frequencies obtained by the HDFn cells. This data provides the entire responses of the frequency-dependent changes in cell related changes. This can provide appropriate frequency for HDFn cell study. The lesser than 16,000 Hz and higher than 32,000 Hz frequencies represent the electrode or electrolyte and culture medium and the working electrode interface involved. Accordingly, both ends of frequency the 24000 Hz frequency (denoted with arrow; figure 3a) area provides optimum and major sensitive to reflect the associated impedance changes happened for the HDFn cells. So this frequency was utilized for further monitoring.

In the light-induced HDFn cells toxicity study, the light was exposed continuously for 10 hours. The normalized impedance data is shown in figure 3b. In cell-free control shows no signal changes and cell covered control electrode showed any changes in normal impedance growth values. But, in the light exposed test cells showed significant impedance changes in after 5 min followed by continuous impedance drop compared to control wells. Further this viability changes were confirmed by MTT assay. The cell viability did not affected in the initial light treatments, but in the continuous light exposures cell viability activities were decreased, and were shown in figure 3c. In the ECIS responses the cellular activities were changed in the initial light exposure compare to MTT assay. The ECIS provides the real physical information by the light exposed cells, which shows the onset of responses of initial light. Then we measured the level of ROS in light exposed cells; here, the HDFn cells responded in the same manner of ECIS physical responses which the impedance was decline in increased ROS production. These light exposed ROS productions were shown in Figure 3d; the initial exposure time of the ROS levels show a minimum difference, but after continuous light exposure the ROS production level was increased.

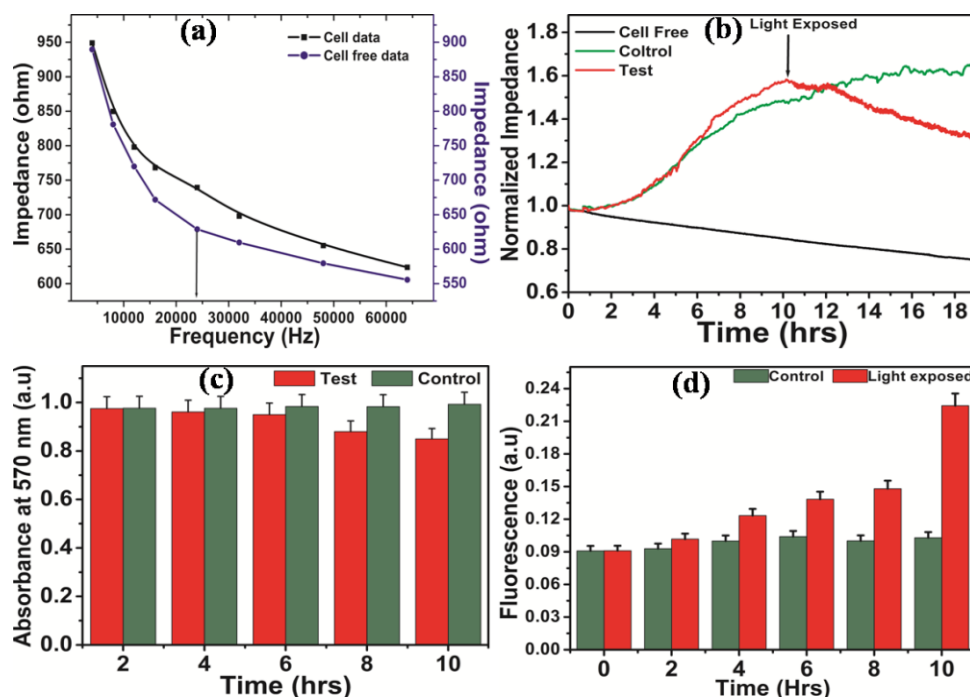


Figure 3. Light-induced characteristic responses of HDFn cells. (a), The frequency-dependent impedance; cell covered and cell-free data, to get the particular best signal output for HDFn cell study. (b), Normalized value of 10 hrs light exposed HDFn cell responses. (c), HDFn cells viability was determined after each times points. (d) The level of ROS is increased with continuous light exposure.

3.2 Nanoparticle Characterizations

Nanoparticles were prepared by a simple emulsion solvent evaporation method using two polymers such as PLGA 50:50 and 85:15 ratios with permeation enhancer. The resulted nanoparticles were imaged with FE-SEM and were shown in figure 4a and b respectively, and it confirms that the prepared nanoparticles were coated with a surfactant along with permeation enhancer. Here we utilised optimal reaction conditions for preparations of nanoparticle. From the optimized method we developed a stable, uniform, and smooth surface nanoparticle with anionic surfactant and oleic acid suitable concentrations. Both the ratio of PLGA produces the uniform mono-dispersed beta-carotene loaded nanoparticles, which were shown in figure 4a and b insets. Beta-carotene loaded nanoparticles with two different ratios of preparation were found to be 122 and 155 nm in size. The surface topography analysis was done by bio-AFM, and the root mean square roughness found to be 22.34 and 14.32 nm respectively. For the drug entrapment efficacy the 50:50 ratio shows high encapsulation (93.41 ± 0.86), but the 85:15 ratio containing preparations shows low drug entrapment efficacy (87.12 ± 0.34) for this method. Polydispersity index shown that uniform size distribution with index of 0.312 - 0.281, which decreased with decreased acid ratio. The highest entrapment results from a lesser proportion ratio. All together the 50:50 ratio of PLGA formulation showed the good drug entrapment efficacy. So this formulation was chosen for future study.

3.3 Evaluation of Cytotoxicity Effect and Drugs Screening

In this study, we used beta-carotene loaded nanoparticles to establish the non-toxic safer drug concentrations. For the high throughput screening, the ECIS data shows the normalized impedance profile of HDFn cell response of cells during culturing in different dosage forms of drug loaded nanoparticles, which is shown in figure 5a. The high concentrations of nanoparticle dosage form (more than $50 \mu\text{M}$) showed low impedance growth; where the impedance value was significantly lower than the control due to the decreased cell mortality. In the case of lower concentration of nanoparticle, the growth values enhanced due to the cell survival compatibility and this concentration of nanoparticle was well tolerated in HDFn cells. Here maximum number of cells attached on electrode and shows maximum cell activity. In parallel, the fifty percent inhibition concentrations (ECIS_{50}) of dosage were calculated from the ECIS data. The inhibition curve figure 5b showed the ECIS_{50} inhibition concentration of β -carotene loaded nanoparticle was found to be $50 \mu\text{M}$, at 6 hours

exposure. Less than 50 μM concentration of β -carotene loaded nanoparticle was found to be effective and safe doses which is well tolerated and were utilized for radiation protectivity study.

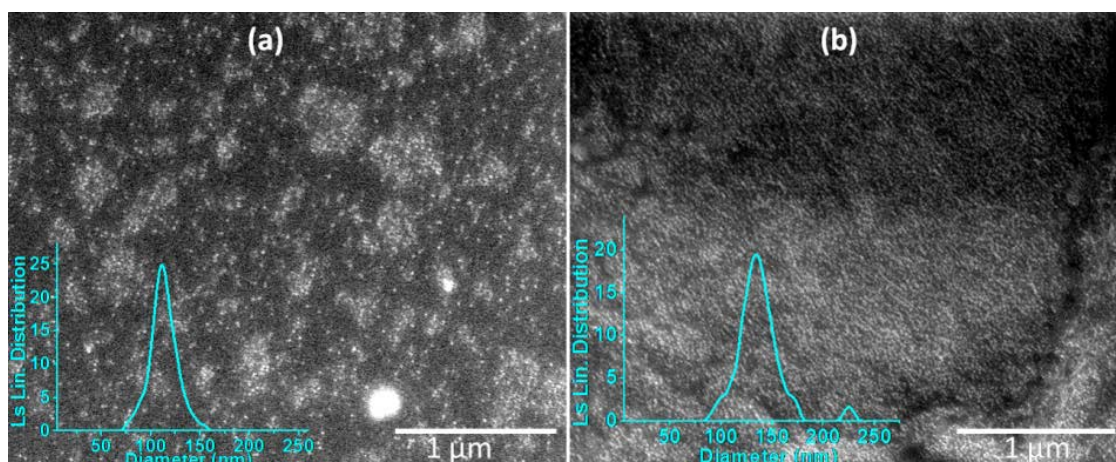


Figure 4. Nanoparticles size analysis by FE-SEM and DLS; inset images. (a), PLGA 50:50 beta-carotene loaded nanoparticles formulation. (b), PLGA 85:15 beta-carotene loaded nanoparticles.

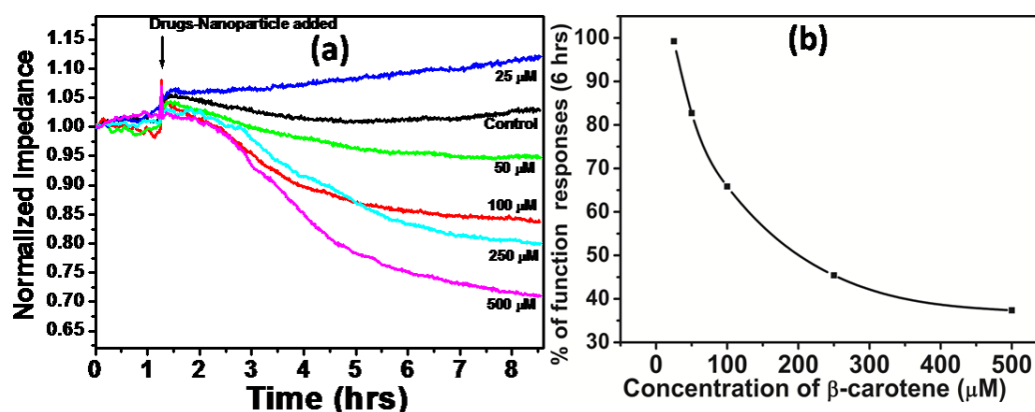


Figure 5. The ECIS toxicity based measurement of β -carotene loaded nanoparticle formulation screening. (a), Impedance profile of HDFn cells exposed to various concentrations of formulation (b), Inhibition curves derived from impedance profile at 6 hours after administration.

3.4 Protective Effect of Nanoparticle on Light-induced HDFn Cells by ECIS

For the efficient cell protective effect, we utilized well tolerated concentration (less than 50 μM) of β -carotene loaded nanoparticle, which were screened by high throughput drug screening. Here the normalized impedance value of cellular resistance was reported. Beta-carotene encapsulated nanoparticle was loaded while the normal cell growth on microelectrode, and was effectively uptake by the cells (after 4 hours; images were taken by fluorescence microscopy and were successfully internalized, the data are not shown), after nanoparticle internalization the light was turned on, then the cellular physical information changes were monitored. Control impedance shows no changes on cell growth, and light exposed control shows a continuous decline in impedance value which cells were impedes their normal growth. After β -carotene loaded nanoparticle treatment the 50 and 40 μM concentration shows the effective protectiveness against light radiation which has been shown in figure 6a and b. Low concentration does not provide the effective treatments. Also the raw β -carotene shows no effect after light radiation treatments; it makes clear that the raw drug may irradiated by the light radiation.

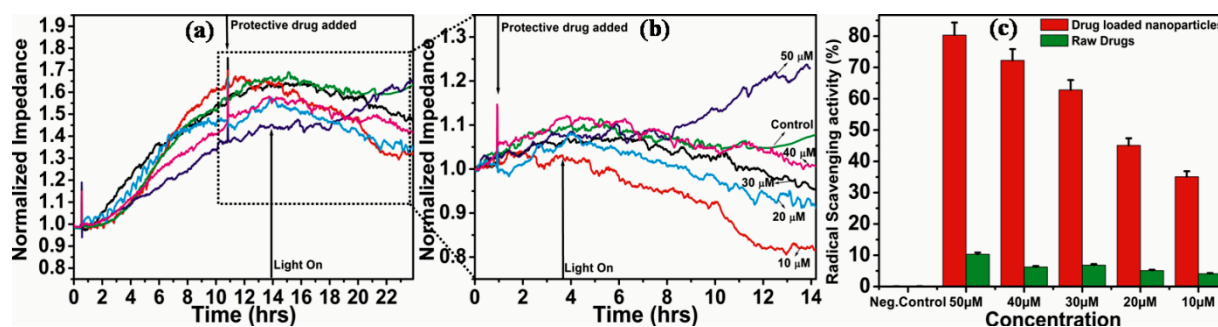


Figure 6. Protective effect against light radiation induced HDFn cells under ECIS system. (a and b), The light radiation causes decline in cellular responses, and during drugs treatment the cells were protected. (c), Evaluation of radical scavenging activity of drugs.

Then the scavenging activities were assessed on the basis of the DPPH assay and were compared (figure 6c). The inhibition activity of the beta-carotene loaded nanoparticle was increase with increasing concentration. However, the 50 μM concentrations show higher inhibition with more than 75 %, and followed by lower concentration. In the case of raw drugs there is no much inhibition activity during the light radiation; it clearly shows that the beta-carotene raw drug have been irradiated. The real-time ECIS cell responses were coincides with the scavenging activity.

4. CONCLUSION

This study employed the modified synthesis protocol to fabricate permeation-enhanced beta-carotene loaded nanoparticle, and the physiochemical characterization shows 50:50 ratio PLGA formulation produced high encapsulated particles with uniform distribution. This formulation was utilized for the study of HDFn light radiation induced toxicity using real-time ECIS system, and it provides 50 and 40 μM concentration of beta-carotene loaded 50:50 ratios PLGA nanoparticle formulation gives better cell tolerant, cures, survival and activities. During light radiation the HDFn cells elevates ROS and continuous with continuous light radiation. This ROS elevation is cures by the antioxidant loaded nanoparticle, which will protect by the light radiation. However, promote this effective analytical model and treatments further studies are required to investigate the expression and regulations of cellular proteins. Overall this artificial light setup with real-time ECIS system has been extensively used for high throughput screening of light radiation protective agents for retard or cure cell damage in early stage, which can enable a platform for skin interventions.

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