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$[Ru(bipy)_3]^{2+}$ nanoparticle-incorporate dental light cure resin to promote photobiomodulation therapy for enhanced vital pulp tissue repair

Rodrigo C. Mosca[1,2], Nicholas Young[2], Carlos A. Zeituni[1], Praveen R. Arany[2]
[1]Energetic and Nuclear Research Institute (IPEN/CNEN – SP) – CTR – Radiation Technology Center, São Paulo, Brazil; [2]Oral Biology & Biomedical Engineering School of Dental Medicine, Engineering & Applied Sciences, University at Buffalo, NY, USA;

ABSTRACT

The use of nanoparticle on dental light cure resin is not new, currently several compounds (nanoadditives) are used to promote better communication between the restorative material and biological tissues. The interest for this application is growing up to enhance mechanical proprieties to dental tissue cells regeneration. Bioactive nanoparticles and complex compounds with multiple functions are the major target for optimizing the restorative materials. In this work, we incorporate $[Ru(bipy)_3]^{2+}$ nanoparticles, that absorbs energy at 450 nm (blue-light) and emits strongly at ~620 nm (red-light), in PLGA Microspheres and insert it in Dental Light Cure Resin to promote the Photobiomodulation Therapy (PBM) effects to accelerate dental pulp repair by in vitro using cytotoxicity and proliferation assay.

1. INTRODUCTION

Dental pulp trauma is one of the biggest challenges in clinical dentistry with preservation of the vitality of pulp being paramount. In deep carious lesions, when the pulp is not exposed, inflammation is confined to the superficial pulp and deeper pulp tissue remains normal besides some dilated blood vessels [1]. One of the well accept non-invasive, vital pulp therapies is the indirect pulp capping that involves removal of only the softened, necrotic, demineralized, and infected dentin layer and leaving deepest layer of the dentin intact over the pulp tissue. This is followed by the placement of a biocompatible liner and hermetic restoration to provide a seal against microleakage [2]. Several biocompatible liner materials have been used and prime among them is Calcium hydroxide ($Ca(OH)_2$). Despite its long clinical history, it has several limitations namely unsatisfactory adherence to dentin, dissolution over time, and multiple tunnel defects within the dentin bridges [1]. Other alternative materials have been explored such as Mineral trioxide aggregate (MTA). It is a bioactive, biocompatible, antibacterial material with good stability, and excellent sealing ability as the stimulation of dentin-bridge formation is faster allowing pulp healing. It has demonstrated high success rates in clinical procedures, but the poor handling properties, high material cost, and the discoloration potential still remain a challenge for the practitioner [1]. Another material, bioactive tricalcium silicate [Ca_3SiO_5]-based dentin substitute is a new generation biomaterial that has been used for both indirect and direct pulp-capping agent for permanent teeth due to its ability to induce odontogenic differentiation and reparative dentin formation in a very short time period [1-2]. However, its price still remains a major disadvantage for its popular clinical use. The use of low dose light treatments termed Photobiomodulation Therapy has proven to be effective to accelerate healing and promote dentin regeneration. It is well established that monochromatic red and near-infrared light can excite endogenous chromophores present in mitochondrial enzyme, specifically Cytochrome C oxidase (complex IV) which is the last electron acceptor in the electron transport chain and modulates mitochondrial respiration rate [3-7]. The direct consequences of enhanced mitochondrial activity lead to higher ATP/ADP ratios and transitory mild oxidative stress (ROS). Both these effects activate several signaling cascades (e.g. AP1 and NFk-B pathways) that can induce cellular proliferation, migration, or apoptosis. These cellular responses can lead to modulation of inflammatory processes, impair pain signaling and optimize tissue healing and regeneration [6].

The use of nanoparticle within dental resin has been pursued previously and several compounds (nanoadditives) were used to improved restorative material and biological tissue interactions for dental tissue regeneration [8]. Polymeric microspheres (poly-lactic-*co*-glycolic acid – PLGA) has been used as drug carriers by encapsulating them into the co-polymer matrix allowing sustained drug release for prolonged biological actions [9]. Specific nanoparticles can be combined within these microspheres that can absorb and emit specific wavelengths of light capable of promoting PBM [10]. Tris(2,2′-bipyridine)ruthenium(II) hexafluorophosphate or $[Ru(bipy)_3]^{2+}$ is a complex used as a photosensitizer and

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red-emitting chromophore with relatively long excited-state lifetimes [11]. It absorbs energy at 450 nm (blue) and emits strongly at ~620 nm (red) in water and this emission lifetimes are approximately 600 ns [12]. In this project, we examined the ability of $[Ru(bipy)_3]^{2+}$ (NPRuBIPY) doped PGLA microspheres inserted into dental light (blue) cured composite resin to promote PBM effects using *in vitro* cytotoxicity and proliferation assay in a pre-odontoblast cell line.

2. MATERIAL AND METHODS

$Ru(bipy)_3]^{2+}$ acetate-loaded nanospheres

$Ru(bipy)_3]^{2+}$ acetate-loaded nanosphers were prepared by a single emulsion and evaporation technique using 5% PLG and Poly(vinyl acetate) 1% PVA / 7% ethyl acetate (all from Sigma-Aldrich). Then, 80Mn $[Ru(bipy)_3]^{2+}$ solution (1 mL) was added to 100 mL ethyl acetate containing PLGA polymer (85/15) and aqueous solution containing PVA (0.5% w/v) by stirring overnight. The solution was sonicated at 20% W for 45 sec. The NPRuBIPY were collected by centrifugation at 10000 rpm for 5 min followed by subsequent washing with deionized water, filtration and lyophilizing (FIGURE 1).

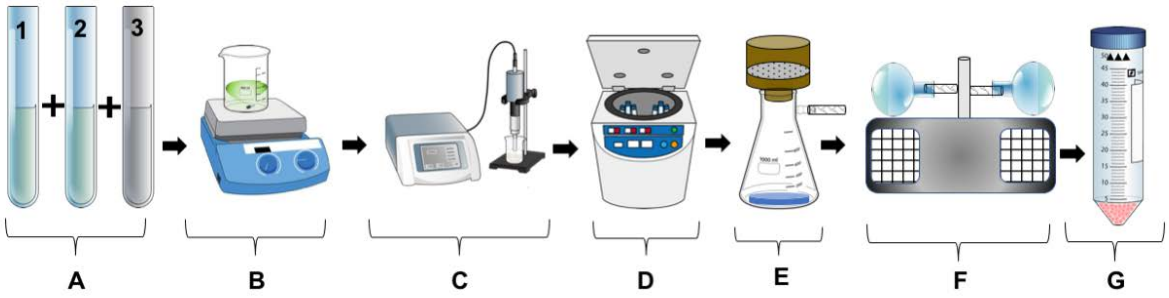

FIGURE 1 – Sequence for $Ru(bipy)_3]^{2+}$ acetate-loaded nanosphers: (**A**) Mixture of PLG+PVA (1) with Ethyl Acetate (2) and 80Mn $[Ru(bipy)_3]^{2+}$ solution; stirring overnight (**B**); Solution was Sonicated (**C**) and centrifugation (**D**). After collected the supernatant it was washed, and filtration as made (**E**). Overnight lyophilizing was performed (**F**) to collect the NPRuBIPY (**G**).

Size distribution of NPRuBIPY was analyzed by dispersing the particles in an aqueous solution of Tween-20 (0.5% v/v) also, the surface morphology by scanning electron microscopy (SEM) and the characterization by Energy Dispersive X-Ray Analyzer (EDX).

Cellular Assays

MDPC-23 pre-odontoblasts were plated (5×10^6 cells) in 25 cm^2 culture flasks. Cell cultures will initially be fed with a mixture of 60% Dulbecco's Modified Eagle's Medium (DMEM), 30% Ham F12 and 10% fetal bovine serum (U.S.A. origin) and supplemented with 4 mML- glutamine and 100 IU/ml penicillin/100 g/ml streptomycin antibiotic solution (all from GIBCO-BRL Life Technologies). Further, 0.18 mM adenine, 5 g/ml insulin, 0.4 g/ml hydrocortisone, 0.1 nM cholera toxin and 2 nM tri-iodothyronin (all from Sigma Chemical Co., St. Louis, MO, U.S.A.) were supplemented. Cell were passaged 3 times per week with 0.25% trypsin-EDTA (GIBCO-BRL Life Technologies).

Light Setup

Using the Blue LED (~ 450 nm wavelength, 152 mW) and Red LED (~645 nm wavelength, 242 mW) and measured in power meter (UDT® Instruments model 25000MD Integrated Sphere) we setup the exactly distances to irradiate the 12 well plate equally once at time and then, both at the same time, following the calculation below:

Blue light: At d = 15 inches high the diameter of the beam was 19.5 in (.495 meters)

$$\frac{1}{2}D = r = .2475m$$

$$A = \pi r^2 = .192\ m^2$$

$$Irradiance = \frac{Power}{Area} = \frac{152mW}{.192\ m^2} = 791.67\frac{mW}{m^2}$$

For a constant Irradiance of 791.67 mW/m^2 using Red light:

Red Light:

$$I = 791.67\ \frac{mW}{m^2} = \frac{242\ mW}{A}$$

$$A = \frac{(242\ mW)}{791.67\frac{mW}{m^2}} = .306\ m^2$$

$$A = .306m^2 = \pi r^2$$

$$r = \sqrt{\frac{.306m^2}{\pi}} = .312\ m$$

$$D = 2r = .624\ m\ or\ (24.6\ inches)$$

Six groups are divided as follow in 12 well plates as follows: Group 1 and 2 wwre Controls Group with MDPC-23 alone and MDPC-23 with NPRuBIPY; Group 3 was MDPC-23 cells treated with Blue LED (irradiance 4J/cm^2); group 4 was MDPC-23 cells treated with Red LED (irradiance 4J/cm^2); group 5 was MDPC-23 cells treated with both Blue and Red LED (irradiance 4J/cm^2) and finally, group 6 was MDPC-23 cells with NPRuBIPY treated with Blue LED (irradiance 4J/cm^2).
The NPRuBIPY containing plates were prepared by dissolving 58 μg in 3600 μL of Chloroform (Millipore Sigma Chemical Co., St. Louis, MO, U.S.A) and 300 μL of this solution was added to each well and allowed to evaporate overnight in a fume hood to create a thin layer at the bottom of the well.

Proliferation Assessment.
MDPC-23 cells were trypsinized, cells were counted, serially diluted in complete DMEM media and seeded into 96-well black plates at densities 150 000 cells per well (100 μl/well). Following 24h and 48h of treatments, 10% v/v of Alamar Blue (BioRad) was added and cells were allowed to incubate for 3 hours. Absorbance and fluorescence (Excitation/Emission: 570/590 nm) was assessed using a spectrophotometer (LS-50B, Perkin-Elmer. Norwalk, CT). Background fluorescence measurements were determined from wells containing dye reagent in culture medium but no cells. All assays were performed in triplicates and read after 24 and 48h after the light irradiate. The mean and standard deviation for all fluorescence measurements were calculated and subsequently corrected for background and expressed as 'relative fluorescence units (RFUs).

Statistical Analysis.
To investigate differences in cells' number between each test group and controls, we used ANOVA test with multiple comparisons with Control Group.

3. RESULTS AND DISCUSSION

Characterization of NPRuBIPY

We used two LED fluorescent channels (Blue Excitation/Emission: 355±40 nm/ 433±36 nm; Red Excitation/Emission: 556±20 nm/ 615±61 nm) and one LED Brightfield Channel (535±85 nm emission) to capture images.

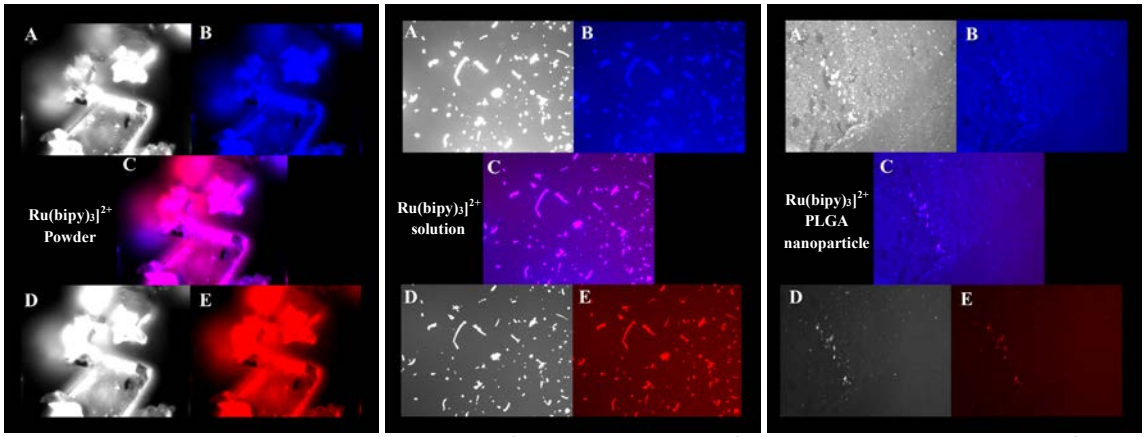

FIGURE 2 - Fluorescent microscopic (100x) images of $[Ru(bipy)_3]^{2+}$ Powder (1); $[Ru(bipy)_3]^{2+}$ Solution (2) and PLGA $[RU(BIPY)_3]^{2+}$ nanosphers (3) at different intensities. Images 1 to 3 - A and D acquired in B&W raw; B excitation at blue light ~450 nm; E excitation at red light ~620 nm and C merged red and blue images (C).

The $[Ru(bpy)_3]^{2+}$ luminescence has maximum intensity at λ= 612 nm when excited by 450 nm and has long excited-state lifetimes [13] as observed in Figure 2.

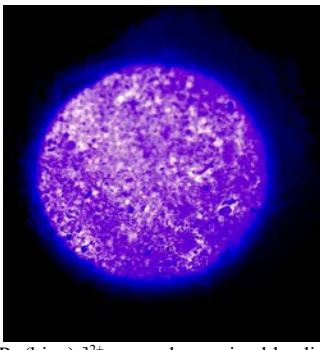

FIGURE 3 - Fluorescent macroscopic image of PLGA $[Ru(bipy)_3]^{2+}$ nanosphers using blue light (~450 nm).

Macroscopic image of PLGA $[Ru(bipy)_3]^{2+}$ nanosphers using the blue light excitation show the red-light emission (Figure 3). SEM analysis demonstrated the morphology of nanosphers (Figure 4A) and elemental analyses with EDS demonstrated presence of Ruthenium-loaded nanospheres (Figure 4B).

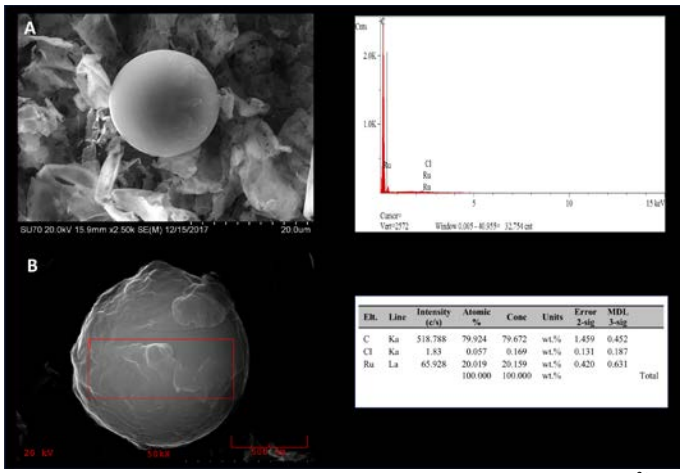

FIGURE 4 – SEM (20 kV) analysis (**A**) and Energy Dispersive X-Ray Analyzer (EDX) of PLGA $[Ru(bipy)_3]^{2+}$ (**B**).

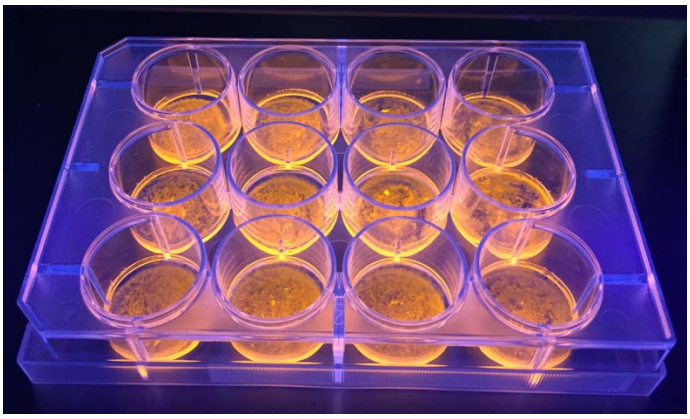

FIGURE 5 - PLGA $[Ru(bipy)_3]^{2+}$ placed in 12 well-plate irradiate with blue light (~450 nm) ready to cells culture.

Future studies are examining the cell viability and differentiation in these new material systems. These results open a wide range of novel biomaterials applications for dentistry, including combination with routinely used dental light (blue) cured composites. It is hoped these new systems are use the combination of Red and Blue light for Photobiomodulation therapy.

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