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Gamma Radiation Interacts with Melanin to Alter its Oxidation-Reduction Potential and Results in Electric Current Production

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Abstract

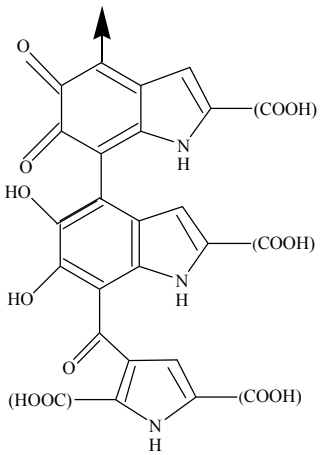
The presence of melanin pigments in organisms is implicated in radioprotection and in some cases, enhanced growth in the presence of high levels of ionizing radiation. An understanding of this phenomenon will be useful in the design of radioprotective materials. However, the protective mechanism of microbial melanin in ionizing radiation fields has not yet been elucidated. Here we demonstrate through the electrochemical techniques of chronoamperometry, chronopotentiometry and cyclic voltammetry that microbial melanin is continuously oxidized in the presence of gamma radiation. Our findings establish that ionizing radiation interacts with melanin to alter its oxidation-reduction potential. Sustained oxidation resulted in electric current production and was most pronounced in the presence of a reductant, which extended the redox cycling capacity of melanin. This work is the first to establish that gamma radiation alters the oxidation-reduction behavior of melanin, resulting in electric current production. The significance of the work is that it provides the first step in understanding the initial interactions between melanin and ionizing radiation taking place and offers some insight for production of biomimetic radioprotective materials.

Keywords: **Melanin; Gamma radiation; Current production; Radioprotection**

1. Introduction

Melanins are complex polymers found in species of all biological kingdoms with a multifaceted utility related to physiology such as protection from visible and UV light [1,2], decreased oxidative stress [3], energy transduction and Fe(III) reduction [4-6]. However, the most fascinating and the least explored function of melanin is related to its interaction with ionizing radiation. Melanin plays a role in decreasing radiosensitivity of human melanoma cells [7] and melanized microbial species thrive in highly radioactive environments such as cooling pools of nuclear reactors, the stratosphere, space stations and inside the damaged nuclear reactor at Chernobyl [8]. Furthermore, certain melanized microbes seem to dominate the environments characterized by elevated levels of ionizing radiation such as pyomelanin-producing bacteria found in uranium-contaminated soils [9] and melanized fungi in radio-contaminated soils showing directional growth towards radiation sources (radiotrophism) [10]. Recently we demonstrated that ionizing radiation changes the electronic structure of melanin and enhances the growth of several melanized fungal species, suggesting a role for melanin in this process [11], specifically the physico-chemical interaction between melanin and ionizing radiation. Melanin pigments are diverse in structure and function in regard to effects from ionizing radiation [7,12] and offer potential as manufactured radioprotective materials [12].

Melanin pigments are composed of quinone moieties that are believed responsible for its redox behavior (Scheme 1). The polymeric structure of melanins permits oxidation and reduction to occur simultaneously. Perhaps the most interesting aspect of any radio protective material is its requirement to withstand the oxidizing impact of ionizing radiation indefinitely, without bleaching. Here we investigated the electrochemical response of the pigment eumelanin to ionizing radiation with a carbon paste/melanin electrode. The results establish that gamma radiation can interact with melanin thus providing key supportive evidence for the initial interactions of this pigment with electromagnetic radiation in such processes as radiosynthesis.



Scheme 1. Structure of eumelanin oligomer.

2. Materials and Methods

2.1. Melanin

Eumelanin produced by the fungus *Cryptococcus neoformans* grown in presence of L-DOPA melanin precursor was used in this study. The growth of melanized cells was followed by acid hydrolysis resulting in production of hollow melanin shells dubbed "ghosts" (as they preserve the shape of the cell) was performed as in [2].

2.2. Electrodes

In this work we utilized the carbon paste (CP) electrode, which was previously used for studying melanin electrochemistry [6, 15]. Dry melanin ghosts were crushed lightly to a powder with a clean glass rod and mixed by weight with CP (Bioanalytical Systems, BAS, West Lafayette, IN) at melanin /CP ratios of 10:90, 20:80 and 30:70. The melanin/CP mixtures were packed into electrode housings (BAS) as previously described [6, 15]. The packed electrodes were stored in sterile, pH 7, 50 mM/L, phosphate buffered saline (PBS) at 25°C for at least one week to allow the melanin to hydrate. Since fungi are known to produce extracellular reductants [13,14] we performed our studies in the presence and absence of a reductant, in order to gain a better understanding of the potential role of extracellular reductants on the long-term radioprotective properties of melanin. When used as a reductant,1 mM/L ascorbate (final concentration) was incorporated with PBS and electrodes were exposed to this solution for at least 24 hrs to allow ascorbate to diffuse into the electrode material. Cyclic voltammetry was used to confirm that sufficient ascorbate was in contact with the electrode material by demonstrating reduced conditions.

2.3. Electrochemical cell

Electrochemical measurements were performed in a three-electrode geometry with a CP or melanin/CP working electrode, a Pt counter electrode (BAS) and a Ag/AgCl, 3M NaCl reference (BAS), all immersed in 50 mM/L, pH7.0, PBS with or without 1mM/L ascorbate. Electrodes were contained in glass vials fitted with screw on caps and teflon septa at either end of the vial. Electrodes were positioned via holes in the septa. The working electrodes were positioned facing up to prevent gas bubble accumulation on their surface (Fig. 1).

2.4. Electrochemistry

Electrochemical analyses were conducted on a VersaSTAT MC Multichannel Potentiostat/Galvanostat (Princeton Applied Research) employing 2 channels simultaneously. For gamma irradiation studies, the cables from the potentiostat were run through access ports of a 25 cm x 25 cm x 100 cm chamber (J. L. Shepherd Model 484). Vials with electrodes were positioned in a stainless steel rack and attached to leads from the potentiostat. Dose rate was determined by distance of the electrodes from the ^{60}Co gamma source inside the chamber for each experiment. For some experiments that

required a high current response to aid in quantification, racks were designed to fit as close as possible to the gamma source for maximum radiation dose.

Chronoamperometry was performed with electrodes poised at -700 mV and irradiation began after electrode current stabilized. Current production as a result of gamma ~~irradiaiton~~ irradiation was determined by the difference in current from the start of irradiation and at 60 and 90 min. Chronopotentiometry was conducted with electrodes poised at 1 mA. Cyclic voltammetry was conducted at a sweep rate of 100 mV/s from -1 V to 1 V for multiple cycles. Cyclic voltammograms were recorded prior to and throughout irradiation. Selected voltammograms were used to illustrate changes as a result of ionizing radiation exposure. All experiments were repeated 2 to 3 times with representative data illustrated here.

3. Results and Discussion

3.1. Current production resulting from melanin irradiation

Gamma irradiation of melanin/ CP mixtures packed into electrode housings (Fig. 1) resulted in greater current production from stable electrodes consisting of CP (80% w:w) plus melanin (20%) compared to only CP electrodes (Fig. 2 and Table 1). The increased current from electrodes poised at -700 mV was a result of oxidation of the electrode materials during irradiation. The sustained current of the melanin electrode relative to CP alone indicated that the melanin remained oxidized for an extended time after irradiation ceased. This is likely due to the presence of stable free radicals in melanin [11]. Current production from electrodes was directly proportional to melanin concentration (10-30%) and irradiation time (Table 1) and corroborates previous studies with eumelanin [7].

Table 1 here.

3.2. Effect of gamma irradiation on melanin potential.

During gamma irradiation, oxidation of 10% melanin/90% CP continued to increase under a continuous 1 mA current supplied to the electrode (Fig. 3). In contrast, the potential of the control, without melanin, decreased. These results demonstrated that with

a 1 mA input, melanin demonstrated a significant capacity for sustained oxidation in radiation fields.

3.3. Current production resulting from melanin irradiation in the presence of a reducing agent

Next, we concentrated on melanin oxidation during exposure to ionizing radiation. We hypothesized that given the redox cycling nature of melanin, the addition of a reductant such as ascorbate would enhance the redox cycling capacity of melanin during radio-oxidation. In this case ascorbate represents an extracellular reductant that could be produced by fungi [13,14]. Therefore, we repeated the experiments (in 3.1) by placing the electrodes in 50 mM/L, pH 7, PBS with 1 mM/L ascorbate. Although some radicals are produced by ascorbate during irradiation, these radicals do not react with melanin [16].

In the presence of ascorbate, current production was greater than in PBS alone (Fig. 4). The reductive properties of ascorbate likely contributed to the reductive behavior of melanin by providing electrons supplemental to that of the electrode. Although the ascorbate, which is a known free radical scavenger, was present in much higher concentrations than estimates of free radicals formed as a result of water radiolysis (17), its presence did not abrogate the oxidation of melanin during gamma irradiation. This observation could suggest that other free radicals from radiolysis were involved in the oxidation of melanin (18). Similarly, melanin oxidation resulting from a loss of Compton electrons during Compton scattering could not be discounted. Current production by the electrode in ascorbate was monitored well after irradiation ceased in order to track this phenomenon. Current production was maintained for over 2.75 h and was likely due to the presence of stable free radicals in melanin [11], especially after exposure to ionizing radiation.

3.4. Cyclic voltammetry studies during gamma irradiation

Without irradiation, cyclic voltammetry demonstrated an oxidation peak at about 100 mV (vs Ag/AgCl) and a broad reduction peak (Fig. 5a). These peaks are similar to those previously reported for oxidized melanin using synthetic melanin films [19]. A slight reduction peak at about -100 mV (vs Ag/AgCl) was also observed in the present study. Following 17 min of irradiation, the oxidation peak increased while the cathodic, or reduction response demonstrated diminished peak current. This trend continued into 33 min of irradiation with a slight increase in peak current at 100 mV and a more defined reduction response with two reduction peaks emerging, near -100 mV and -400 mV. Similar reduction peaks at various pH values were reported previously for synthetic L-DOPA melanin [13].

Cyclic voltammetry demonstrated a dramatically increased oxidation of melanin in ascorbate solution during gamma irradiation (Fig. 5b). In the presence of ascorbate, melanin was more reduced (Fig. 5b), as evident in the oxidation peak at about 100 mV (vs Ag/AgCl) and the reduction peak at about -100 mV (vs Ag/AgCl). The addition of ascorbate also resulted in increased peak current at 100 mV (vs Ag/AgCl) from both electrodes with a substantial increase in oxidation (Fig. 5b). These data suggest a 2-step reduction from semiquinone to hydroquinone, followed by a 1-step oxidation to the

quinone state. The continued decrease in reduction peaks (Figs. 5a and 5b) indicated that reduced components of the melanin molecule were continuously oxidized during radiation and the increased peak current of the anodic peak corroborates this scenario.

4. Conclusions

The sustained radioprotective properties of melanin afforded to organisms may be useful as biomimetic materials, provided the mechanisms of radioprotection are understood. Here we demonstrated that irradiation of melanin resulted in melanin oxidation over time as measured by current production and was proportional to the amount of melanin irradiated. The increased oxidation of melanin in the presence of ascorbate during exposure to ionizing radiation was due to the reductive properties of ascorbate. Maintaining the radioprotective properties of melanin-like materials is key for long-term functioning of the material and this property is likely optimized in microorganisms. For instance, fungi produce numerous extracellular reductants capable of melanin reduction [13,14], hence, electron transfer from the cell membrane to melanin during radiation exposure could provide sufficient reducing power to allow melanin to undergo continuous oxidation. Future studies targeting microbial growth and stress responses to ionizing radiation are key to better understanding the use and maintenance of melanin as a radioprotective material.

Acknowledgments

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Figure Legends

Figure 1. Diagram of electrode configuration used in radiation studies.

Figure 2. Gamma radiation-induced melanin oxidation. Increased current production of a 20% melanin/80% CP electrode (black line), poised at -700 mV (vs. Ag/ AgCl) during irradiation with 300 Gy/h ^{60}Co gamma radiation. The CP (control) electrode response is shown in the gray line. Both electrodes were submerged in pH 7.0 PBS.

Figure 3. Changes in melanin oxidation-reduction potential upon ^{60}Co exposure. CP with 10% melanin electrodes (black line) and CP controls (gray line) in pH 7 PBS were poised at a constant current of 1 mA during 600 Gy/h ^{60}Co gamma irradiation.

Figure 4. Gamma radiation-induced melanin oxidation with and without 1 mM/L ascorbate. 20% melanin/80% CP electrode was submerged in pH 7.0 PBS with the reductant ascorbate (black line) relative to pH 7.0 PBS alone (gray line) during exposure to 300 Gy/h ^{60}Co gamma radiation. Both electrodes were poised at -700 mV (vs. Ag/AgCl). Background current from CP electrode exposed to identical conditions was subtracted.

Figure 5. Cyclic voltametry of 20% melanin/80% CP electrodes exposed to 4000 Gy/h of ^{60}Co gamma radiation for; 0 min. (black line); 17 min. (dashed line); and 33 min. (gray line). Background from CP electrode (control) was subtracted. a) pH 7.0 PBS; b) pH 7.0 PBS with 1 mM/L ascorbate. Scan rate was 100 mVs^{-1}. All scans began at -1 V.

Vitae

Dr Charles E. Turick is currently a Science Fellow at the Savannah River National Laboratory and holds a BS degree in Biology and a MS and PhD in Microbiology. Dr. Turick's research experience at SRNL involves microbial ecology and physiology related to biogeochemistry and energy production. He is also an adjunct professor at Clemson University, has authored numerous peer-reviewed publications and several book chapters as well as 4 patents in the fields of biochemical engineering and environmental microbiology. Dr. Turick's research interests include mechanisms of electron transfer between microorganisms and inorganic compounds, including solid phase metal oxides and electrodes.

Dr Ekaterina Dadachova received her BSc in Chemistry and PhD in Physical Chemistry from Moscow State University in Russia. Currently she is an Associate Professor of Nuclear Medicine and Microbiology and Immunology and Sylvia and Robert S. Olnick Faculty Scholar in Cancer Research at the Albert Einstein College of Medicine in New York. She has published 100 peer-reviewed articles, 4 book chapters, and has 3 granted US patents. Her laboratory is working on targeted radionuclide-based therapies, in particular on radioimmunotherapy of infectious diseases, metastatic melanoma and cancers of viral origin, and also on melanin-based radioprotectors for patients undergoing radiation therapy.

Charles Edward Milliken is a Senior Scientist with the Biotechnology Section at Savannah River National Laboratory in Aiken, SC. He earned Bachelor's and Master's degrees in Biochemistry and Molecular Biology from Boston University in 1998 and has been a biotechnologist for nearly 15 years, focusing on environmental microbiology. Recently, he has been developing analytical methods for chromatography and microscopy for various bioenergy projects at SRNL, including microbial fuel cell design and application.

Dr Amy Ekechukwu, a Senior Science Fellow at the Savannah River National Laboratory, received her PhD in Analytical Electrochemistry from Duke University. She has applied her expertise to a wide range of technical challenges throughout the DOE complex from developing methods to monitor high level waste tank chemistry, developing and deploying field instrumentation for environmental monitoring and remediation, addressing the complex issues associated with measuring beryllium in the workplace, and developing novel materials and applications for fuel cells. She has been granted eleven U.S. patents that involve instrumentation and sensors and has authored over 70 publications and presentations.

Dr Arturo Casadevall is the Leo and Julia Forchheimer Professor of Microbiology & Immunology at the Albert Einstein College of Medicine of Yeshiva University. He received his B.A. in Chemistry from Queens College, City University of New York, and his M.D. and Ph.D. in Biochemistry from New York University. He has published over 440 scientific papers and coauthored a book on *Cryptococcus neoformans*. His research is focused on microbial pathogenesis and antibody structure-function. His groundbreaking work in the field of infectious diseases has been recognized by many, including the NIH, which presented him with a Merit Award in 2007.

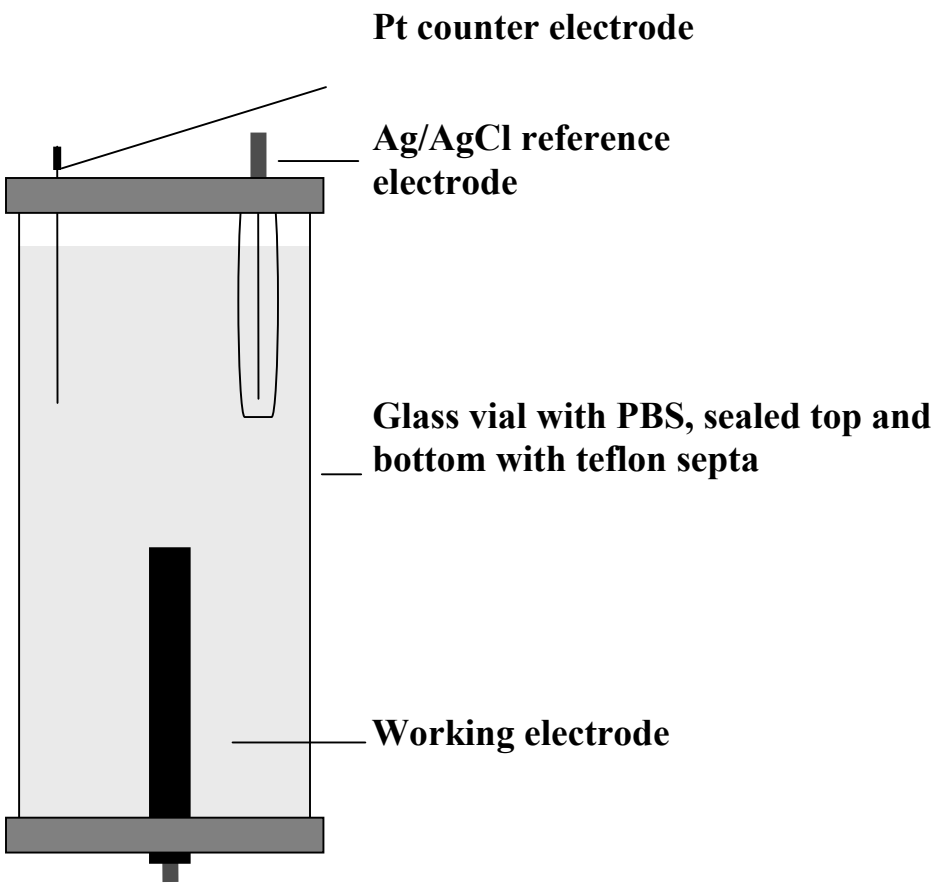
Pt counter electrode
Ag/AgCl reference electrode
Glass vial with PBS, sealed top and bottom with teflon septa
Working electrode

Figure 1

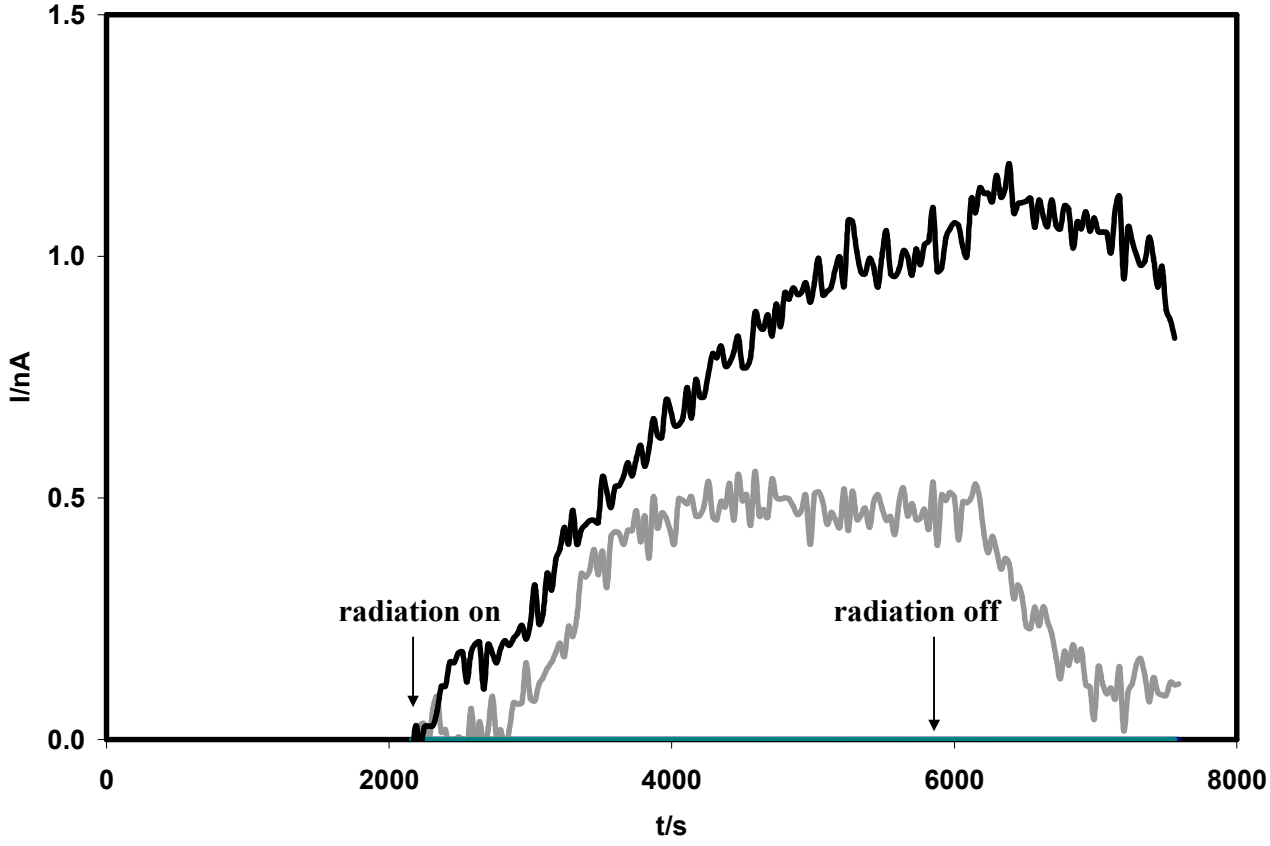
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Figure 2.

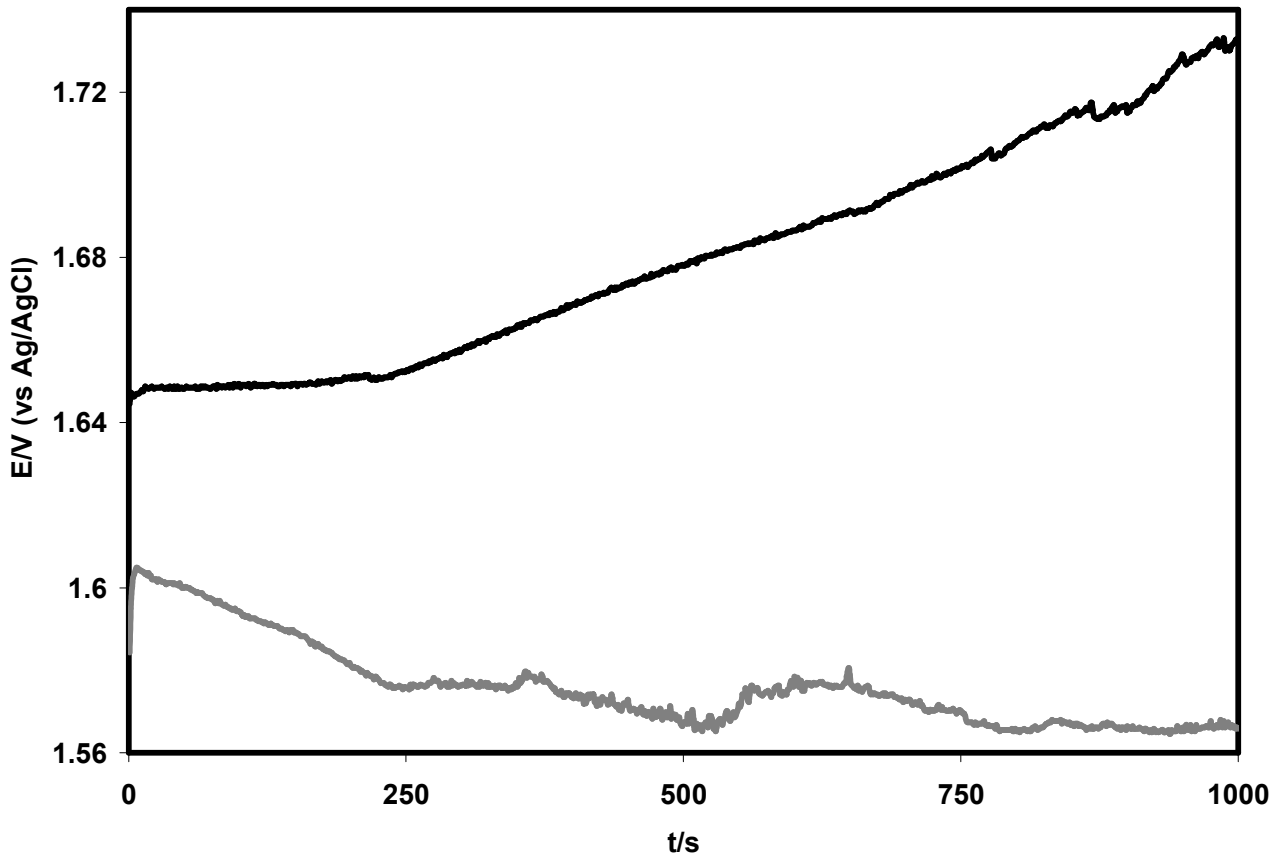

Figure 3.

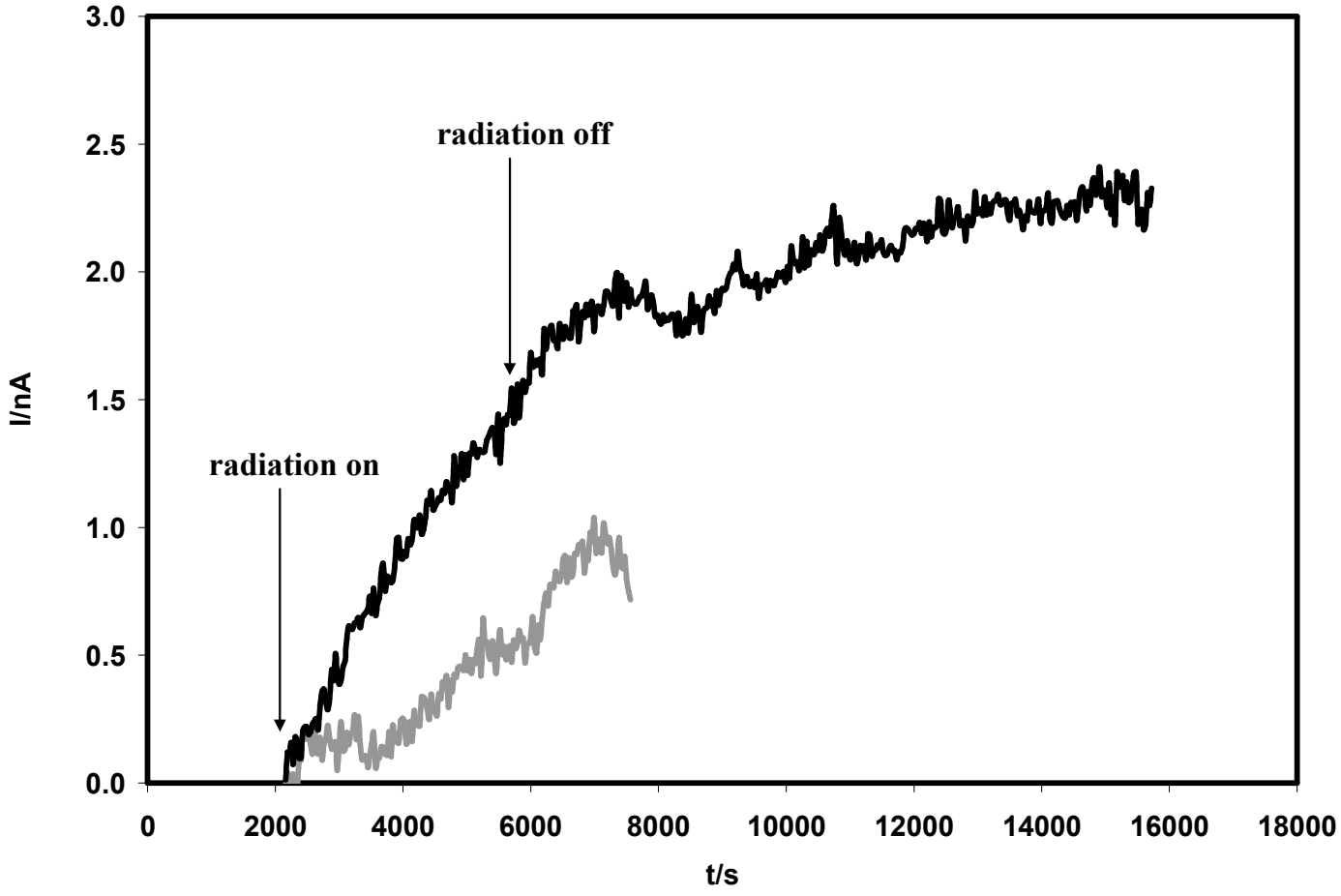
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Figure 4.

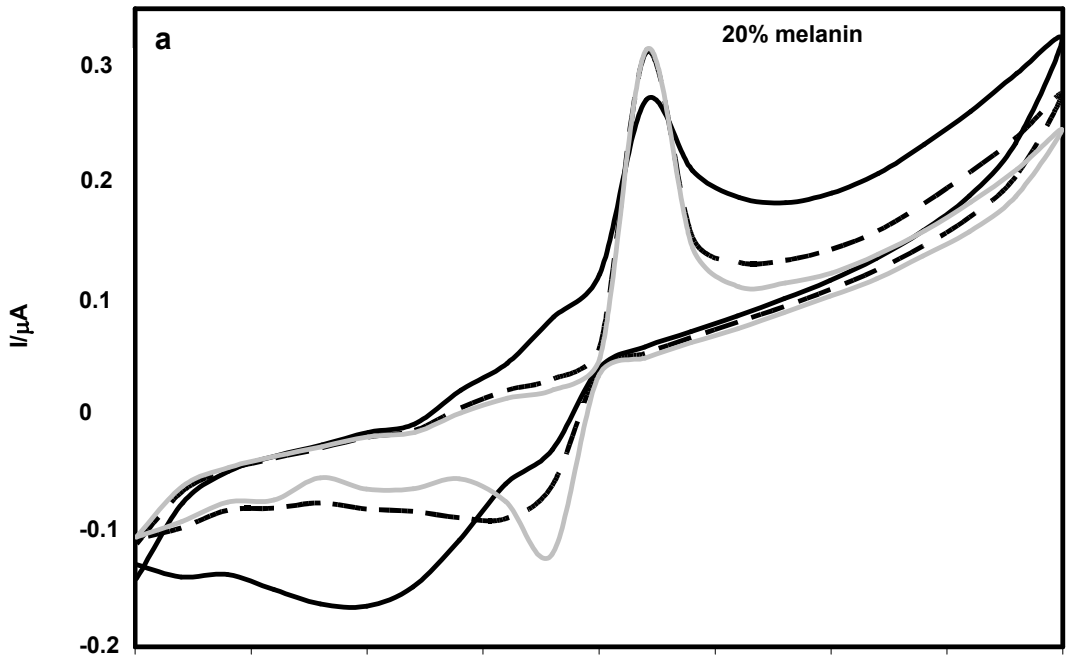

Figure 5a.

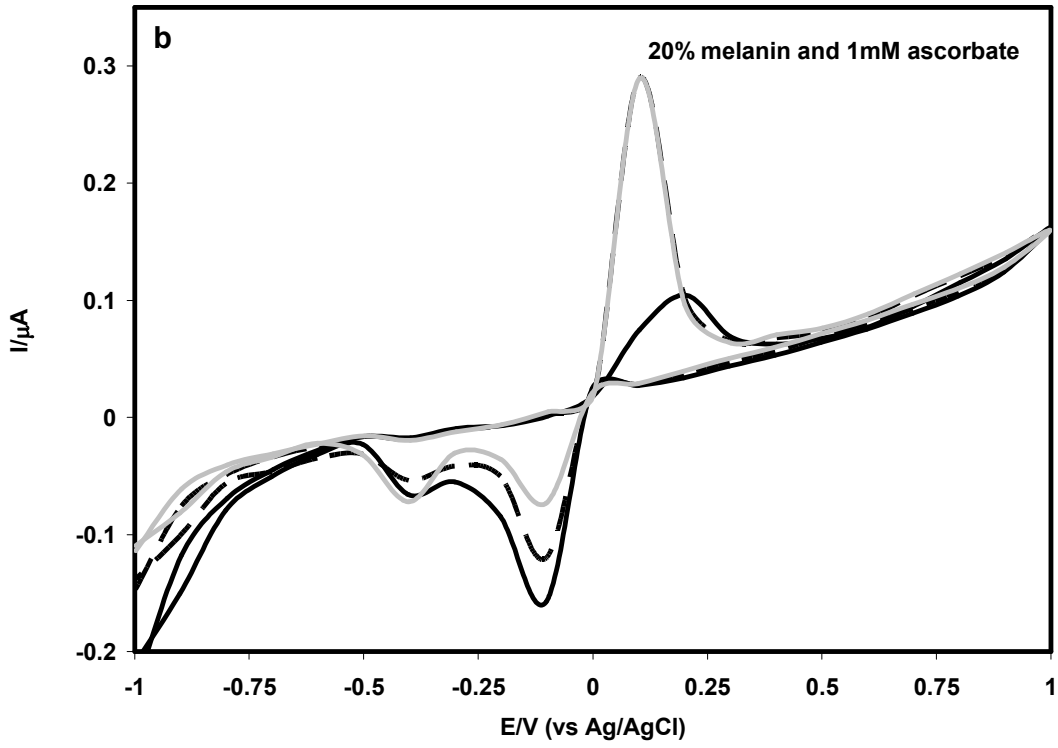
b
20% melanin and 1mM ascorbate
0.3
0.2
0.1
0
-0.1
-0.2
I/μA
-1
-0.75
-0.5
-0.25
0
0.25
0.5
0.75
1
E/V (vs Ag/AgCl)

Figure 5b

Table 1
Change in current production as a function of melanin concentration and time of exposure to 4000 Gy/h ^{60}Co gamma radiation. CP electrode (control) and 10-30% melanin/CP electrode poised at -700 mV in pH 7 PBS throughout the study.

Electrode material	Exposure (t/m)	ΔI/μA
100% CP	60	0.52 (0.06)*
10% melanin; 90% CP	60	0.72 (0.08)
20% melanin; 80% CP	60	0.89 (0.10)
30% melanin; 70% CP	60	1.22 (0.14)
20% melanin; 80% CP	90	0.93 (0.11)
30% melanin; 70% CP	90	1.45 (0.17)

* Standard deviation