Tuning of the pro-oxidant and antioxidant activity of trolox through the controlled release from biodegradable poly(trolox ester) polymers

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Abstract: In a variety of biomedical applications (e.g., tissue engineering, drug delivery, etc.), the role of a bioactive material is to serve as a platform by which one can modulate the cellular response into a desired role. Of the methods by which one may achieve this control (e.g., shape, structure, binding, growth factor release), the control of the cellular redox state has been under evaluated. Ideally, the ability to tune the redox state of a cell provides an additional level of control over a variety of cellular responses including, cell differentiation, proliferation, and apoptosis. Yet, in order to achieve such control, it is important to know both the overall oxidative status of the cell and what molecular targets are being oxidized. In this work, poly (trolox ester) nanoparticles were evaluated for their ability to either inhibit or induce cellular oxidative stress in a dose-dependent fashion. This polymer delivery form possessed a unique ability to suppress protein oxidation, a feature not seen in the free drug form, emphasizing the advantage of the delivery/dosage formulation has upon regulating cellular response.

Key Words: oxidative stress, antioxidant polymers, trolox, nanoparticles, biocompatibility


INTRODUCTION

It is well known that, depending upon the setting, the degradation of biodegradable materials can result in a localized inflammatory response. This inflammatory response is often the result of accumulated degradable byproducts, inducing cellular oxidative stress.1–4 This observation has led to several groups developing antioxidant coupled biomaterials as a means of inhibiting localized biomaterial related inflammation.5–10 While this work has demonstrated an ability to suppress inflammation, little is known about the chemical targets in which these materials augment oxidative stress. For instance, during inflammation, activation of macrophages and endothelial cells results in the formation of the NADPH oxidase complex, which converts oxygen into the highly reactive superoxide anion, O2−.11 The enzyme, superoxide dismutase (SOD), converts O2− into hydrogen peroxide (H2O2). H2O2 can then react with transient, redox active reduced metal ions (Fe2+, Cu+, etc.) to form hydroxyl radicals (OH·). O2− can also react with nitric oxide (NO) to form peroxynitrite (ONOO−). Collectively, these reactive oxygen species (ROS) and reactive nitrogen species (RNS), respectively, can further react with and oxidatively damage cellular proteins and lipids thereby producing oxidative stress markers such as protein carbonyl, 3-nitrotyrosine (3NT) and 4-hydroxy-2-trans-nonenal (HNE).12,13 The latter can covalently bind to Cys, His, and Lys residues on proteins via Michael addition, changing the structure and function of protein.14 However, small molecule antioxidants can terminate different reactive species, for example, water soluble antioxidants like gallic acid, vitamin C, trolox and so forth eliminate radicals generated in the cytosolic cellular compartment, while hydrophobic antioxidants like vitamin E (tocopherol), β-carotene, and so forth reduce lipid peroxidation. Antioxidants can modulate the redox state of the cell by controlling the levels of ROS and RNS.15 Modulation of this redox state can induce various cell responses like cell proliferation, differentiation, inflammation, apoptosis, and so forth.16–19 Of the tools available for modulating cellular behavior (e.g., growth factor release, cytokine/drug release, structural cues), redox status remains an underdeveloped yet exciting mechanism for controlling cellular response to biomaterials. As such, a knowledge of which chemical targets are affected can provide insight into which settings an oxidation sensitive biomaterial is most useful.

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In our previous work, we had synthesized poly(trolox ester), a biodegradable polymer of trolox that upon degradation results in release of active antioxidant trolox. Trolox [(+)6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid], a synthetic and water-soluble analogue of α-tocopherol (Vitamin E), has been shown to have antioxidant protective effect against oxidative stress injury. When formulated as a synthetic and water-soluble analogue of Vit E, trolox has been shown to have antioxidant protective effect against oxidative stress injury.23–25 In the current study, it was found that poly(trolox ester) provided protection against cellular oxidative stress in an in vitro model where cobalt nanoparticles were used to induce cellular oxidative stress. While this work was a promising proof of concept, trolox is known to possess a concentration dependent antioxidant and pro-oxidant effect (Fig. 1).23–25 In the current study, it was found that the toxicity of trolox resulting from its pro-oxidant effect can be reduced by the slow release of trolox through biodegradation of poly(trolox ester). Further, it was found that the method of delivery altered what chemical target was protected from oxidation. Specifically, there was a dose-dependent suppression of protein oxidation (as monitored by protein carbonyl formation) for poly(trolox ester) nanoparticles and not free soluble trolox. This work details the importance of oxidized product analysis and highlights the advantages that the mechanism of delivery can have upon the therapeutic response. This result emphasizes the unique potential for antioxidant polymers like poly(trolox ester) in a variety of biomedical applications, including wound healing, improving implant response and tissue engineering applications.

MATERIALS AND METHODS

Materials

All reagents were used as received without any further purification. The (+)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenylyltetrazolium bromide (MTT), and Pluronic F-68 were purchased from Sigma-Aldrich (St. Louis, MO). The 2’,7’-dichlorodihydrofluorescein diacetate (DCF-DA) and Live/Dead® cell viability assay were purchased from Invitrogen (Carlsbad, CA). All solvents were either obtained from Sigma-Aldrich or Fisher Scientific. Anti-nitrotyrosine antibody, Anti-dinitrophenylhydrazine (DNPH) protein antibody, and HNE anti-body were purchased from Intergen (Purchase, NY) and Millipore (Billerica, MA).

Poly(trolox ester) nanoparticle formulation

PTx-1000 and PTx-2500 nanoparticles were formulated as previously described.8 Briefly, polymer solution in acetone (10 mg mL⁻¹ PTx-1000 and 2 mg mL⁻¹ PTx-2500) was added to a pluronic F-68 solution in PBS while stirring. The resulting nanoparticle solution was left open overnight under stirring conditions to allow evaporation of the acetone. To remove excess surfactant, the nanoparticle suspension was centrifuged at 22,000 rpm for 2 h. Supernatant was discarded and the pellet was resuspended in 40 mL phosphate buffered saline (PBS). Centrifugation was repeated two more times and cell media was used for final resuspension of the pellet. Nanoparticle size was measured using dynamic light scattering on a Malvern Zetasizer Nano (Westborough, MA). Nanoparticles free of excess surfactant and of size 180–200 nm were used for all the studies.

Cell line

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza. Cells were cultured in EGM-2 medium with 2% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO₂ (v/v). All the studies with HUVECs have been conducted with cells from passage 3–5 and at 90% confluency.

Measuring oxidative stress in cells using DCF fluorescence

HUVECs were seeded onto a 96-well plate at a density of 25,000 cells cm⁻² and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. After 24 h, medium in each well was replaced by 100 μL of treatment solution [trolox solution or poly(trolox ester) nanoparticle suspension in media] and 100 μL of 10 μM DCF-DA solution in media. Fluorescence was then measured at various time points using a bottom-reading GENios Pro fluorescence spectrophotometer (Tecan, Switzerland) at excitation and emission wavelengths of 485 and 535 nm, respectively. Wells were incubated at 37°C throughout the study and briefly taken out of the incubator for fluorescence measurements at each time point.

Cytotoxicity of trolox, poly(trolox ester) nanoparticles, and nanoparticle leachouts

Cytotoxicity of trolox was determined using a standard MTT assay according to manufacturer’s protocol. Active reductase enzymes in the cell convert MTT into a colored formazan product which is then measured using UV spectrophotometry. HUVECs were seeded onto a 96-well plate at a density
of 25,000 cells cm$^{-2}$. After 24 h, media in the wells was removed and replaced by trolox solution in cell media. Freshly prepared 250 mM trolox stock solution in DMSO was diluted in cell media to prepare trolox solutions. Another 24 h later, trolox solution was removed and cells were washed twice with 200 μL PBS. About 200 μL of 0.5 mg mL$^{-1}$ MTT solution in PBS was then added to each well and the 96-well plate was incubated at 37°C. After 5 h, MTT solution was gently removed from the wells and 100 μL of DMSO was added to dissolve the formazan product. The absorbance intensity was recorded at 570 nm for formazan and at 690 nm for background using a Cary-50 Bio UV-visible spectrophotometer equipped with a Cary 50 MPR microplate reader (Varian, Santa Clara, CA).

Toxicity of poly(trolox ester) nanoparticles to mouse pulmonary microvascular endothelial cells (MPMVEC) as measured by MTS assay (modification of MTT assay) has been reported previously. Hence, in this study, cytotoxicity of PTx-1000 and PTx-2500 nanoparticles was determined using Live/Dead Viability Assay (Molecular Probes) according to manufacturer’s protocol. HUVECs were seeded onto a 24-well plate at a cell density of 25,000 cells cm$^{-2}$. After 24 h, cell media was replaced with 0.5 mL of nanoparticle suspensions in cell media. After another 24 h, nanoparticle solution was removed from each well and cells were washed twice with 2 mL of PBS. Cells were then stained with two-color fluorescence Live/Dead assay. They were then imaged via fluorescence microscopy where the live cells fluoresced green and the dead cells fluoresced red. The live and dead cells were counted using NIS-Elements software (Nikon Instruments, Melville, NY). The cell viability was then calculated as the number of live cells over the total number of live and dead cells.

To study the cytotoxicity of nanoparticle leachouts, HUVECs were seeded onto a 24-well plate at a cell density of 25,000 cells cm$^{-2}$. After 24 h, media was taken out of each well. Cell media (250 μL) was added to the well and a porous insert (Nunc™ cell culture insert with 0.02 μm pore size Anapore™ membrane) was placed in each well. Another 250 μL of media or nanoparticle suspension was added to the insert. Cell viability was measured after 24 h using Live/Dead assay as described above.

Measurement of protein carbonyls, 3-nitrotyrosine (3NT), and protein bound 4-hydroxy-2-trans-nonenal (HNE) as markers of oxidative stress
HUVECs were seeded on to a six-well plate at a cell density of 25,000 cells cm$^{-2}$. After 24 h, cell media from the well was replaced by 2 mL of treatment solution in media. Twenty-four hours after the treatment, solution above the cells was removed and cells were washed twice with chilled PBS. Cells were then scraped and centrifuged. Cell pellet was then lysed using a cell lysis buffer, the latter prepared by mixing RIPA buffer (pH = 8.0) and protease inhibitor cocktail (Amresco, Solon, OH) using manufacturer’s protocol.

Levels of protein carbonyl, 3NT and HNE were measured by slot blot technique. Briefly, for protein carbonyl levels, each sample was derivatized by incubating with 5 μL of 12% SDS and 10 mM solution of 2,4-dinitrophenylhydrazine (DNPH) in 2N HCl for 20 min at room temperature followed by a 7.5 μL addition of a neutralization solution (2M Tris in 30% glycerol). The sample was then used for slot blot analysis. Whereas, for 3NT and protein bound HNE levels, samples were denatured in 5 μL of 12% SDS solution and Laemmli sample buffer. Specific antibodies were used against protein carbonyl, 3NT or HNE protein modifications and colorimetric technique was used for detection as described previously.

Statistical analysis
Treatment comparisons were made using analysis of variance (ANOVA) followed by post hoc Student’s t test. Contrasts were considered significantly different at $P < 0.05$. Data are reported as mean ± standard errors.

RESULTS

Monitoring oxidative stress level in the cells using DCF fluorescence
Effect of trolox on the oxidative stress levels in the cells was measured using DCF fluorescence (used as a marker of oxidative stress) in HUVECs (Fig. 1). Fluorescence intensities as percent of control at 27-h time are compared in Figure 1. Trolox at lower concentrations suppresses oxidative stress in the cells as indicated by reduced fluorescence as compared to the control. At concentrations of trolox from 50 to 125 μg mL$^{-1}$, the DCF fluorescence in the cells is lower than control, but higher as compared to fluorescence at 25 μg mL$^{-1}$. DCF fluorescence increases with increasing trolox concentration in the range of 50–1000 μg mL$^{-1}$, where the fluorescence at 1000 μg mL$^{-1}$ is almost eight times that of the untreated (0 μg mL$^{-1}$ trolox) wells.

In a similar study, PTx-1000 and PTx-2500 nanoparticles followed by DCF-DA solution were added to HUVECs and fluorescence was measured after 27 h (Fig. 2). In the case of PTx-1000 nanoparticles, DCF fluorescence at 27 h decreases with increasing nanoparticle concentration up to 1000 μg mL$^{-1}$. At concentrations of 2000 and 4000 μg mL$^{-1}$ of PTx-1000 nanoparticles, an increase in the fluorescence is observed compared to the fluorescence at 1000 μg mL$^{-1}$. DCF fluorescence is increasingly suppressed with an increase in PTx-2500 nanoparticle concentration.

Cytotoxicity of trolox, poly(trolox ester) nanoparticles, and their leachouts
HUVECs were treated with trolox solutions of different concentrations for 24 h and the cell viability was measured using MTT assay (Fig. 3). Trolox at concentrations up to 500 μg mL$^{-1}$ does not have any significant toxicity as compared to the control. However, cell viability at 1000 μg mL$^{-1}$ significantly decreased to just above 70%.

To study the cytotoxicity of poly(trolox ester) nanoparticles, PTx-1000 and PTx-2500 NPs were added to HUVECs at different concentrations and cell viability was measured after 24 h using Live/Dead assay (Fig. 4). In the concentration range studied here, cell viability did not change significantly as compared to control.
To study the cytotoxicity of poly(trolox ester) nanoparticle leachouts, poly(trolox ester) nanoparticles suspended at concentration of 1 mg mL\(^{-1}\) were physically separated from HUVECs using a porous membrane support with pore size of 20 nm. Viability of the HUVECs exposed to poly(trolox ester) leachouts did not change significantly as compared to control (Fig. 5).

**DISCUSSION**

Concentration dependent biphasic behavior of antioxidants in both *in vitro* and *in vivo* settings has been illustrated in the literature.\(^{21,23–25,28–32}\) In a healthy cell/tissue, there is a balance between the rate at which ROS and RNS are generated and the rates at which antioxidant defense mechanisms terminate the reactive species. Any change in the antioxidant reservoir concentration or rate of generation of free radicals can change the redox state of the cell/tissue. A change in redox state can induce different cellular responses ranging from cellular apoptosis to cell differentiation.\(^{18,33,34}\)

This two-way antioxidant and pro-oxidant effect can be used in tissue engineering applications to control the oxidative stress level in the cells and thereby modulate cell response. Polymers composed of antioxidants linked through hydrolysable bonds provide a means of controlling release of these antioxidants and thereby affect the redox state of the cell.

Phenolic antioxidants (A-OH) like trolox can react with and terminate free radical species (R.) resulting in a stable phenoxyl radical (A-O.). In a normal cell/tissue, various small molecule antioxidants exert antioxidant effects synergistically where other antioxidants with reduction potential lower than A-O. can regenerate A-OH. The mechanisms by which antioxidants can act as pro-oxidants vary depending on that particular antioxidant and its environment. Some of the mechanisms that could result in pro-oxidant effects are depletion of glutathione,\(^{29,32}\) the presence of reduced transition metal (Fe, Cu) ions,\(^ {28,30,35}\) blocking several biomolecular targets like kinases and other proteins, and so forth. Even though the biphasic effect of trolox represented by the U-shaped bar graph in Figure 1(b) has been reported in the literature,\(^ {25,36–38}\) the exact mechanism for its pro-oxidant activity is not known. Trolox (T-OH) can react with a free radical (R) to form trolox phenoxyl radical (T-O.) and R-H. The resulting T-O. is more stable as compared to R and can trap another free radical to give a trolox quinone.\(^ {39–41}\) If trolox is present in excess as compared to the rate of generation of free radicals, the system will have equivalent...
amount of trolox converted into trolox phenoxyl radical which can in turn oxidize species that have lower redox potential.

DCF fluorescence is a widely used model to study oxidative stress injury. DCF-DA (2',7'-dichlorodihydrofluorescein diacetate), a non-fluorescent ester form of the dye, is taken up by the cells and cleaved to non-fluorescent DCFH (2',7'-dichlorodihydrofluorescein) by active esterases in the cell. DCFH can then react with free radicals to result in fluorescent DCF (2',7'-dichlorofluorescein) a marker of oxidative stress in the cells. DCF fluorescence data in Figure 1 suggest that the critical trolox concentration for HUVECs at which trolox starts showing pro-oxidant effects is approximately between 25 and 50 µg/mL.

Even though the DCF fluorescence model is simple and widely used, it is an indirect and general marker of overall oxidative stress in the cell and does not provide information regarding which specific oxidative species are responsible for oxidation of DCFH, nor what cellular components are at risk of damage. Indeed, DCFH can not only be oxidized by variety of oxidative species like ONOO⁻, OH, lipid peroxides, thiol radicals, and so forth, but could also be oxidized by antioxidant radicals (T-O). DCF fluorescence data should therefore be interpreted with caution. As compared to the DCF fluorescence, markers of oxidative stress like protein carbonyl, 3NT and protein-bound HNE are direct evidence of damage occurred to proteins, enzymes and lipids at cellular levels and are quantitative. Also, 3-NT and protein-bound HNE are markers specific for protein damage by RNS and lipid peroxidation, respectively. Generalized protein oxidation can be detected through monitoring the extent of protein carbonyl content contained within the cell, with an increase indicating an elevation in protein oxidation. Proteins obtained after treatment of HUVECs with free trolox, PTx-1000 and PTx-2500 nanoparticles were analyzed for their protein carbonyl, 3-NT and protein-bound HNE content. Both PTx-1000 and PTx-2500 nanoparticles at 1 mg mL⁻¹ showed a significant difference in protein carbonyl content, suggesting a unique anti-oxidant protective effect. Neither antioxidant nor pro-oxidant effect of free trolox was observed using any of the three markers, suggesting that the DCF fluorescence increase observed may be a “false positive,” further emphasizing the importance of secondary validation when monitoring cellular oxidative stress.

The advantage of having antioxidant polymers like poly (trolox ester) is that they can be used to deliver antioxidants in gradual and controlled manner as compared to initial pulse dose of antioxidants. Our previous work on poly (trolox ester) suggests the polymer undergo enzymatic degradation to release active antioxidants. As shown in Figure 2, treatment of HUVECs with PTx-1000 and PTx-2500 NPs resulted in suppression of DCF fluorescence in a concentration dependent manner. To rule out the possibility of this suppression of fluorescence as a result of cell death, cytotoxicity of trolox monomer, PTx-1000 and PTx-2500 nanoparticles was studied.
determined. As shown in Figure 3, trolox has significant cytotoxicity at concentration of 1000 μg mL⁻¹. While toxicity of trolox at higher concentrations was thought to be a result of its pro-oxidant effect as observed by DCF fluorescence studies, the lack of oxidative products (Fig. 6) suggest an alternate mechanism for this cell death.

PTx-1000 and PTx-2500 have very little to no cytotoxicity to HUVECs as indicated by the cell viability data in Figure 4. This study conforms with our previous findings regarding poly(trolox ester) nanoparticles having very little to no cytotoxicity to mouse pulmonary microvascular endothelial cells (MPMVEC), where cell viability was measured using the MTS assay. However, cytotoxicity could also result from degradation products or the leachouts from poly(trolox ester) nanoparticles. To determine cytotoxicity of leachouts, poly(trolox ester) nanoparticles were suspended in a porous support above a confluent layer of HUVECs. The porous support (Nunc cell culture inserts, 0.02 μm Anapore membrane) had a pore size of ~20 nm which would prevent nanoparticles of 180–200 nm from interacting with cells. However, water soluble leachouts from nanoparticles can diffuse through the membrane and interact with the

FIGURE 6. Monitoring of oxidative stress levels in HUVECs Cells were treated with free trolox, PTx-1000 and PTx-2500 at two different concentrations for 24 h. Cells were then lysed and collected protein was analyzed for (a) protein carbonyl content, (b) 3-NT levels, and (c) protein-bound HNE levels using immunochemical methods. One-way ANOVA was performed on all the data sets. The trend is significant only for the protein carbonyl levels, but not for 3-NT and HNE levels. (n = 3, M ± SE).

cells. Poly(trolox ester) nanoparticle leachouts do not have any significant cytotoxicity as shown in Figure 5. Insignificant cytotoxicity of poly(trolox ester) nanoparticles and leachouts suggests that the suppression of DCF fluorescence by PTx-1000 and PTx-2500 treatment is a result of the antioxidant effect. The antioxidant effect of poly(trolox ester) was also verified by the protein carbonyl data, where PTx-1000 and PTx-2500 nanoparticles at higher concentrations suppressed protein carbonyl content in HUVECs. PTx-1000 nanoparticles suppress DCF fluorescence more as compared to PTx-2500 nanoparticles. This conceivably can result from a difference in the degradation rate of the polymers and hence trolox being released at difference rates. PTx-1000 is more hydrophilic as compared to PTx-2500 and would degrade faster due to its lower molecular weight. In a previously published data set, a similar trend was observed where PTx-1000 nanoparticles provided more protection from oxidative stress injury in a in vitro model as compared to PTx-2500 nanoparticles. Increase DCF fluorescence at higher PTx-1000 nanoparticle concentrations of 2000 and 4000 μg mL⁻¹ indicates the pro-oxidant effect of PTx-1000. Biphasic DCF monitored antioxidant and pro-oxidant
behavior of trolox could be recreated using PTx-1000 nanoparticles, where PTx-1000 acts as a pro-oxidant at very high concentrations.

CONCLUSIONS
Poly(trolox ester) nanoparticles affect the redox state of the cells as confirmed by DCF fluorescence and protein carbonyl measurements in HUVECs. The polymer form of trolox possessed a unique ability to suppress protein oxidation not seen with the free trolox samples, emphasizing the importance of delivery route in modulating the potential therapeutic effect of antioxidant drugs. While DCF demonstrated a biphasic antioxidant/pro-oxidant effect of trolox, monitored cellular oxidative stress processes did not exhibit this effect. Because of the slow release of trolox through its biodegradation, poly(trolox ester) is an effective means of modulating cellular redox states. This capability has far reaching implications in the use of antioxidant polymers as a means of controlling cell status for a variety of biomedical, pharmaceutical, and tissue engineering applications.

REFERENCES


