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(54) Title: WATER SOLUBLE FULLERENE FORMULATIONS AND METHODS OF USE THEREOF

(57) Abstract: The present invention provides water soluble fullerene formulations and methods of use thereof.



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**Water Soluble Fullerene Formulations and Methods of Use
Thereof**

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This application claims priority under 35 U.S.C. §119(e) to U.S. Provisional Patent Application No. 61/432,639, filed January 14, 2011. The foregoing
10 application is incorporated by reference herein.

This invention was made with government support under Grant No. RR021937 awarded by the National Institutes of Health. The government has certain rights
15 in the invention.

FIELD OF THE INVENTION

The present invention relates generally to the antioxidants. More specifically, the present invention
20 relates to compositions and methods for the delivery of therapeutic antioxidants to a patient.

BACKGROUND OF THE INVENTION

The unique physical and chemical properties of
25 fullerene (e.g., C₆₀) have elicited broad research interest from different areas since it was discovered in 1985 (Kroto et al. (1985) Nature 318:162-3; Kroto et al. (1991) Chem. Rev., 91:1213-35). Over the past 20 years, fullerene has been investigated as a radical scavenger,
30 due to its highly unsaturated structure and excellent electron-receptor properties (Bosi et al. (2003) Eur. J. Med. Chem., 38:913-23; Krusic et al. (1991) Science 254:1183-5; McEwen et al. (1992) J. Am. Chem. Soc., 114:4412-4; Markovic et al. (2008) Biomaterials
35 29:3561-73; Nakamura et al. (2003) Acc. Chem. Res., 36:807-15). It has been reported that one fullerene

molecule can readily react with at least 15 benzyl radicals or 34 methyl radicals to form stable radical or non-radical adducts (Krusic et al. (1991) Science 254:1183-5). Many detrimental biological free radicals, such as superoxide (O_2^-), hydroxyl radical (OH), singlet oxygen (1O_2) and nitrogen-based radicals can be efficiently scavenged by fullerene and fullerene derivatives (Yin et al. (2009) Biomaterials 30:611-21; Misirkic et al. (2009) Biomaterials 30:2319-28; Lin et al. (2001) Appl. Magn. Reson., 20:583-4; Dugan et al. (1997) Proc. Natl. Acad. Sci., 94:9434-9; Wang et al. (1999) J. Med. Chem., 42:4614-20). However, the extreme hydrophobicity and potential toxicity of fullerene limit its application as a therapeutic antioxidant (Nakamura et al. (2003) Acc. Chem. Res., 36:807-15). To overcome these barriers, two major categories of strategies have been developed in the last two decades: (1) synthesis of water-soluble fullerene derivatives which maintain the radical scavenging capability, such as carboxyfullerene (C3) (Dugan et al. (1997) Proc. Natl. Acad. Sci., 94:9434-9; Lamparth et al. (1994) J. Chem. Soc. Chem. Commun., 14:1727-8.) and poly-hydroxyfullerene (fullerenol) (Dugan et al. (1997) Proc. Natl. Acad. Sci., 94:9434-9; Chiang et al. (1994) J. Org. Chem., 59:3960-8); and (2) solubilization of pristine fullerene using polymer, surfactant, cyclodextrin, liposome, solvent exchange or nanomilling (Misirkic et al. (2009) Biomaterials 30:2319-28; Kato S, Kikuchi et al. (2010) J. Photochem. Photobiol. B., 98:144-51; Andrievsky et al. (1995) J. Chem. Soc. Chem. Commun., 12:1281-2; Ungurenasu et al. (2000) J. Med. Chem., 43:3186-8; Samal et al. (2000) Chem. Commun., 13:1101-2; Yamakoshi et al. (1994) J. Chem. Soc. Chem. Commun., 4:517-8; Shinohara et al. (2009) Toxicol. Lett., 191:289-96; Xiao et al.

(2010) Biomaterials 31:5976-85). For example, a carboxylated fullerene derivative (C₆₀ tris-malonic acid) was reported to be able to protect neurons from apoptosis induced by glutamate receptor-mediated excitotoxicity, and is now being commercially developed as a therapy for neurodegenerative diseases (Dugan et al. (1997) Proc. Natl. Acad. Sci., 94:9434-9; Ali et al. (2008) Nanomedicine 4:283-94; Ali et al. (2004) Free Radic. Biol. Med., 37:1191-202). Several pristine fullerene formulations, such as fullerene-poly (N-vinyl pyrrolidone) (PVP) complex (Radical Sponge®) or fullerene-containing vegetable squalane (LipoFullerene®), have been approved as the antioxidant ingredients in cosmetic products and are sold in some countries (Xiao et al. (2010) Biomaterials 31:5976-85; Xiao et al. (2005) Biomed. Pharmacother., 59:3518; Xiao et al. (2006) Bioorg. Med. Chem. Lett., 16:1590-5; Lens et al. (2009) Recent Pat. Biotechnol., 3:118-23).

20 SUMMARY OF THE INVENTION

In accordance with the instant invention, particles comprising at least one fullerene and at least one oxazoline polymer are provided wherein the fullerene is complexed with the oxazoline polymer. Compositions comprising the particles, optionally with at least one pharmaceutically acceptable carrier or cosmetically acceptable carrier are also provided. The fullerene may be derivatized or pristine. In a particular embodiment, the fullerene is C₆₀ or a mixture of fullerenes comprising C₆₀. The oxazoline polymer may be a homopolymer or a random or block copolymer. In a particular embodiment, the oxazoline polymer comprises 2-ethyl-2-oxazoline.

In accordance with the instant invention, methods for treating, inhibiting, and/or preventing an oxidative stress associated disease or disorder in a subject are provided. The methods comprise administering to the
5 subject at least one composition of the instant invention.

BRIEF DESCRIPTIONS OF THE DRAWING

Figure 1A provides the structures of PVP. Figure
10 1B provides the structure of POx, where R = ethyl for PEtOx and R = ethyl or butyl for P(EtOx-co-BuOx). Figure 1C provides the proposed charge-transfer complex formation between fullerene and POx.

Figure 2A provides a picture of the aqueous
15 dispersion of C₆₀-P(EtOx-co-BuOx). Figure 2B provides the UV-vis spectra of P(EtOx-co-BuOx) and C₆₀-P(EtOx-co-BuOx). Figure 2C provides the infrared spectra of PEtOx and C₆₀-PEtOx. Figure 2D provides a picture of the aqueous dispersion of C₆₀-PVP (10kD). Figure 2E provides
20 the UV-vis spectra of PVP and C₆₀-PVP.

Figure 3A provides the representative size distributions determined by dynamic light scattering (DLS) for C₆₀-PEtOx and C₆₀-P(EtOx-co-BuOx). Figures 3B and 3C provide atomic force microscopy (AFM) images of
25 C₆₀-POx complexes: C₆₀-PEtOx (Fig. 3B) and C₆₀-P(EtOx-co-BuOx) (Fig. 3C). Figures 3D and 3E provide the size distribution of C₆₀-PVP complexes determined by DLS (Fig. 3D) and AFM (Fig. 3E). For AFM, C₆₀-polymer complexes were deposited on the 1-(3-aminopropyl)silatrane (APS)
30 mica. Concentration of the complexes is 100 μM in C₆₀.

Figures 4A and 4B provide representative transmission electron microscopy (TEM) images of C₆₀-PEtOx complex (Fig. 4A) and C₆₀-PVP complex (Fig. 4B) obtained with negative staining using 1% of uranyl

acetate. Concentration of the complex is 100 μM in C_{60} . The fullerene domains of the complex are marked by arrows.

Figures 5A and 5B show dose-dependent superoxide scavenging by C_{60} -polymer complexes as determined by electron paramagnetic resonance (EPR) spectroscopy. Figure 5A: 0.025 mM hypoxanthine (HX) and 10 mU xanthine oxidase (XO) were used as the superoxide source. Figure 5B: 0.02 mM riboflavin 50-monophosphate sodium salt dehydrate (FMN) was used as the superoxide source.

Figures 5C-5G provide representative EPR spectra: HX + XO alone (Fig. 5C); HX + XO in the presence of 100 μM in C_{60} of C_{60} -PVP (Fig. 5D); HX + XO in the presence of 40 μM in C_{60} of C_{60} -PEtOx (Fig. 5E); HX + XO in the presence of 100 μM in C_{60} of C_{60} -P(EtOx-co-BuOx) (Fig. 5F); and HX + XO in the presence of 400 U/ml SOD1 (Fig. 5G). Each sample was incubated at room temperature for 5 minutes.

Figure 6 provides the cytotoxicity of C_{60} -polymer complexes in MDCK (Fig. 6A), Hep G2 (Fig. 6B), and CATH.a neuronal cells (Fig. 6C). Cells were incubated with complexes at different concentrations of C_{60} for 24 hours. Cytotoxicity was determined by the cell counting assay. $**p < 0.01$, $n = 3$.

Figure 7 shows the neuronal uptake and sub-cellular distribution of fullerene after 24 hour exposure of CATH.a neuronal cells to 100 μM C_{60} complexes. Figure 7A provides the UV-vis spectra of supernatant of cell lysates. The absorbance maximum of fullerene at 340 nm is marked by arrows. The absorbance was normalized by the cellular protein concentration determined by MicroBCA™ assay. The spectrum of the control group was subtracted. Figure 7B provides the immunofluorescence images of CATH.a neuronal cells treated with C_{60} -PVP or C_{60} -PEtOx complexes. Cells untreated with C_{60} -polymer

complexes are shown for comparison. Mitochondria were stained with MitoTracker® Red. Fullerene was detected with anti-fullerene antibody and then stained with Alexa Fluor® 488 labeled secondary antibody. Figure 7C

5 provides TEM images of CATH.a neuronal cells treated with C₆₀-PEtOx complex. Fullerene was detected with anti-fullerene antibody and 25 nm colloidal gold-labeled secondary antibody. The colloidal gold was marked by arrows.

10 Figure 8A shows superoxide scavenging by C₆₀-POx complex in CATH.a neuronal cells. Cells were treated with 100 µM of C₆₀-PEtOx complex for 24 hours. 200 µM of methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) was added as the cell-permeable spin probe. Superoxide
15 was stimulated by treatment of cells by 20 µM of menadione for 1 hour. The signal was recorded by EPR spectroscopy over 10 minutes. EPR amplitude was normalized to total cell numbers. ** p<0.01 and * p<0.05, n = 3. Figure 8B shows the intracellular
20 superoxide scavenging by C₆₀-polymer complexes in CATH.a neuronal cells. CATH.a neurons were treated with various concentrations of C₆₀-PVP or C₆₀-PEtOx complexes for 24 hours. 200 µM of CMH was added as the cell-permeable spin probe. Superoxide was generated by
25 stimulating neurons with 100 nM of Ang II. CMH radical signal was recorded by EPR spectroscopy over 10 minutes. EPR signal was normalized to total cell number. **p < 0.01 and *p < 0.05, n = 3.

30 DETAILED DESCRIPTION OF THE INVENTION

Elevated levels of reactive oxygen species (ROS), including superoxide, hydroxyl radical, and hydrogen peroxide (H₂O₂) have been associated with the pathogenesis of numerous diseases, such as hypertension,

heart failure, arthritis, cancer, and multiple neurodegenerative disorders (Pelicano et al. (2004) Drug Resist. Updat., 7:97-110; Mapp et al. (1995) Br. Med. Bull., 51:419-36; Dhalla et al. (2000) J. Hypertens., 18:655-73; Halliwell, B. (1992) J. Neurochem., 59:1609-23). More specifically, considerable evidence has shown that the intracellular signaling pathway of angiotensin II (Ang II), a vasoconstrictor peptide that increases sympathetic nerve activity causing an elevation of blood pressure, involves an increase in superoxide. In neurons, the Ang II-induced increase in superoxide modulates ion channel activity and increases neuronal excitations which are linked to neuro-cardiovascular diseases like hypertension and heart failure (Zimmerman et al. (2004) Prog. Biophys. Mol. Biol., 84:125-49; Welch, W.J. (2008) Hypertension 52:51-6; Laursen et al. (1997) Circulation 95:588-93; Zimmerman et al. (2005) Hypertension 45:717-23; Yin et al. (2010) Am. J. Physiol. Cell Physiol., 298:C857-65). Therefore, scavenging superoxide in the central nervous system (CNS) is a rational strategy for the treatment of Ang II-dependent cardiovascular diseases.

Fullerene, the third allotrope of carbon, has been referred to as a "radical sponge" because of its powerful radical scavenging activities. However, the hydrophobicity and toxicity associated with fullerene limits its application as a therapeutic antioxidant. Numerous water-soluble fullerene derivatives have been synthesized and exhibited excellent ROS scavenging capabilities in several disease models. Further, fullerene, as an electron-receptor, can be solubilized by polymers containing electron-donors (like amide groups) to form water-soluble charge-transfer complexes (Ungurenasu et al. (2000) J. Med. Chem., 43:3186-8).

These fullerene-polymer complexes, of which C₆₀-PVP has been the most widely studied, usually have high aqueous solubility, high colloidal stability, narrow size distribution and low toxicity, which are suitable for biological and pharmaceutical applications (Ungurenasu et al. (2000) J. Med. Chem., 43:3186-8; Xiao et al. (2005) Biomed. Pharmacother., 59:351-8). Compared to functionalized fullerenes, the application of pristine fullerene provides ease of manufacturing, low cost, and high yield. Furthermore, evidence shows that after being properly formulated, pristine fullerene is also a therapeutic agent for ROS-related diseases and other biomedical applications. For example, the water-soluble C₆₀-PVP complex (Radical Sponge®) has been successfully commercialized by a Japanese company as an antioxidant for skin protection (Xiao et al. (2006) Bioorg. Med. Chem. Lett., 16:1590-5). Recently, it has also been reported that fullerene formulated with sodium dodecyl sulfate, γ -cyclodextrin or ethylene vinyl acetate-ethylene vinyl versatate copolymer can efficiently protect L929 cells from the cytotoxicity induced by nitric oxide (Misirkic et al. (2009) Biomaterials 30:2319-28). This study, however, used mechanochemical assisted formulation of C₆₀-based nanoparticles, usually characterized by high PDI, which is an impediment for their regulatory approval. Furthermore, it is likely that the C₆₀ was chemically modified due to high shear stress and energy released upon the mechanochemical formulation procedure. Therefore, efficient and biocompatible excipients allowing formulation of fullerene in mild conditions are needed to advance fullerene-based products to a broader clinical use.

Herein, the limitations of other fullerene compositions are overcome by generating water-soluble

nanoformulations of fullerene. Specifically, the biocompatible polymer poly(2-alkyl-2-oxazoline)s (POx) is shown to form water-soluble, antioxidant-active complexes with fullerene. One type of C₆₀-PVP (PVP 10 kDa) and two types of C₆₀-POx (PEtOx 5 kDa, and P(EtOx-co-BuOx) 8 kDa) complexes were prepared and their physicochemical properties and superoxide scavenging activities were quantitatively measured and compared. More specifically, the C₆₀-polymer complexes were characterized by UV-vis spectroscopy, infrared spectroscopy (IR), dynamic light scattering (DLS), atomic force microscopy (AFM) and transmission electron microscopy (TEM). Cellular uptake and intracellular distribution of the selected formulations in catecholaminergic (CATH.a) neurons were examined by UV-vis spectroscopy, immunofluorescence and immunogold labeling. Electron paramagnetic resonance (EPR) spectroscopy was used to determine the ability of the C₆₀-polymer complexes to scavenge superoxide. Their cytotoxicity was evaluated in three different cell lines. C₆₀-POx and C₆₀-PVP complexes exhibited similar physicochemical properties and antioxidant activities. C₆₀-poly(2-ethyl-2-oxazoline) (PEtOx) complex, but not C₆₀-PVP complex, were efficiently taken up by CATH.a neurons and attenuated the increase in intra-neuronal superoxide induced by angiotensin II (Ang II) stimulation. These results show that C₆₀-POx complexes are non-toxic, neuronal cell permeable, superoxide scavenging antioxidants that can be used for the treatment of brain-related diseases associated with increased levels of superoxide.

POx polymers have been studied for their formulation, drug delivery, and other biomedical and pharmaceutical applications (Luxenhofer et al. (2010)

Biomaterials 31:4972-9; Hoogenboom, R. (2009) Angew Chem. Int. Ed. Engl., 48:7978-94). PMeOx and PEtOx have shown high biocompatibility and similar properties as PEG (Gaertner et al. (2007) J. Control Release, 119:291-300). However, PEG as a polyether is prone to oxidation and can elicit oxidative stress by itself (Sung et al. (2009) J. Cell. Physiol., 218:549-57; Sung et al. (2010) Soft Matter 6:5196-205). Herein, it is demonstrated that POx can form water-soluble and biocompatible nano-complexes with fullerene. The type of polymer used in such formulations has profound effects on the physicochemical properties of the resulting C₆₀-based nanoparticles, as well as their intracellular uptake and superoxide scavenging ability. For example, the complex formed by a more hydrophobic POx, P(EtOx-co-BuOx), has smaller particle size and lower scavenging activity than those of C₆₀-PEtOx complex. Without being bound by theory, P(EtOx-co-BuOx) might form dense nanoscale networks around C₆₀ molecules (or clusters), which limits the accessibility of superoxide radicals to the fullerene. In contrast, a more hydrophilic PEtOx polymer may form more swollen, bulky, and accessible fullerene-based aggregates.

It has been reported that nanosized aggregates of pristine fullerene cause oxidative damage to the cell membranes and are substantially more toxic than highly water-soluble C₆₀ derivatives (Sayes et al. (2004) Nano Lett., 4:1881-7). It is demonstrated herein that when properly formulated with water-soluble polymers, both PVP and POx, fullerene does not exhibit any substantial toxicity in kidney, hepatocyte and neuronal cells in a broad dose range. The hydrophilic polymers used in these formulations can effectively mask fullerene from

contact with cell membranes and substantially attenuate its cytotoxicity.

The pathogenesis of Ang II-dependent cardiovascular diseases, including hypertension and heart failure, involves an increase in intracellular ROS produced by NADPH oxidases and/or mitochondria (Yin et al. (2010) Am. J. Physiol. Cell. Physiol., 298:C857-65). Recent evidence has shown that fullerene may be used as a scavenger of Ang II-induced ROS in the disease conditions. For example, it has been reported that a water-soluble fullerene vesicle alleviated Ang II-induced oxidative stress in endothelial cells (Maeda et al. (2008) Hypertens. Res., 31:141-51). In the present study, C₆₀-PEtOx complex showed a considerable intracellular superoxide scavenging effect in CATH.a neuronal cells stimulated with 100 nM of Ang II. Notably, the previously developed C₆₀-PVP system was not active under the same conditions. Differential neuronal uptake is one major reason for different intracellular ROS scavenging activities of these two complexes. Indeed, the neuronal uptake of C₆₀-PEtOx complex is at least two times greater than that of C₆₀-PVP complex. The microstructures of these complexes may play an important role in the process of neuronal membrane binding and uptake. Another possible reason is that compared to PVP, POx may have more favorable interaction and non-specific binding with cell membranes. Recently it has been reported that the cellular binding and internalization of a model protein, horseradish peroxidase was significantly increased after this protein was conjugated with POx copolymers (Tong et al. (2010) Mol. Pharm., 7:984-92). In the present study, the greater uptake of C₆₀-PEtOx complex in neuronal cells reinforces the significance of such formulations as ROS

scavengers. The analysis of intracellular distribution after 24 hours of C₆₀-PEtOx complex incubation with cells by TEM imaging showed that fullerene was distributed in both cytoplasm and nuclear region but not in mitochondria. Similar localization was previously found in skin keratinocytes treated with C₆₀-liposome complex (Kato et al. (2010) J. Photochem. Photobiol. B, 98:144-51). Notably, the distribution of POx-based fullerene complexes may be drastically different from that of some water-soluble fullerene derivatives. For example, carboxylated fullerene was localized into mitochondria and inhibited mitochondrial ROS formation (Chirico et al. (2007) Exp. Dermatol., 16:429-36; Foley et al. (2002) Biochem. Biophys. Res. Commun., 294:116-9). Here, the lack of mitochondrial localization indicates that C₆₀-PEtOx complex can efficiently scavenge the ROS stimulated in the cytoplasm. Indeed, C₆₀-PEtOx efficiently scavenged 60% of superoxide induced by Ang II stimulation, but only 20-30% of superoxide induced by menadione. This may be explained by different mechanisms of action of these two ROS generators. It is well known that Ang II induces the increase of superoxide in both cytoplasm and mitochondria, while menadione generates superoxide via redox cycling in the mitochondrial electron transport chain (Zimmerman, M.C. (2004) Prog. Biophys. Mol. Biol., 84:125-49; Yin et al. (2010) Am. J. Physiol. Cell. Physiol., 298:C857-65; Cadenas et al. (2000) Free Radic. Biol. Med., 29:222-30). Altogether, the data indicates that POx-based fullerene formulations are stable, biocompatible and efficient superoxide scavengers in neuronal cells.

In the present study, new types of C₆₀-polymer complexes were prepared and characterized by various techniques. Their physicochemical properties,

antioxidant activities and cytotoxicity were evaluated and compared. The results show that C₆₀-PEtOx exhibited greater cellular uptake and significant intracellular superoxide scavenging activity in CATH.a neuronal cells, which is not found in C₆₀-PVP complex. Efficient scavenging of intracellular superoxide induced by Ang II stimulation indicates the therapeutic use of C₆₀-POx complexes in Ang II-related cardiovascular diseases like hypertension and heart failure. It is also noteworthy that the safety of POx has been validated and the homopolymer PEtOx has been applied as an excipient in cosmetic industry. Therefore, this study also highlights the applications of C₆₀-POx complexes as cosmetic formulations and treatments of other ROS-related human diseases.

I. Compositions

In accordance with the instant invention, compositions are provided comprising at least one fullerene (e.g., at least one type) and at least one oxazoline polymer. In a particular embodiment, the fullerene and oxazoline polymer are complexed to form nanoparticles. The compositions may further comprise at least one carrier, particularly a pharmaceutically acceptable carrier.

As used herein, the term "fullerene" refers to a molecule containing carbon atoms arranged in a closed cage. Fullerenes may comprise from 20 to about 500 or more carbon atoms, particularly from 60 to 200 carbon atoms. In a general notation, C_x represents a fullerene cage having x carbon atoms. Fullerenes are typically spherical, but may form other shapes such as carbon nanotubes. For example, the fullerene may be a tubular structure with hemispherical caps at each end of the

tube. Examples of fullerenes include, without limitation, C₆₀, C₇₀, C₇₆, C₈₂, C₈₄, C₉₆, C₂₄₀, and C₅₄₀. In a particular embodiment, the fullerene is C₆₀ and/or C₇₀. The fullerene may comprise chemical moieties attached to
5 the exterior or incorporated within the cage. In a particular embodiment, the fullerene is pristine fullerene.

The instant invention may also encompass the use of hyperfullerenes in place of the fullerene.

10 Hyperfullerenes comprise one or more fullerene molecules inside another, i.e., hyperfullerene structures comprise one structure contained within a second larger structure.

The compositions of the instant invention may
15 comprise about 0.01% to about 10% (w/w) fullerene, particularly about 0.1% to about 5% fullerene, more particularly about 0.5% to about 1.0% fullerene.

In a particular embodiment, the particles of the instant invention (fullerene plus polymer) have an
20 average size about 50 nm to about 500 nm, particularly about 100 nm to about 200 nm. In a particular embodiment, the particles of the instant invention have a narrow size distribution. For example, the particles may have a polydispersity index (PDI) of about 0.15 -
25 about 0.25.

The compositions of the instant invention may further comprise at least one therapeutic agent. The core of pristine fullerene is hydrophobic and can be used to carrier hydrophobic therapeutic agents. In a
30 particular embodiment, the fullerene is derivatized with a linker or moiety, e.g., a hydrophilic moiety, to allow for the attachment of therapeutic agents. Therapeutic agents include, without limitation, polypeptides, peptides, glycoproteins, nucleic acids, synthetic and

natural drugs, peptoides, polyenes, macrocycles, glycosides, terpenes, terpenoids, aliphatic and aromatic compounds, small molecules, and their derivatives and salts. While the therapeutic agents are exemplified
5 herein, any bioactive agent may be administered to a patient, e.g., a diagnostic or imaging agent.

As stated hereinabove, the fullerene of the instant invention is complexed with at least one oxazoline polymer. In a particular embodiment, the oxazoline
10 polymer is a water-soluble, biocompatible polymer. The fullerene may be formulated with a poly(2-oxazoline) (POx) homopolymer (e.g., poly(2-alkyl-2-oxazoline)s such as poly(2-ethyl oxazoline; PEtOx) or an oxazoline copolymer (e.g., P(EtOx-co-BuOx)). Examples of
15 oxazoline polymers are provided in PCT/US11/31542

The oxazoline copolymer may be a random copolymer or a block copolymer. Block copolymers are most simply defined as conjugates of at least two different polymer segments. The simplest block copolymer architecture
20 contains two segments joined at their termini to give an A-B type diblock. Consequent conjugation of more than two segments by their termini yields A-B-A type triblock, A-B-A-B-type multiblock, or even multisegment A-B-C- architectures. If a main chain in the block
25 copolymer can be defined in which one or several repeating units are linked to different polymer segments, then the copolymer has a graft architecture of, e.g., an A(B)_n type. More complex architectures include for example (AB)_n (wherein n is about 1 to about
30 100) or A_nB_m starblocks which have more than two polymer segments linked to a single center. An exemplary block copolymer of the instant invention has the formula A-B or B-A, wherein A is a hydrophilic polymer segment and B is a hydrophobic polymer segment. Another exemplary

block copolymer has the formula A-B-A. Block copolymers structures include, without limitation, linear copolymers, star-like block copolymers, graft block copolymers, dendrimer based copolymers, and

5 hyperbranched (e.g., at least two points of branching) block copolymers. The segments of the block copolymer may have from about 2 to about 1000, about 2 to about 300, or about 2 to about 100 repeating units or monomers.

10 Well-defined poly(2-oxazoline) block copolymers of the instant invention may be synthesized by the living cationic ring-opening polymerization of 2-oxazolines. The synthetic versatility of poly(2-oxazoline)s allows for a precise control over polymer termini and

15 hydrophilic-lipophilic balance (HLB). Block length, structure, charge, and charge distribution of poly(2-oxazoline)s may be varied. For example, the size of the hydrophilic and/hydrophobic blocks may be altered, triblock polymers may be synthesized, star-like block

20 copolymers may be used, polymer termini may be altered, and ionic side chains and/or ionic termini may also be incorporated. Ionic side chains (e.g., comprising -R-NH₂ or R-COOH, wherein R is an alkyl) may be incorporated into the hydrophilic (preferably) or hydrophobic block.

25 The polymers of the instant invention may also comprise units or blocks from other polymers (e.g., hybrid oxazoline polymers) such as polyethyleneoxide (PEG), polyester, polylactic acid, poly(lactide-co-glycolide), poly(lactic-co-glycolic acid), poly(acrylic acid),

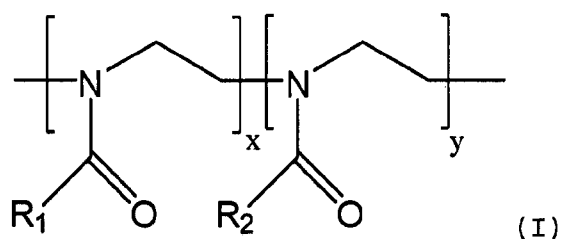
30 poly(methacrylic acid), poly(ethyleneimine), polycaprolactone, chitosan, poly(2-(N,N-dimethylamino)ethyl methacrylate), or polyamino acid (e.g. polyaspartate, poly(glutamic acid), poly(lysine) or poly(aspartic acid)).

Poly(2-oxazoline)s (also known as 2-substituted 4,5-dihydro oxazoles) are polysoaps and depending on the residue at the 2-position of the monomer can be hydrophilic (e.g., methyl, ethyl) or hydrophobic (e.g. propyl, pentyl, nonyl, phenyl, and the like) polymers. Moreover, numerous monomers introducing pending functional groups are available (Taubmann et al. (2005) *Macromol. Biosci.*, 5:603; Cesana et al. (2006) *Macromol. Chem. Phys.*, 207:183; Luxenhofer et al. (2006) *Macromol.*, 39:3509; Cesana et al. (2007) *Macromol. Rapid Comm.*, 28:608). Poly(2-oxazoline)s can be obtained by living cationic ring-opening polymerization (CROP), resulting in well-defined block copolymers and telechelic polymers of narrow polydispersities (Nuyken, et al. (1996) *Macromol. Chem. Phys.*, 197:83; Persigehl et al. (2000) *Macromol.*, 33:6977; Kotre et al. (2002) *Macromol. Rapid Comm.*, 23:871; Fustin et al. (2007) *Soft Matter*, 3:79; Hoogenboom et al. (2007) *Macromol.*, 40:2837). Several reports indicate that hydrophilic poly(2-oxazoline)s are essentially non-toxic and biocompatible (Goddard et al. (1989) *J. Control. Release*, 10:5; Woodle et al. (1994) *Bioconjugate Chem.*, 5:493; Zalipsky et al. (1996) *J. Pharm. Sci.*, 85:133; Lee et al. (2003) *J. Control. Release*, 89:437; Gaertner et al. (2007) *J. Control. Release*, 119:291). Using lipid triflates or pluritriflates, lipopolymers (Nuyken, et al. (1996) *Macromol. Chem. Phys.*, 197:83; Persigehl et al. (2000) *Macromol.*, 33:6977; Kotre et al. (2002) *Macromol. Rapid Comm.*, 23:871; Fustin et al. (2007) *Soft Matter*, 3:79; Hoogenboom et al. (2007) *Macromol.*, 40:2837; Punucker et al. (2007) *Soft Matter*, 3:333; Garg et al. (2007) *Biophys. J.*, 92:1263; Punucker et al. (2007) *Phys. Rev. Lett.*, 98:078102/1; Luedtke et al. (2005) *Macromol. Biosci.*, 5:384; Purmcker et al. (2005)

J. Am. Chem. Soc., 127:1258) or star-like poly(2-oxazoline)s are readily accessible. Additionally, various poly(2-oxazoline)s with terminal quaternary amine groups have been reported, which interact strongly
5 with bacterial cell membranes (Waschinski et al. (2005) Macromol. Biosci., 5:149; Waschinski et al. (2005) Biomacromol., 6:235).

The copolymers of the instant invention may comprise hydrophilic and hydrophobic oxazolines.
10 Examples of hydrophilic 2-oxazolines include, without limitation, 2-methyl-2-oxazoline, 2-ethyl-2-oxazoline, and mixtures thereof. Examples of hydrophobic 2-oxazolines include oxazolines with hydrophobic substituents (e.g., an alkyl or an aryl) at the 2-
15 position of the oxazoline ring including, without limitation 2-butyl-2-oxazoline, 2-propyl-2-oxazoline, and mixtures thereof. In a particular embodiment, the biocompatible, water soluble polymer is a homopolymer of 2-ethyl-2-oxazoline or a copolymer (random or block)
20 comprising 2-ethyl-2-oxazoline. In a particular embodiment, the copolymer is at least as hydrophilic as the P(EtOx-co-BuOx) polymer of the instant invention or as hydrophilic as the 2-ethyl-2-oxazoline homopolymer.

In a particular embodiment of the instant
25 invention, the polymer of the instant invention is represented by the formula provided in Figure 1B, wherein R (e.g., an alkyl) is a singular entity for a homopolymer or varies for a copolymer (random or block). When the copolymer is a block copolymer, the polymer
30 comprises the formula:



wherein x and y are independently selected between 1 and about 300, particularly about 5 to about 150, and more particularly about 10 to about 100; and wherein R₁ and R₂ are an alkyl. In a particular embodiment, R₁ or R₂ is -CH₂CH₃. In a particular embodiment, R₁ or R₂ is butyl (including isopropyl, sec-butyl, or tert-butyl) or propyl (including isopropyl). In yet another embodiment, R₁ or R₂ is -CH₂-CH₂-CH₂-CH₃ or -CH₂-CH₂-CH₃.

II. Methods

The compositions of the instant invention efficiently scavenge reactive oxygen species (ROS) and intracellular superoxide. Notably, the particles exhibit considerably high cellular uptake in neuron cells and efficiently attenuate the increase of intracellular superoxide level induced by different free radical stimulants including angiotensin II and menodione. Accordingly, the compositions of the instant invention attenuate the oxidative stress induced by different stimulants and can be used as effective antioxidant therapies.

In accordance with the instant invention, methods are provided for the treatment of reactive oxygen species (ROS)-related diseases. Elevated levels of reactive oxygen species (ROS), including superoxide, hydroxyl radical, and hydrogen peroxide (H₂O₂) have been associated with the pathogenesis of numerous diseases, such as hypertension, heart failure, arthritis, cancer, neurodegenerative disorders, and cardiovascular diseases

(e.g., angiotensin-II induced cardiovascular diseases). The instant invention encompasses methods of inhibiting, treating, and/or preventing oxidative stress associated diseases or disorders (caused by reactive oxygen species (ROS)) comprising the administration of at least one composition of the instant invention to a subject in need thereof. In a particular embodiment, the oxidative stress associated disease or disorder is selected from the group consisting of atherosclerosis, ischemia/
10 reperfusion injury, stroke, traumatic brain injury, restenosis, hypertension (including in chronic heart failure), heart failure, cardiovascular diseases, cancer, inflammation, an inflammatory disease or disorder, an acute respiratory distress syndrome (ARDS),
15 asthma, inflammatory bowel disease (IBD), a dermal and/or ocular inflammation, arthritis, metabolic disease or disorder, obesity, diabetes, neurological disorders and other disorders of the central nervous system, multiple sclerosis, neurocardiovascular
20 disease/dysregulation, and neurodegenerative disease or disorder (e.g., Alzheimer's disease, Huntington's disease, Parkinson's disease, Lewy Body disease, amyotrophic lateral sclerosis, and prion disease). The compositions of the instant invention may also be used
25 for immune enhancement and as an anti-aging agent.

In a particular embodiment, the method comprises administering at least one other antioxidant to the subject. The additional antioxidant may be administered simultaneously or consecutively with the compositions of
30 the instant invention. The additional antioxidant may be in the same composition as the particles of the instant invention or contained in separate compositions (e.g., with its own carrier). The instant invention encompasses a kit comprising at least one first

composition comprising the particles of the instant invention and at least one second composition comprising an additional antioxidant.

In accordance with another aspect of the instant invention, cosmetic compositions are provided comprising at least one fullerene, at least one oxazoline polymer, and at least one cosmetically acceptable carrier (e.g., for topical administration). In a particular embodiment, the fullerene containing particles of the instant invention are used in the cosmetic compositions described in U.S. Patent Application Publication No. 2005/0136079. The topical compositions of the present invention may be made into a wide variety of product types such as, without limitation, liquids, lotions, powders, creams, salves, gels, milky lotions, sticks, sprays (e.g., pump spray), aerosols, ointments, pastes, mousses, dermal patches, controlled release devices, and other equivalent forms. The instant invention also encompasses methods of inhibiting, treating, and/or preventing oxidative stress associated diseases or disorders (e.g., of the skin; for reducing damage by free radicals) through the topical application of a composition of the instant invention.

As stated hereinabove, the topical compositions of the instant invention may comprise the fullerene/polymer particles of the instant invention and at least one cosmetically acceptable carrier. "Cosmetically acceptable" refers to entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction when administered to an animal, particularly a human. A cosmetically acceptable carrier may be a pharmaceutically acceptable carrier. Cosmetically acceptable carriers are preferably approved by a

regulatory agency of the Federal or a state government. General types of acceptable topical carriers include, without limitation, emulsions (e.g., microemulsions and nanoemulsions), gels (e.g., an aqueous, alcohol, 5 alcohol/water, or oil (e.g., mineral oil) gel using at least one suitable gelling agent (e.g., natural gums, acrylic acid and acrylate polymers and copolymers, cellulose derivatives (e.g., hydroxymethyl cellulose and hydroxypropyl cellulose), and hydrogenated 10 butylene/ethylene/styrene and hydrogenated ethylene/propylene/styrene copolymers), solids (e.g., a wax-based stick, soap bar composition, or powder (e.g., bases such as talc, lactose, starch, and the like), liposomes (e.g., unilamellar, multilamellar, and 15 paucilamellar liposomes, optionally containing phospholipids), stabilizers, penetration enhancers, chelating agents (e.g., EDTA, EDTA derivatives (e.g., disodium EDTA and dipotassium EDTA), iniferine, lactoferrin, and citric acid), and excipients.

20 The topical (cosmetic) compositions of the invention may further comprise at least one additional agent. The additional agents include, without limitation, sunscreens (e.g., organic or inorganic sunscreens such as cinnamate compounds (e.g., 25 methoxyoctylcinnamate), titanium dioxide, zinc oxide, iron oxide, zirconium oxide, p-aminobenzoic acid, anthranilates, salicylate esters, dihydroxycinnamic acids, trihydroxy-cinnamic acids, diphenylbutadiene, stilbene, dibenzalacetone, benzalacetophenone, 30 naphtholsulfonates, di-hydroxynaphthoic acids, o- and p-hydroxybiphenyl disulfonates, coumarin derivatives, diazoles, quinine salts, quinoline derivatives, hydroxy- or methoxy-substituted benzophenones, uric and violuric acids, tannic acids, hydroquinone, benzophenones, and

derivatives and salts thereof), anti-aging agents (sunscreens, anti-oxidants (e.g., vitamins such as ascorbic acid, vitamin B, biotin, pantothenic acid, vitamin D, vitamin E and vitamin C), sodium bisulfite, yeast extract, ginkgo biloba, bisabolol, panthenol, alpha hydroxy acids, and oligosaccharides (e.g., melibiose)), chemotherapeutic and/or chemopreventative agents (e.g., plactaxel, cisplatin, docetaxol, carboplatin, vincristine, vinblastine, methotrexate, cyclophosphamide, CPT-11, 5-fluorouracil (5-FU), gemcitabine, estramustine, carmustine, adriamycin (doxorubicin), etoposide, arsenic trioxide, irinotecan, and epothilone derivatives), steroids, anti-inflammatory agents (e.g., steroidal (e.g., corticosteroids (e.g., hydrocortisone), hydroxyltriamcinolone, alpha-methyl dexamethasone, dexamethasone-phosphate, beclomethasone dipropionate, clobetasol valerate, desonide, desoxycorticosterone acetate, dexamethasone, dichlorisone, deflorasonediacetate, diflucortolone valerate, fluadronolone, fluclarolone acetonide, fludrocortisone, flumethasone pivalate, fluosinolone acetonide, fluocionide, flucortine butylester, flucortolone, flupredidene (flupredylidene) acetate, flurandronolone, halcinonide, hydrocortisone acetate, hydrocortisone butyrate, methylprednisolone, triamcinolone acetonide, cortisone, cortodoxone, flucetonide, fludrocortisone, difluorosone diacetate, fluradrenalone acetonide, medrysone, amciafel, amcinafide, betamethasone and its esters, chlorprednisone acetate, clocortelone, clescinolone, dichlorisone, difluprednate, flucloronide, flunisolide, fluoromethalone, fluperolone, fluprednisolone, hydrocortisone valerate, hydrocortisone cyclopentylpropionate, hydrocortamate, meprednisone,

paramethasone, prednisolone, prednisone, beclomethasone dipropionate, and triamcinolone) and non-steroidal anti-inflammatory agents (e.g., salicylates, acetic acid derivatives, fenamates, propionic acid derivatives and
5 pyrazoles)), pigment modulating agents (e.g., depigmenting agents such as lipoic acid, arbutim, dihydrolipoic acid, resveratrol, ascorbic acid, kojic acid, hydroquinone, isoflavones, retinoids (e.g., retinol, retinoic acid, and retinyl palmitate),
10 tyrosinase inhibitors, melanosome transfer inhibitors, selective cytotoxic agents for melanocytes, and natural extracts (e.g., licorice extract, gatuline A (pilewort extract), and micromerol (butylene glycol and apple extract))), exfoliating agents (e.g., organic hydroxy
15 acids (e.g., alpha and beta hydroxy acids), salicylic acid, glycolic acid, lactic acid, 5-octanoyl salicylic acid, hydroxyoctanoic acid, hydroxycaprylic acid, lanolin fatty acids, sulphhydryl compounds, protease or peptase enzymes (natural and bio-engineered), mimetic
20 compounds that mimic hydroxyl acids, and bioactive metals (e.g., manganese, tin, and copper), and natural soy-based products), lipid molecules (e.g., sphingosine-1-phosphate and lysophosphatidic acid), amino acids (e.g., arginine and lysine), vitamin A, vitamin D,
25 bradykinins, substance P, calcium gene-related peptide (CGRP), insulin, vascular endothelial growth factor (VEGF), thrombin, antibodies to platelet endothelial cells surface marker, compounds specifically binding adhesion molecules (e.g., ICAMs, NCAMs, PECAMs), extra-
30 cellular matrix proteins (e.g., glycosaminoglycans), fibrous proteins (e.g., collagen; elastin, fibronectins, and laminin), growth factors (e.g., platelet derived growth factors (PDGF), epidertnal growth factor (EGF), keratinocyte growth factor (KGF), vascular endothelial

growth factors (VEGFs), fibroblast growth factors (FGFs), transforming growth factors (TGFs), and insulin-like growth factor-1 (IGF-1)) tumor necrosis factor-alpha (TNF-alpha), tumor necrosis factor-beta (TNF-beta), and thymosin B4), anti-irritation agents (e.g., methyl nicotinate, corticosteroids, ascorbic acid, and acetic acid), anti-cellulite agents (e.g., xanthine compounds such as caffeine, theophylline, theobromine, and aminophylline), anti-fungal agents (e.g.,
10 terbinafine hydrochloride, nystatin, amphotericin B, griseofulvin, ketoconazole, miconazole nitrate, flucytosine, fluconazole, itraconazole, clotrimazole, benzoic acid, salicylic acid, and selenium sulfide), anti-bacterial agents (e.g., antibiotics, penicillins,
15 cephalosporins, carbacephems, cephamycins, carbapenems, monobactams, aminoglycosides, glycopeptides, quinolones, tetracyclines, macrolides, fluoroquinolones, and derivatives thereof), moisturizing agents (e.g., polyhydric alcohols (e.g., glycerin, propylene glycol,
20 1,3-buthyleneglycol, polyethylene glycol, sorbitol, isoprene glycol, and POB methyl glucoside), saccharides (e.g., trehalose, pullulan, and maltose), and biological polymers (e.g., sodium hyaluronate, chondroitin sodium sulfate, collagen, elastin, amino acids, sodium lactate,
25 pyrrolidone sodium carboxylate, and urea), emollients (e.g., lanolin, spermaceti, mineral oil, paraffin, petrolatum, white ointment, white petroleum, yellow ointment, vegetable oils, waxes, cetyl alcohol, glycerin, hydrophilic petrolatum, isopropyl myristate,
30 myristyl alcohol, and oleyl alcohol), antioxidants (e.g., water-soluble antioxidants (e.g., sulfhydryl compounds, sulfhydryl derivatives (e.g., sodium metabisulfite and N-acetyl-cysteine), lipoic acid, dihydrolipoic acid, resveratrol, acetyl-cysteine

(iniferine), lactoferrin, ascorbic acid, and ascorbic acid derivatives (e.g., ascorbyl palmitate and ascorbyl polypeptide)), oil-soluble antioxidants (butylated hydroxytoluene, retinoids (e.g., retinol and retinyl palmitate), tocopherols (e.g., tocopherol acetate), tocotrienols, and ubiquinone), and natural extracts (e.g., extracts containing resveratrol, flavonoids and isoflavonoids and derivatives thereof (e.g., genistein and diadzein) such as grape seed, green tea, pine bark, propolis, and legume extracts), anti-swelling agents (e.g., lanolin, aloe vera extract, hydrocortisone, and menthol), anesthetics (e.g., ambucaine, amolanone, amylcaine, benoxinate, benzocaine, betoxycaine, biphenamine, bupivacaine, butacaine, butamben, butanilicaine, butethamine, butoxycaine, carticaine, chloroprocaine, cocaethylene, cocaine, cyclomethycaine, dibucaine, dimethisoquin, dimethocaine, diperodon, dyclonine, ecogonidine, ecogonine, etidocaine, euprocin, fenalcomine, formocaine, hexylcaine, hydroxyteteracaine, isobutyl p-aminobenzoate, ketamine, leucinocaine, levexadrol, lidocaine, mepivacaine, meprylcaine, metabutoxycaine, methyl chloride, myrtecaine, naepaine, octacaine, orthocaine, oxethazaine, parenthoxycaine, phenacaine, phenol, piperocaine, piridocaine, polidocanol, pramoxine, prilocalne, procaine, propanocaine, proparacaine, propipocaine, propoxycaine, pseudococaine, pyrrocaine, ropivacaine, salicyl alcohol, tetracaine, tolycaine, trimecaine, zolamine, and pharmaceutically acceptable salts thereof), anti-viral agents (e.g., amantadine hydrochloride, rimantadin, acyclovir, famciclovir, foscarnet, ganciclovir sodium, idoxuridine, ribavirin, sorivudine, trifluridine, valacyclovir, vidarabin, didanosine, stavudine, zalcitabine, zidovudine, interferon alpha, and

edoxudine), nutrients (e.g., vitamins (e.g., vitamin A, vitamin B, vitamin C, and vitamin E), essential amino acids, and essential fatty acids), cosmetic agents, coloring agents, and fragrances.

5 Protocols and procedures which facilitate formulation of the topical compositions of the invention can be found, for example, in Cosmetic Bench Reference, Cosmetics & Toiletries, Allured Publishing Corporation, Illinois, 2005 and in International Cosmetic Ingredient
10 Dictionary and Handbook. 10th ed. Edited by Gottschalck and McEwen. Washington, Cosmetic, Toiletry and Fragrance Association, 2004.

 The compositions comprising the particles of the instant invention may be conveniently formulated for
15 administration with any pharmaceutically/cosmetically acceptable carrier(s). For example, the complexes may be formulated with an acceptable medium such as water, buffered saline, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the
20 like), dimethyl sulfoxide (DMSO), oils, detergents, suspending agents or suitable mixtures thereof. The concentration of the particles in the chosen medium may be varied and the medium may be chosen based on the desired route of administration of the pharmaceutical
25 preparation. Except insofar as any conventional media or agent is incompatible with the nanoparticles to be administered, its use in the pharmaceutical preparation is contemplated.

 The dose and dosage regimen of particles according
30 to the invention that are suitable for administration to a particular patient may be determined by a physician considering the patient's age, sex, weight, general medical condition, and the specific condition for which the nanoparticles are being administered and the

severity thereof. The physician may also take into account the route of administration, the pharmaceutical carrier, and the particle's biological activity. The dosage ranges for the administration of the compositions of the invention are those large enough to produce the desired effect (e.g., curing, relieving, treating, and/or preventing the disease or disorder, the symptoms of it, or the predisposition towards it). The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counter indications.

Selection of a suitable pharmaceutical preparation will also depend upon the mode of administration chosen. For example, the particles of the invention may be administered by direct injection or intravenously. In this instance, a pharmaceutical preparation comprises the nanoparticle dispersed in a medium that is compatible with the site of injection.

Particles of the instant invention may be administered by any method. For example, the particles of the instant invention can be administered, without limitation parenterally, subcutaneously, orally, topically, pulmonarily, rectally, vaginally, intravenously, intraperitoneally, intrathecally, intracerebrally, epidurally, intramuscularly, intradermally, or intracarotidly. In a particular embodiment, the nanoparticles are administered topically, intravenously or intraperitoneally. Pharmaceutical preparations for injection are known in

the art. If injection is selected as a method for administering the particle, steps must be taken to ensure that sufficient amounts of the molecules or cells reach their target cells to exert a biological effect.

5 Dosage forms for oral administration include, without limitation, tablets (e.g., coated and uncoated, chewable), gelatin capsules (e.g., soft or hard), lozenges, troches, solutions, emulsions, suspensions, syrups, elixirs, powders/granules (e.g., reconstitutable
10 or dispersible) gums, and effervescent tablets. Dosage forms for parenteral administration include, without limitation, solutions, emulsions, suspensions, dispersions and powders/granules for reconstitution. Dosage forms for topical administration include, without
15 limitation, creams, gels, ointments, salves, patches and transdermal delivery systems.

Pharmaceutical compositions containing a particle of the present invention as the active ingredient in intimate admixture with a pharmaceutically acceptable
20 carrier can be prepared according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., intravenous, oral, direct injection, intracranial, and
25 intravitreal.

A pharmaceutical preparation of the invention may be formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form, as used herein, refers to a physically discrete
30 unit of the pharmaceutical preparation appropriate for the patient undergoing treatment. Each dosage should contain a quantity of active ingredient calculated to produce the desired effect in association with the selected pharmaceutical carrier. Procedures for

determining the appropriate dosage unit are well known to those skilled in the art.

Dosage units may be proportionately increased or decreased based on the weight of the patient.

- 5 Appropriate concentrations for alleviation of a particular pathological condition may be determined by dosage concentration curve calculations, as known in the art.

- 10 In accordance with the present invention, the appropriate dosage unit for the administration of nanoparticles may be determined by evaluating the toxicity of the molecules or cells in animal models. Various concentrations of nanoparticles in pharmaceutical preparations may be administered to mice,
15 and the minimal and maximal dosages may be determined based on the beneficial results and side effects observed as a result of the treatment. Appropriate dosage unit may also be determined by assessing the efficacy of the nanoparticle treatment in combination
20 with other standard drugs. The dosage units of nanoparticle may be determined individually or in combination with each treatment according to the effect detected.

- The pharmaceutical preparation comprising the
25 nanoparticles may be administered at appropriate intervals, for example, at least twice a day or more until the pathological symptoms are reduced or alleviated, after which the dosage may be reduced to a maintenance level. The appropriate interval in a
30 particular case would normally depend on the condition of the patient.

The instant invention encompasses methods of treating a disease/disorder comprising administering to a subject in need thereof a composition comprising a

nanoparticle of the instant invention and, particularly, at least one pharmaceutically acceptable carrier. The instant invention also encompasses methods wherein the subject is treated via ex vivo therapy. In particular, 5 the method comprises removing cells from the subject, exposing/contacting the cells in vitro to the nanoparticles of the instant invention, and returning the cells to the subject. In a particular embodiment, the cells comprise macrophage. Other methods of 10 treating the disease or disorder may be combined with the methods of the instant invention may be co-administered with the compositions of the instant invention.

The instant also encompasses delivering the 15 nanoparticle of the instant invention to a cell in vitro (e.g., in culture). The nanoparticle may be delivered to the cell in at least one carrier.

III. Definitions

20 "Pharmaceutically acceptable" indicates approval by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

25 A "carrier" refers to, for example, a diluent, adjuvant, preservative (e.g., Thimersol, benzyl alcohol), anti-oxidant (e.g., ascorbic acid, sodium metabisulfite), solubilizer (e.g., Tween 80, Polysorbate 80), emulsifier, buffer (e.g., Tris HCl, acetate, 30 phosphate), antimicrobial, bulking substance (e.g., lactose, mannitol), excipient, auxiliary agent or vehicle with which an active agent of the present invention is administered. Pharmaceutically acceptable carriers can be sterile liquids, such as water and oils,

including those of petroleum, animal, vegetable or synthetic origin. Water or aqueous saline solutions and aqueous dextrose and glycerol solutions may be employed as carriers, particularly for injectable solutions.

- 5 Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin (Mack Publishing Co., Easton, PA); Gennaro, A. R., Remington: The Science and Practice of Pharmacy, (Lippincott, Williams and Wilkins); Liberman, et al.,
10 Eds., Pharmaceutical Dosage Forms, Marcel Decker, New York, N.Y.; and Kibbe, et al., Eds., Handbook of Pharmaceutical Excipients, American Pharmaceutical Association, Washington.

The term "treat" as used herein refers to any type
15 of treatment that imparts a benefit to a patient afflicted with a disease, including improvement in the condition of the patient (e.g., in one or more symptoms), delay in the progression of the condition, etc. In a particular embodiment, the treatment of an
20 oxidative stress related disease or disorder results in at least a reduction in the amount of (the scavenging of) reactive oxygen species (ROS).

A "therapeutically effective amount" of a compound or a pharmaceutical composition refers to an amount
25 effective to prevent, inhibit, treat, or lessen the symptoms of a particular disorder or disease.

As used herein, the term "therapeutic agent" refers to a chemical compound or biological molecule including, without limitation, nucleic acids, peptides, proteins,
30 and antibodies that can be used to treat a condition, disease, or disorder or reduce the symptoms of the condition, disease, or disorder.

As used herein, the term "small molecule" refers to a substance or compound that has a relatively low

molecular weight (e.g., less than 4,000, less than 2,000, particularly less than 1 kDa or 800 Da).

Typically, small molecules are organic, but are not proteins, polypeptides, or nucleic acids, though they
5 may be amino acids or dipeptides.

As used herein, the term "polymer" denotes molecules formed from the chemical union of two or more repeating units or monomers. The term "block copolymer" most simply refers to conjugates of at least two
10 different polymer segments, wherein each polymer segment comprises two or more adjacent units of the same kind.

As used herein, the term "antioxidant" refers to compounds that neutralize the activity of reactive oxygen species or inhibit the cellular damage done by
15 the reactive species or their reactive byproducts or metabolites. The term "antioxidant" may also refer to compounds that inhibit, prevent, reduce or ameliorate oxidative reactions. Examples of antioxidants include, without limitation, vitamin E, vitamin C, ascorbyl
20 palmitate, vitamin A, carotenoids, beta carotene, retinoids, xanthophylls, lutein, zeaxanthin, flavones, isoflavones, flavanones, flavonols, catechins, ginkgolides, anthocyanidins, proanthocyanidins, carnosol, carnosic acid, organosulfur compounds,
25 allylcysteine, alliin, allicin, lipoic acid, omega-3 fatty acids, eicosapentaeneic acid (EPA), docosahexaeneic acid (DHA), tryptophan, arginine, isothiocyanates, quinones, ubiquinol, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA),
30 super-oxide dismutase mimetic (SODm), and coenzymes-Q.

The terms "reactive oxygen species," or "oxidative species," as used herein, refer to oxygen derivatives from oxygen metabolism or the transfer of electrons,

resulting in the formation of "free radicals" (e.g., superoxides or hydroxyl radicals).

As used herein, the terms "host," "subject," and "patient" refer to any animal, including humans.

5 As used herein, the term "prevent" refers to the prophylactic treatment of a subject who is at risk of developing a condition (e.g., cancer) resulting in a decrease in the probability that the subject will develop the condition.

10

The following example provides illustrative methods of practicing the instant invention, and is not intended to limit the scope of the invention in any way.

15

EXAMPLE

Materials and methods

Materials

Fullerene (C₆₀), PVP 10 kDa (K15), toluene,
20 chloroform, hypoxanthine (HX), xanthine oxidase (XO),
angiotensin II (Ang II), riboflavin 50-monophosphate
sodium salt dehydrate (FMN) and superoxide dismutase
(SOD1) were purchased from Sigma-Aldrich Co. (St. Louis,
MO). Poly(2-ethyl-2-oxazoline) 5 kDa (PEtOx) was
25 purchased from Polysciences Inc. (Warrington, PA).
Poly(2-ethyl-2-oxazoline)-co-poly (2-butyl-2-oxazoline)
8 kDa (P(EtOx₅₀-co-BuOx₂₀)) was synthesized and purified
as described (Tong et al. (2010) Mol. Pharm., 7:984-92).
Methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH),
30 deferoxamine methanesulfonate salt (DF) and
diethyldithiocarbamic acid sodium (DETC) were from
Noxygen Science Transfer & Diagnostics GmbH (Elzach,
Germany). Fullerene monoclonal antibody (mouse IgG1)
was purchased from Santa Cruz Biotechnology, Inc. (Santa

Cruz, CA). Alexa Fluor® 488 goat anti-mouse IgG and MitoTracker® Red FM were from Invitrogen (Carlsbad, CA). Nunc Lab-Tek II chamber slide system was from Thermo Fisher Scientific (Waltham, MA). 25 nm colloidal gold-labeled anti-mouse IgG was from Aurion (Hatfield, PA).

Devices

A LAMBDA 25 UV-vis spectrometer (PerkinElmer, Waltham, MA) and a Nicolet 380 FT-IR spectrometer (Thermo Scientific, Waltham, MA) were used for the measurement of absorbance and infrared spectra of C₆₀-polymer complexes. Particle size and size distribution were determined by dynamic light scattering (DLS) using Nano series Zetasizer (Malvern Instruments Inc., Westborough, MA). Atomic force microscopy (AFM) was carried out with MFP-3D microscope (Asylum Research, Santa Barbara, CA) mounted on inverted optical microscope (Olympus, Center Valley, PA). Transmission electron microscopy (TEM) was done with Tecnai GZ Spirit TW device (FEI, Hillsboro, OR) equipped with an AMT digital imaging system (Danvers, MA). Electron paramagnetic resonance (EPR) spectroscopy was performed with a Bruker Biospin e-scan spectrometer (Bruker, Billerica, MA). Confocal microscopy was carried out by LSM 510 Meta Confocal imaging system (Carl Zeiss, Peabody, MA).

Preparation of C₆₀-polymer complexes

The aqueous dispersions of C₆₀-polymer complexes were prepared by a thin film hydration method with minor modifications (Ungurenasu et al. (2000) J. Med. Chem., 43:3186-8; Xiao et al. (2006) Bioorg. Med. Chem. Lett., 16:1590-5). Briefly, 1 ml of fullerene in toluene (1 mg/ml) was mixed with 3 ml of PVP or POx in chloroform

and stirred at room temperature (r.t.) for 2 hours. The C₆₀ to polymer molar ratios were varied from 1:0.5 to 1:4. The solvents were removed in vacuo at 60°C to form the thin films. The C₆₀-polymer complexes were readily
5 dispersed in 1 ml of deionized water (DIH₂O), in selected cases using 1-2 minute sonication. Excess of unincorporated fullerene was removed by centrifugation (10,000 rpm, 5 minutes) and filtration through a 0.45 µm filter resulting in a clear yellow colloidal solution.
10 The complexes were dialyzed against water overnight to eliminate the residual toluene and excess of the polymer, and then lyophilized and stored at r.t. for further characterization.

15 *Characterization of C₆₀-polymer complexes*

UV-vis spectroscopy

UV-vis spectra of C₆₀-polymer complexes were recorded and used to determine the concentration of solubilized fullerene by absorbance at 340 nm (the molar
20 absorptivity 49,000 cm l/mol (Yamakoshi et al. (1994) J. Chem. Soc. Chem. Commun., 4:517-8)). The fullerene loadings were expressed as %wt. of the dispersed phase (C₆₀-polymer complex dry powder weighted after lyophilization of the dispersions).

25

Infrared (IR) spectroscopy

IR spectra were recorded using lyophilized powders of free polymers or C₆₀-polymer complexes.

30 Dynamic light scattering (DLS)

Effective hydrodynamic diameters of the particles were measured by photon correlation spectroscopy (i.e. dynamic light scattering, DLS) in a thermostatic cell at a scattering angle of 90° using the same instrument

equipped with a Multi Angle Sizing Option (BI-MAS). Briefly, 0.5 ml of complexes dissolved in DIH₂O at 100 μ M C₆₀ was used for the measurement. All measurements were performed at 25°C. Software provided by the
5 manufacturer was used to calculate the size of the particles and polydispersity indices. All the formulations were prepared and measured three times in a parallel manner. The data are presented as means $\pm$ SEM. For the stability testing, 0.5 ml of complexes with
10 different pH (pH 2-10), ionic strength (0-1 M NaCl), concentration (1-100 μ M) and storage time (1 day - 3 weeks at r.t.) were used.

AFM and TEM imaging

15 For AFM imaging, 10 μ l of C₆₀-polymer complexes were dropped on the positive APS mica. The sample was dried under vacuum for 1 hour and used for AFM scanning. The data are presented as means $\pm$ SEM. For TEM imaging, 10 μ l of C₆₀-polymer complexes were dropped at the top of
20 carbon-coated grid. 1% of uranyl acetate solution was applied as the negative staining. The grid was dried for 3 minutes at r.t. and sent for TEM imaging.

EPR spectroscopy

25 The ability of C₆₀-polymer complexes to scavenge superoxide was measured by EPR spectroscopy, as reported (Rosenbaugh et al. (2010) Biomaterials 31:5218-26). Briefly, superoxide generated by HX-XO or a photosensitizer, FMN, was detected using the CMH spin
30 probe. Samples contained 0.025 mM DF and 0.005 mM DETC buffer (pH 7.4), 0.025 mM CMH, 0.025 mM HX and 10 mU XO (or 0.02 mM FMN instead of HX-XO) and C₆₀-polymer complexes at different concentrations. After complete mixing, each sample was incubated at r.t. for 5 minutes

and 50 μ l of the sample was loaded into a glass capillary, which was then inserted into the capillary holder of the EPR spectrometer. The CMH radical signal was recorded and EPR spectrum amplitude was quantified.

5

Cell culture

CATH.a neuronal cells (ATCC CRL-11179™) were seeded in 6-well plates at 500,000 cells/well or in 24-well plates at 150,000 cells/well (for cytotoxicity studies only) in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 1% penicillin/streptomycin, 4% fetal bovine serum (Invitrogen, Carlsbad, CA) and 8% horse serum (Gibco, Life Tech., Grand Island, NY). As previously described (Yin et al. (2010) Am. J. Physiol. Cell Physiol., 298:C857-65), CATH.a neurons were cultured at 37°C with 95% humidity and 5% CO₂, and differentiated by adding 1 mM of fresh N-6,2'-O-dibutyryl adenosine 3',5'-cyclic-monophosphate (AMP, Sigma-Aldrich Co. St. Louis, MO) every 2 days. The cells were typically cultured for 6-8 days for full expression of Ang II receptors on the cell surface. Madin Darby Canine Kidney (MDCK) cells (ATCC, CCL-34), and human liver carcinoma Hep G2 cells (ATCC, HB-8065) were seeded in 96-well plates at 10,000 cells/well in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum. These cells were cultured at 37°C with 95% humidity and 5% CO₂ for 48 hours.

30 *Cytotoxicity assay*

Cytotoxicity of C₆₀-polymer complexes was determined using a cell counting kit (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) according to the protocol provided by the manufacturer. Briefly, cells

were seeded in 96-well plates (MDCK, Hep G2) or 24-well plates (CATH.a) and cultured as described above until the media was replaced with the fresh one containing different concentrations of C₆₀ complexes. After that
5 the cells were incubated with the complexes for additional 24 hours. The media was then changed for the fresh media containing 10% CCK-8 solution (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). The absorbance at 450
10 nm was recorded in a plate reader (Molecular Devices, Sunnyvale, CA) after 1 hour incubation. The cell viability was calculated as follows: Cell Viability (%) = $(A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{control}} \times A_{\text{blank}}) \times 100\%$. Each treatment was repeated three times and data are presented as means
15 $\pm$ SEM.

Cellular uptake in CATH.a neuronal cells

After 6-8 days culture, in 6-well plates CATH.a neuronal cells were exposed to 100 μ M of C₆₀-polymer
20 complexes in fresh full medium for 24 hours. Then cells were washed twice with 1.0 ml of 0.1M phosphate buffer (pH 7.4) and lysed with 0.5 ml of 1% triton X-100. The UV-vis spectra of cell lysate supernatant were recorded by a spectrometer. The absorbance was normalized by the
25 cellular protein concentration determined by MicroBCA assay (Pierce, Rockford, IL). The spectrum of the control group (without treatment) was subtracted.

Immunofluorescence staining and confocal microscopy

30 The immunofluorescence staining of fullerene has been described (Kato et al. (2010) J. Photochem. Photobiol. B., 98:144-51; Chirico et al. (2007) Exp. Dermatol., 16:429-36). After 6-8 days culture, CATH.a neuronal cells in chamber slides were incubated with 100

μ M C₆₀-polymer complexes in fresh full medium for 24 hours. Cells were washed twice with 1.0 ml of 0.1 M phosphate buffer (pH 7.4), stained with MitoTracker® Red FM for 30 minutes and then fixed with 4% paraformaldehyde for 30 minutes at r.t. After brief blocking, the fixed cells were incubated with fullerene antibody (1:30 dilution) overnight at 4°C and then secondary antibody labeled with Alexa Fluor® 488 (1:200 dilution) for 2 hours at r.t. The slides were sealed with coverslips and images were collected using confocal microscopy.

Immunogold labeling and TEM imaging

The immunogold labeling of fullerene has been described (Chirico et al. (2007) Exp. Dermatol., 16:429-36). Briefly, CATH.a neuronal cells were exposed to 100 μ M of C₆₀-PEtOx complex and cultured as described above. They were then washed twice with 0.1M phosphate buffer and fixed with 2.5% glutaraldehyde in 0.2 M cacodylate at r.t. for 20 minutes. Then cells were postfixed in 1% osmium tetroxide in 0.2 M cacodylate for 1 hour, dehydrated in ethanol solution and embedded in Agar 100 resin. Thin sections were prepared and incubated with fullerene antibody (1:30 dilution) for 1 hour at r.t. and then incubated with 25 nm colloid gold-labeled secondary antibody (1:10 dilution) for 1 hour at r.t. The sections were stained with 1% uranyl acetate and the images were collected using a TEM.

Intracellular EPR spectroscopy

EPR spectroscopy was used to measure the intracellular superoxide levels in CATH.a neuronal cells as described (Rosenbaugh et al. (2010) Biomaterials 31:5218-26). After 6-8 days culture, CATH.a neuronal cells in 24-well plates were incubated with C₆₀-polymer

complexes at different concentrations in fresh full medium for 24 hours. Then, cells were washed twice with 0.5 ml of EPR buffer (pH 7.4), and incubated with 1 ml of EPR buffer containing 200 μ M of CMH as the

5 intracellular spin probe for 1 hour at 37°C. After incubation ca. 0.9 ml of buffer was aspirated from the well, the cells were scraped from the well and re-suspended with the remaining buffer, and 100 μ l of this cell suspension was stimulated with 1 ml of Ang II (100

10 nM) to generate intracellular superoxide. Immediately after full mixing, 50 μ l of cell suspension were loaded into the EPR spectrometer and the generation of superoxide over 10 minutes was recorded. The difference in EPR amplitude between the 1st scan (0 minute) and

15 10th scan (10 minutes) was interpreted as the Ang II-stimulated, superoxide-dependent signal. To control for the difference in cell number between samples, 10 μ l of cell suspension were used for cell counting, and the EPR amplitude of each sample was normalized by cell number.

20 Each treatment was repeated three times and data are presented as means $\pm$ SEM.

Statistical analysis

Statistical analysis was done using one-way ANOVA

25 (LSD multiple comparisons). A minimum p-value of 0.05 was estimated as the significance level.

Results

Characterization of C₆₀-polymer complexes

30 One type of C₆₀-PVP complex (based on PVP 10 kDa) and two types of C₆₀-POx complexes (based on a homopolymer, PEtOx 5 kDa, and a random copolymer, P(EtOx-co-BuOx) 8 kDa) were prepared. The chemical structures of these polymers and the proposed charge-

transfer complex formation between fullerene and POx are shown in Figure 1. The aqueous dispersions of these complexes were optically transparent and revealed the presence of fullerene in the UV-vis spectra (Figure 2).

5 For both C₆₀-PVP and C₆₀-POx complexes, similar spectra and specific absorbance of fullerene around 340 nm were recorded. The net amount of solubilized fullerene increased for both C₆₀-PVP and C₆₀-P(EtOx-co-BuOx) mixtures as the C₆₀: polymer molar ratio increased from 10 1:0.5 to 1:4. However, the loading of fullerene practically did not change and varied from 0.6% to about 1% wt. of dispersed phase. Generally, the maximal loading was observed at 1:1 ratio of the components in the initial mixture (Table 1). Interestingly, the 15 solubilization capacity of the more hydrophobic of the two POx, P(EtOx-co-BuOx), was comparable to PVP having similar molecular weight, while a more hydrophilic POx, PEtOx, incorporated less fullerene than PVP. Furthermore, the most hydrophilic POx, poly(2-methyl-2-oxazoline) (PMeOx), 5 kDa, was incapable of forming 20 stable dispersions with fullerene. These results clearly indicate that the hydrophobicity of POx significantly affects their solubilization capacity for fullerene.

25

Formulations	Particle size (nm) ^a	Polydispersity index (PDI)	Maximal fullerene loading ^d (w/w %)
C ₆₀ -PVP 40kd	220	0.15 -0.20	0.8
C ₆₀ -PVP 10kd	94.9±15.21 ^b	0.227 ± 0.024	1.0
C ₆₀ -PEtOx 5kd	156.5±22.48 ^c	0.217 ± 0.022	0.5
C ₆₀ -P(EtOx-	132.6±5.94 ^c	0.195 ± 0.019	1.0

co-BuOx) 8kd			
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Table 1: Characterization of C₆₀-polymer complexes.

^a Particle size and size distribution do not change upon dilution (1-100 μ M C₆₀), alteration of pH (pH 2-10) or ionic strength (0-1 M NaCl) of the final dispersion.

5 ^b Aggregation is observed after 2 weeks at r.t.

^c For POx, no aggregation is observed upon storage at r.t. for 3 weeks. ^d Weight percent of fullerene in dispersion per dispersed phase.

10 The charge-transfer interaction between PVP and pristine fullerene has been studied (Ungurenasu et al. (2000) J. Med. Chem., 43:3186-8). As shown in Figure 2, an absorption band at about 430 nm is attributed to the formation of C₆₀-PVP charge-transfer complex. This
15 absorption band was also observed at 430nm for C₆₀-POx complex (Figure 2B). It has been reported that there is a red-shift for the carbonyl stretching mode of IR spectrum from 1669 cm⁻¹ for the free PVP to 1662 cm⁻¹ for the complex (Yu-Huei et al. (1999) Fullerene Sci.
20 Techn., 7:807-23). This shift indicates that the amide groups of PVP are involved in the charge-transfer interaction between the polymer and fullerene. In the present study, only a minor red-shift of 2cm⁻¹ was found in the IR spectra of C₆₀-POx complex. In these cases the
25 major peak corresponding to the stretching mode of the carbonyl group (amide I band) of PEtOx shifted from 1629 cm⁻¹ to 1627 cm⁻¹ (Figure 2C).

 The effective size and polydispersity indices (PDI) of the dispersed particles were measured by DLS (Table
30 1). The representative size distributions of C₆₀-PVP and C₆₀-POx particles are shown in Figure 3. C₆₀-POx complexes formed larger particles (ca. 150 nm and 130 nm for C₆₀-PEtOx and C₆₀-P(EtOx-co-BuOx) respectively) compared to C₆₀-PVP complex (ca. 90 nm). For all the
35 polymers used the PDI indices ranged from ca. 0.15 to 0.25. Notably, the particle size and size distribution

did not change upon altering pH or ionic strength, and 100-times dilution of the initial dispersion. Furthermore, they did not aggregate upon storage at r.t. for at least 2 weeks. After this period, aggregation and increase in PDI (to PDI 0.4) was recorded for the C₆₀-PVP complex, while both C₆₀-POx complexes remained stable for an additional week. Despite such remarkable stability in an aqueous dispersion, the fullerene quantitatively recovered and separated from the polymers by extraction into toluene (3:1 v/v).

The representative AFM images are shown in Figure 3. The AFM revealed generally spherical morphology of the particles. In the dry state these particles had mean diameters ranging from 40 nm (C₆₀-PVP) to 96 nm (C₆₀-PEtOx) and mean heights ranging from 5.6 nm (C₆₀-PVP) to 9.7 nm (C₆₀-PEtOx) (Table 2). The spherical morphology was further reinforced by TEM (Fig. 4), which also suggested that particles are rather heterogeneous, consistent with both DLS and AFM. TEM further revealed possible inner-structures within some of the C₆₀-PVP and C₆₀-POx particles exhibited as dense formations with relatively high electron density (the dark regions marked by arrows in Figure 4). This may indicate that these complexes are composed of one (or more) fullerene-rich domain(s) and a hydrated polymer-rich domain.

Formulations	Mean diameter (nm) ^a	Mean height (nm)	mean volume (nm ³)
C ₆₀ -PVP	40.1 ± 1.38	5.6 ± 0.1	9640 ± 356
C ₆₀ -PEtOx	96.0 ± 3.04	9.7 ± 0.5	49,900 ± 3990
C ₆₀ -P(EtOx-co-BuOx)	57.7 ± 2.77	6.1 ± 0.3	28,800 ± 5750

Table 2: AFM characterization of C₆₀-polymer complexes.

Superoxide scavenging of C₆₀-polymer complexes

The radical scavenging capabilities of antioxidants can be quantitatively evaluated by EPR spectroscopy. In 5 this experiment, superoxide was generated using two different sources: (1) an HX + XO catalytic system or (2) a photosensitizer, FMN. Dose-dependent superoxide scavenging was found in all the formulations tested and was independent of the superoxide source (Figures 5A and 10 5B). The representative EPR spectra are also shown in Figures 5C-5G. Treatment with SOD1, which specifically scavenges superoxide, completely abolished the EPR spectrum (Figure 5G); thus, corroborating the fidelity of the assay and showing that the CMH radical detected 15 by EPR spectroscopy is superoxide dependent. The C₆₀-PVP and C₆₀-PEtOx complexes exhibited comparable superoxide scavenging activity while C₆₀-P(EtOx-co-BuOx) was the least effective. The same scavenging effects as shown for C₆₀-polymer complexes were not observed in the 20 presence of the same concentrations of polymers alone.

Cytotoxicity

The possible toxicity of carbon nanomaterials has been a concern for their biological applications (Nel et 25 al. (2006) Science 311:622-7; Mori et al. (2006) Toxicology 225:48-54; Sayes et al. (2004) Nano Lett., 4:1881-7). However, it has been reported that surface functionalization and formulation can efficiently attenuate the toxicity of "naked" fullerene and other 30 carbon nanomaterials such as carbon nanotubes (Sayes et al. (2004) Nano Lett., 4:1881-7; Sayes et al. (2006) Toxicol. Lett., 161:135-42). Therefore cytotoxicity of C₆₀-polymer complexes was comprehensively evaluated using three different cell models: MDCK, Hep G2 and CATH.a

neuronal cells (Figure 6). No cytotoxicity was observed upon incubation of MDCK cells with either C₆₀-PVP or C₆₀-P(EtOx-co-BuOx) complexes for 24 hours (Figure 6A). However, a minor inhibition of cell growth (80% cell viability) was found in the C₆₀-PEtOx treatment groups at the highest concentration, 100 μ M of C₆₀. In Hep G2 cells, C₆₀-PVP and C₆₀-PEtOx exhibited similar toxicity profiles with some toxicity (80% cell viability) at 100 μ M of C₆₀ (Figure 6B). Likewise, C₆₀-PVP and C₆₀-PEtOx were not toxic to CATH.a cells up to 50 μ M, but decreased cell viability to 80% at 100 μ M and were considerably toxic (30% cell viability, $p < 0.01$) at 200 μ M (Figure 6C). Based on these results, C₆₀-PVP and C₆₀-PEtOx complexes were selected for further investigations in the neuron models and the 100 μ M was set as the highest concentration in these studies.

Neuronal cell uptake and distribution

Cellular uptake of C₆₀-PVP and C₆₀-PEtOx into CATH.a neuronal cells was observed, as determined by the UV-vis spectra of cell lysates (Figure 7A). As shown in Figure 7A, after 24 hours of incubation the lysates displayed specific absorption of fullerene in the vicinity of 340 nm. Absorption of fullerene in neurons incubated with C₆₀-PEtOx was considerably greater than that of C₆₀-PVP, which suggests that POx-based formulation enhances binding and/or uptake of C₆₀ in neuronal cells. The intracellular localization of C₆₀ was further examined by immunofluorescence and immunogold labeling (Figures 7B and 7C). Consistent with the UV-vis analysis, CATH.a neuronal cells treated with C₆₀-PEtOx exhibited much greater uptake of C₆₀, than the C₆₀-PVP-treated neurons (Figure 7B). No obvious co-localization of fullerene and Mitotracker® Red was observed, suggesting that

fullerene did not accumulate in the mitochondria after internalization into the cells. The immunogold labeling showed that fullerene was mainly distributed in the cytoplasm and nuclear region (Figure 7C). No fullerene was found in the mitochondria using this method.

Intracellular superoxide scavenging

EPR spectroscopy was used to measure intracellular levels of superoxide in Ang II-stimulated CATH.a neuronal cells. As shown in Figure 8, a significant increase in EPR amplitude, indicating an increase in intracellular superoxide, was detected after 10 minutes of Ang II stimulation (control vs. control + Ang II, $p < 0.01$). Significant superoxide scavenging was observed upon exposure of cells to C₆₀-PEtOx complex at 100 μ M ($p < 0.01$) and 50 μ M ($p < 0.05$). Dose-dependent scavenging of intracellular superoxide was clearly evident in this case. Furthermore, 100 μ M C₆₀-PEtOx significantly reduced intracellular superoxide levels in CATH.a neuronal cells incubated with another superoxide generator, menadione (Figure 8A). In contrast to C₆₀-PEtOx, C₆₀-PVP did not decrease levels of intracellular superoxide (Figure 8B), which may be due to the limited cellular uptake of this formulation (Figure 7).

25

A number of publications and patent documents are cited throughout the foregoing specification in order to describe the state of the art to which this invention pertains. The entire disclosure of each of these citations is incorporated by reference herein.

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications
5 may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

What is claimed is:

1. A composition comprising at least one fullerene and
at least one oxazoline polymer, wherein said fullerene
5 is complexed with said oxazoline polymer.
2. The composition of claim 1 further comprising at
least one pharmaceutically acceptable carrier or
cosmetically acceptable carrier.
- 10 3. The composition of claim 1, wherein said fullerene is
pristine.
4. The composition of claim 1, wherein the fullerene is
15 C₆₀.
5. The composition of claim 1, wherein said oxazoline
polymer is a homopolymer.
- 20 6. The composition of claim 1, wherein said oxazoline
polymer is a random or block copolymer.
7. The composition of claim 1, wherein said oxazoline
polymer comprises 2-ethyl-2-oxazoline.
- 25 8. The composition of claim 7, wherein said oxazoline
polymer is a homopolymer of 2-ethyl-2-oxazoline.
9. The composition of claim 7, wherein said oxazoline
30 polymer is a copolymer comprising 2-ethyl-2-oxazoline.
10. The composition of claim 1, wherein the complexes
comprise about 0.1% to about 5% fullerene.

11. The composition of claim 1 further comprising a
cosmetically acceptable agent selected from the group
consisting of sunscreens, anti-aging agents,
chemotherapeutic agents, steroids, anti-inflammatory
5 agents, exfoliating agents, lipid molecules, amino
acids, vitamins, anti-irritation agents, anti-cellulite
agents, anti-fungal agents, anti-bacterial agents,
moisturizing agents, saccharides, emollients,
antioxidants, anti-swelling agents, anesthetics, anti-
10 viral agents, cosmetic agents, coloring agents, and
fragrances.

12. A method for treating or inhibiting an oxidative
stress associated disease or disorder in a subject in
15 need thereof, said method comprising administering to
said subject at least one composition of claim 1.

13. The method of claim 12, wherein said oxidative
stress associated disease or disorder is selected from
20 the group consisting of hypertension, heart failure,
arthritis, cancer, neurodegenerative disorders, and
cardiovascular diseases.

14. The method of claim 13, wherein said
25 neurodegenerative disease is selected from the group
consisting of Alzheimer's disease, Huntington's disease,
Parkinson's disease, Lewy Body disease, amyotrophic
lateral sclerosis, and prion disease.

30

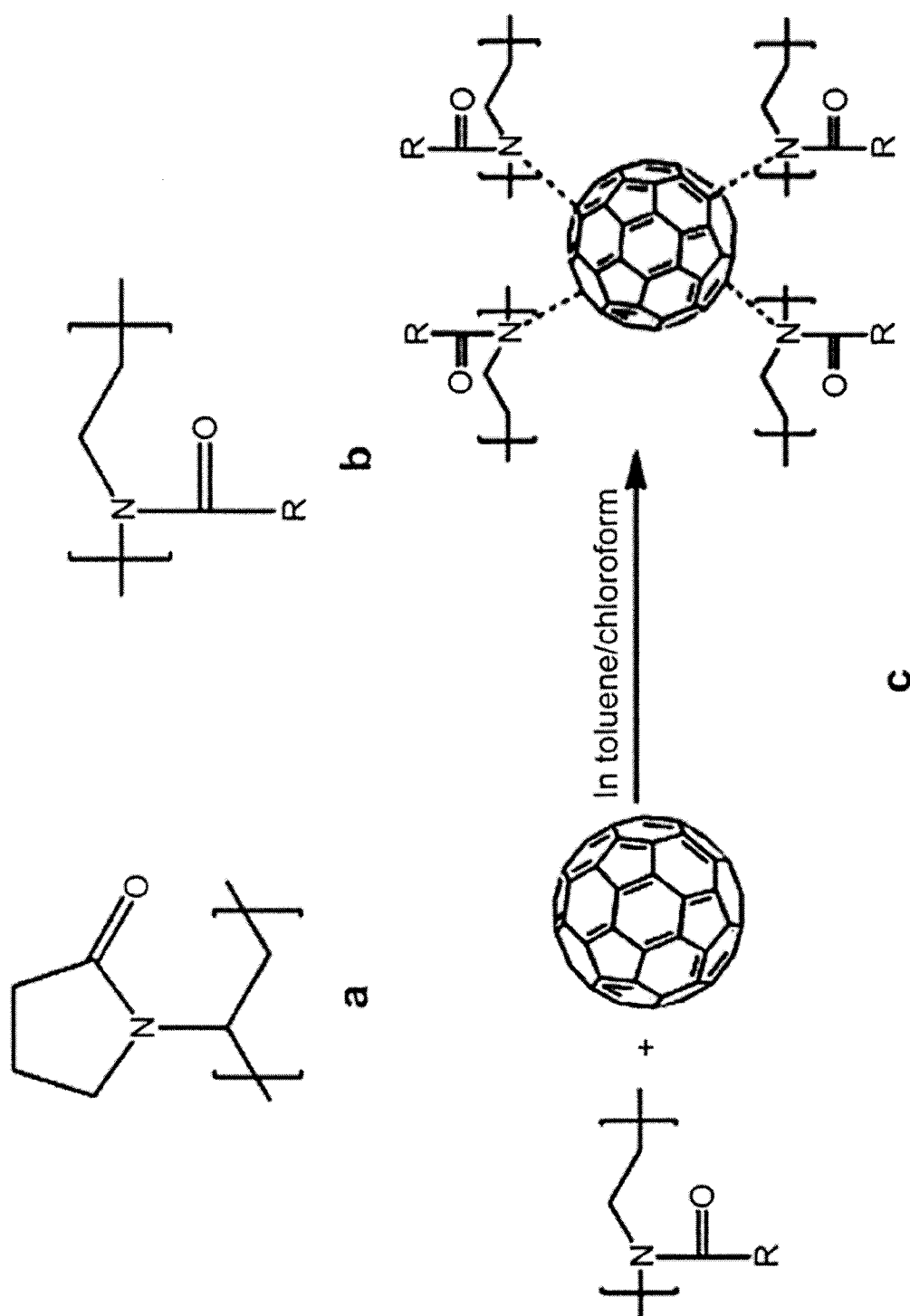


Figure 1

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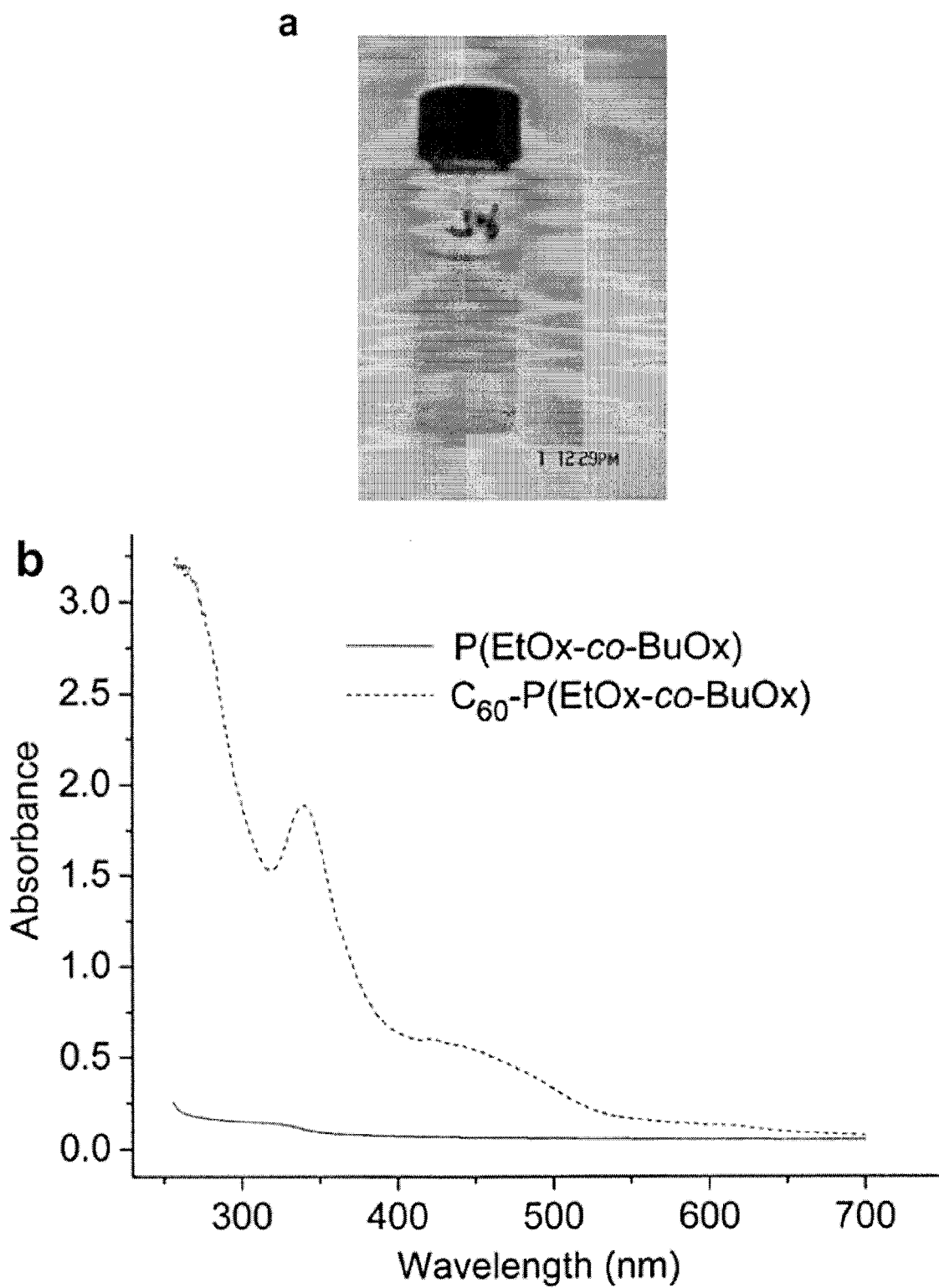


Figure 2

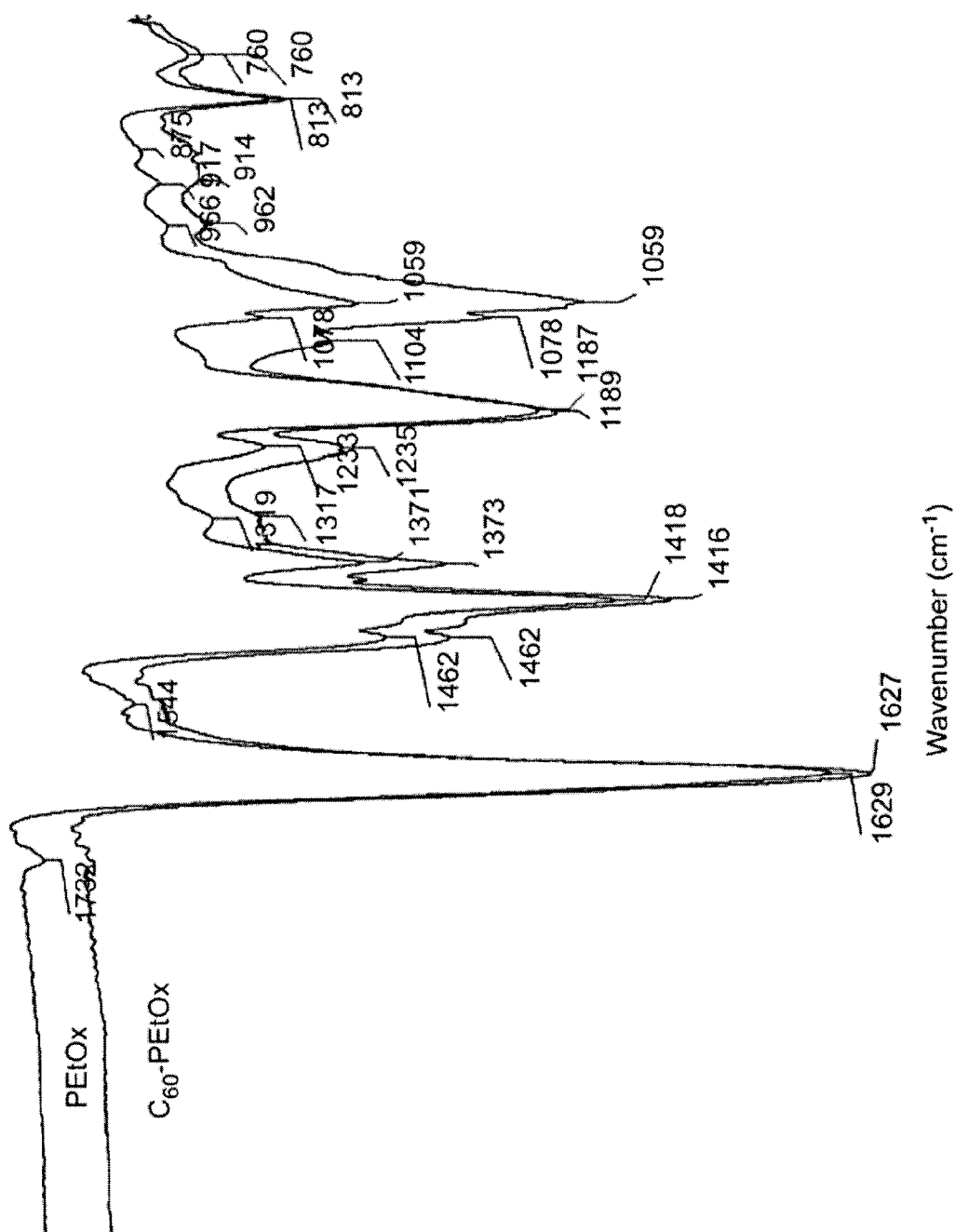


Figure 2C

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Figure 2D

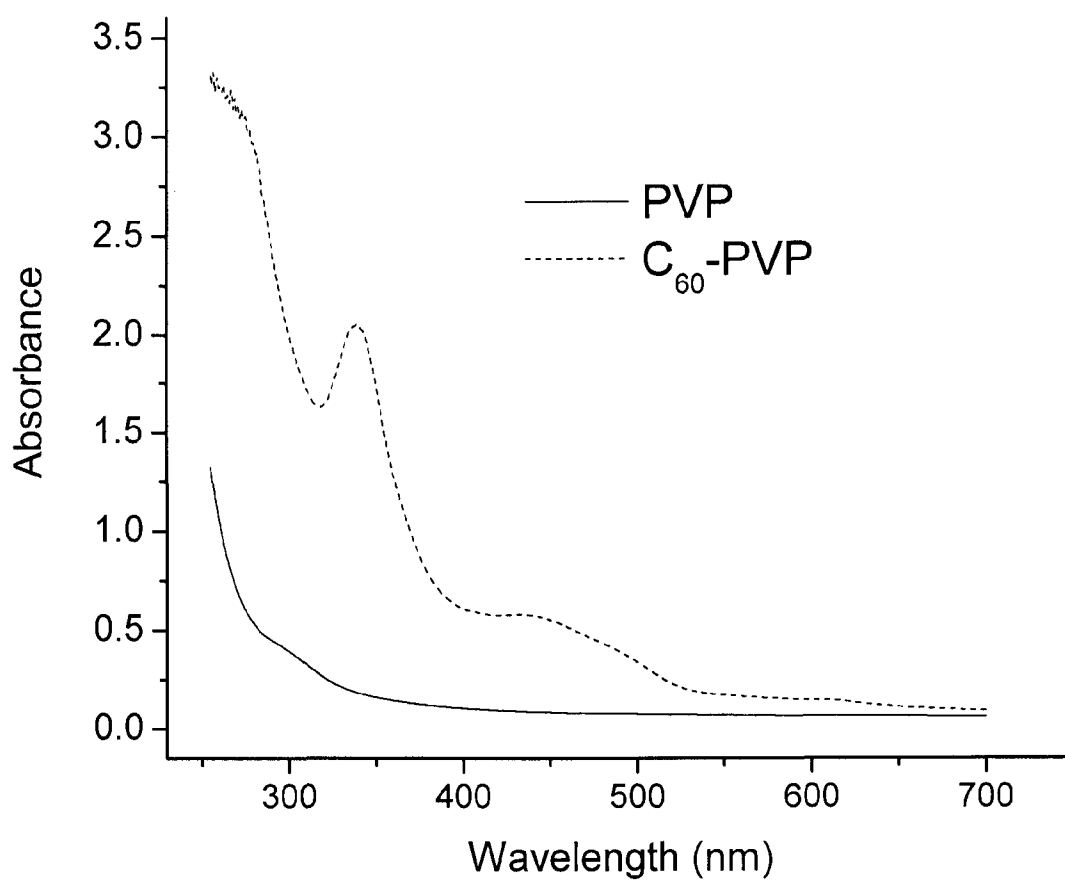


Figure 2E

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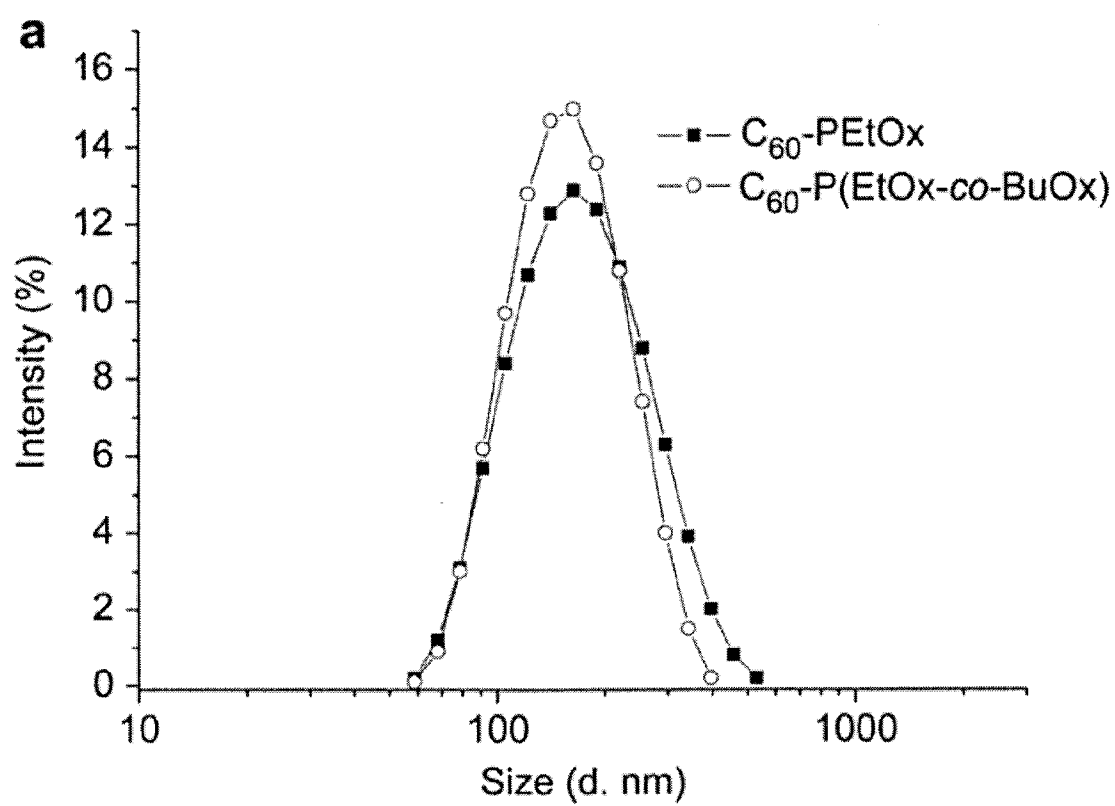


Figure 3A

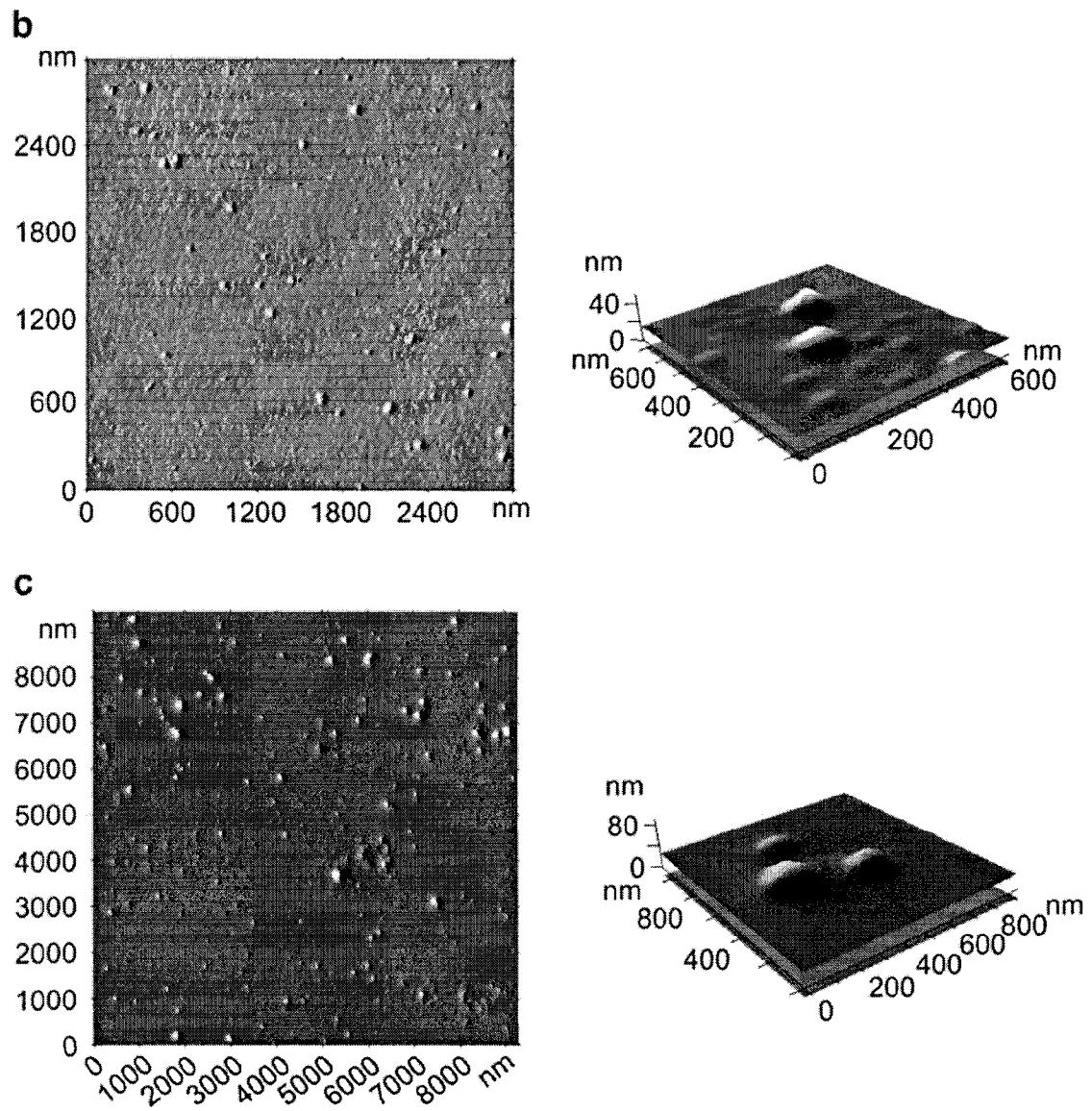


Figure 3

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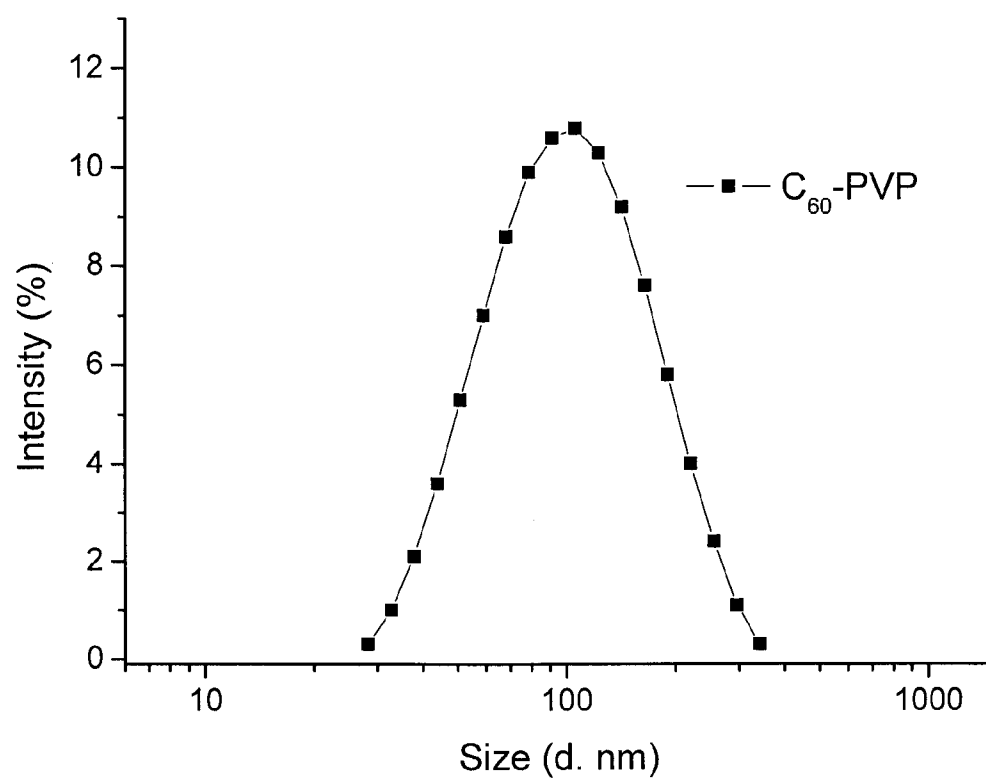


Figure 3D

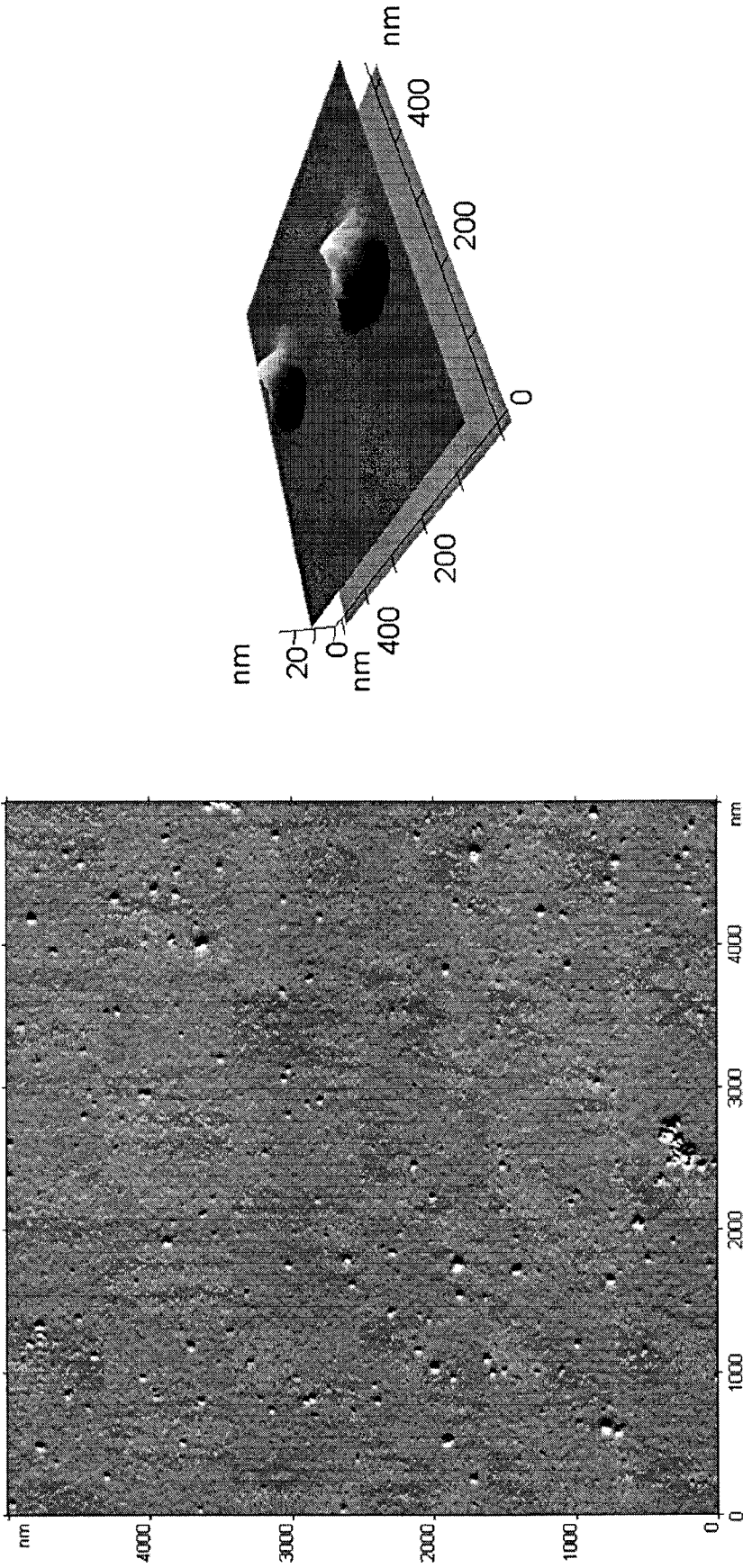


Figure 3E

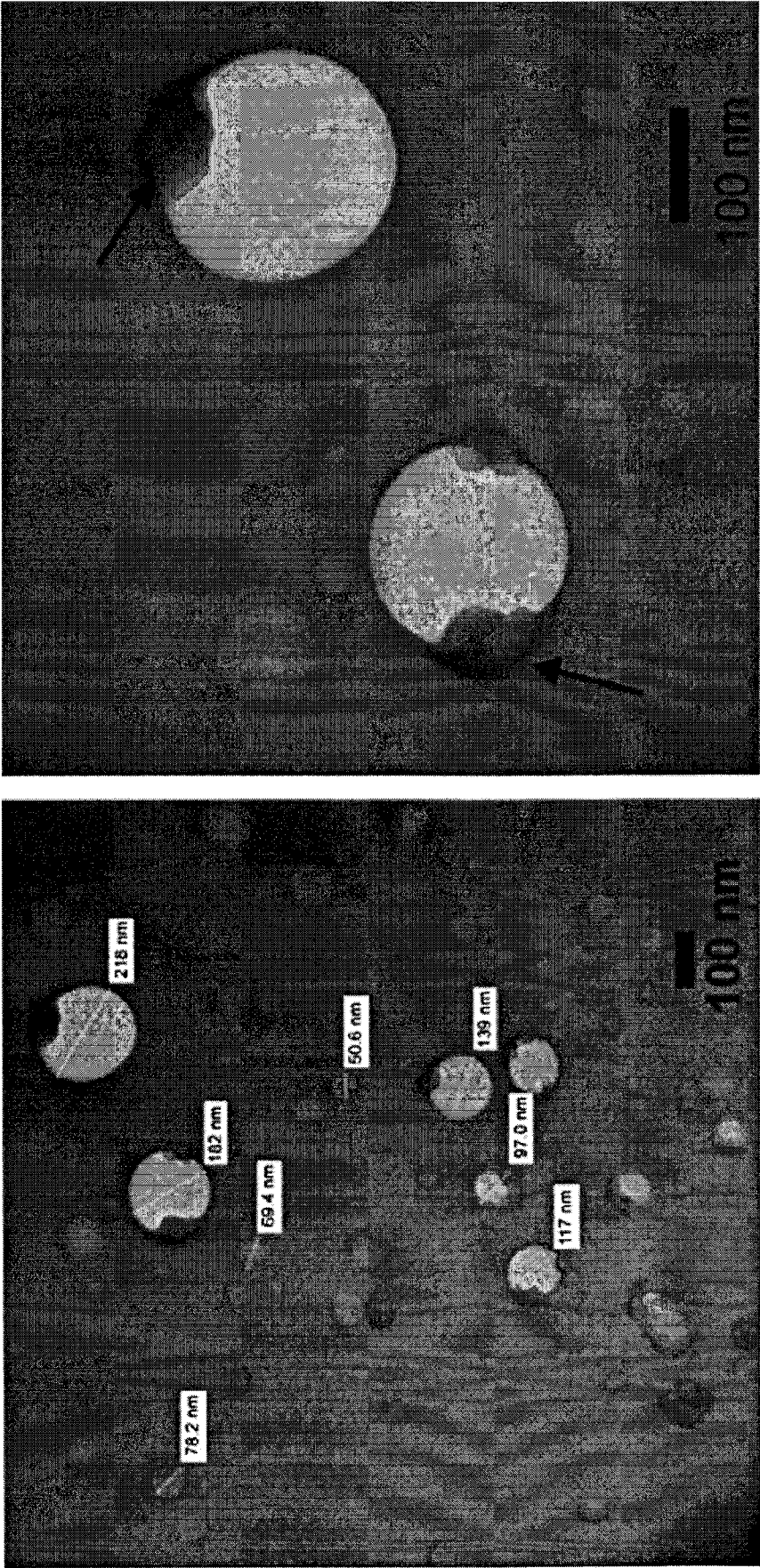


Figure 4A

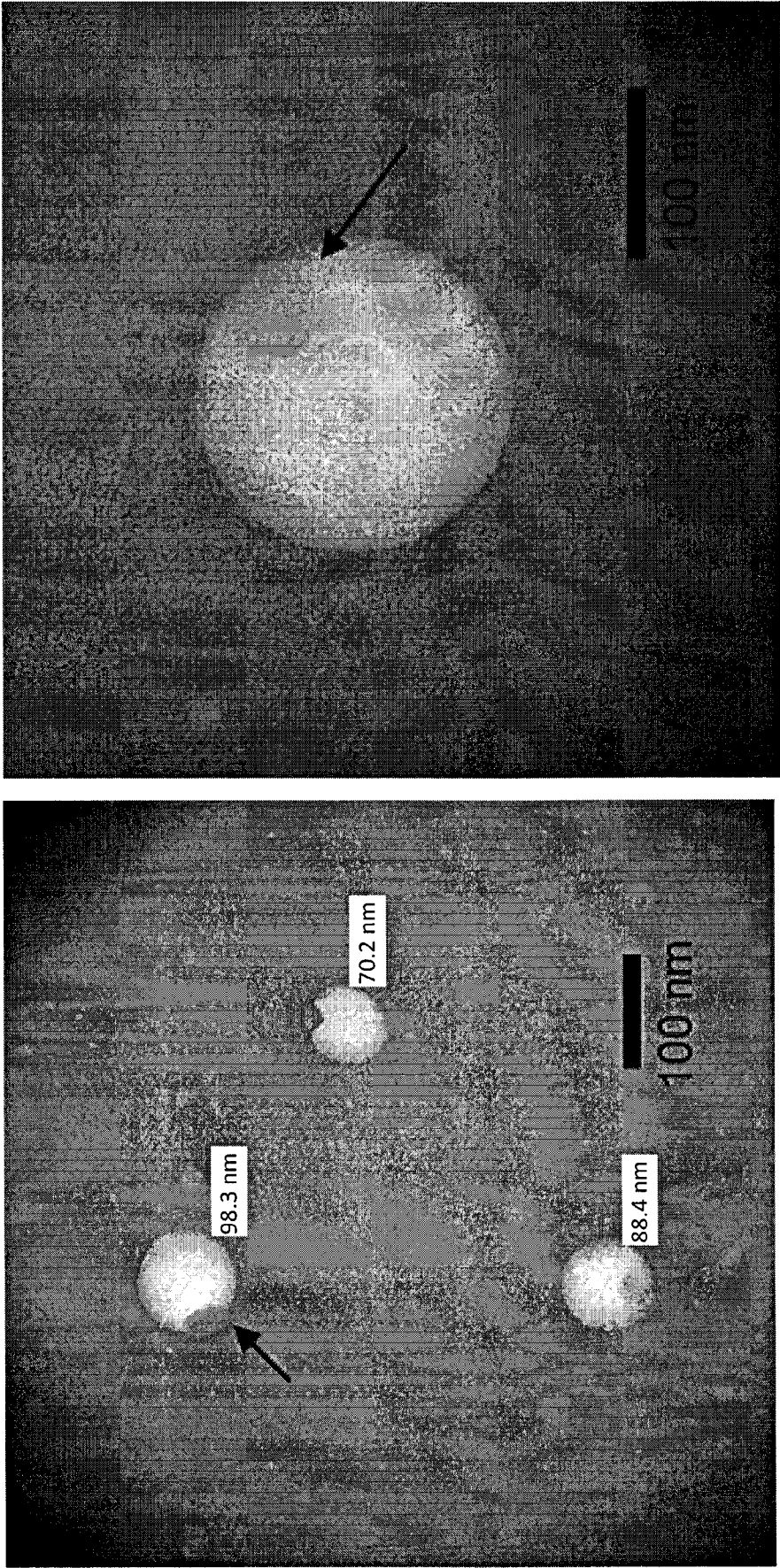


Figure 4B

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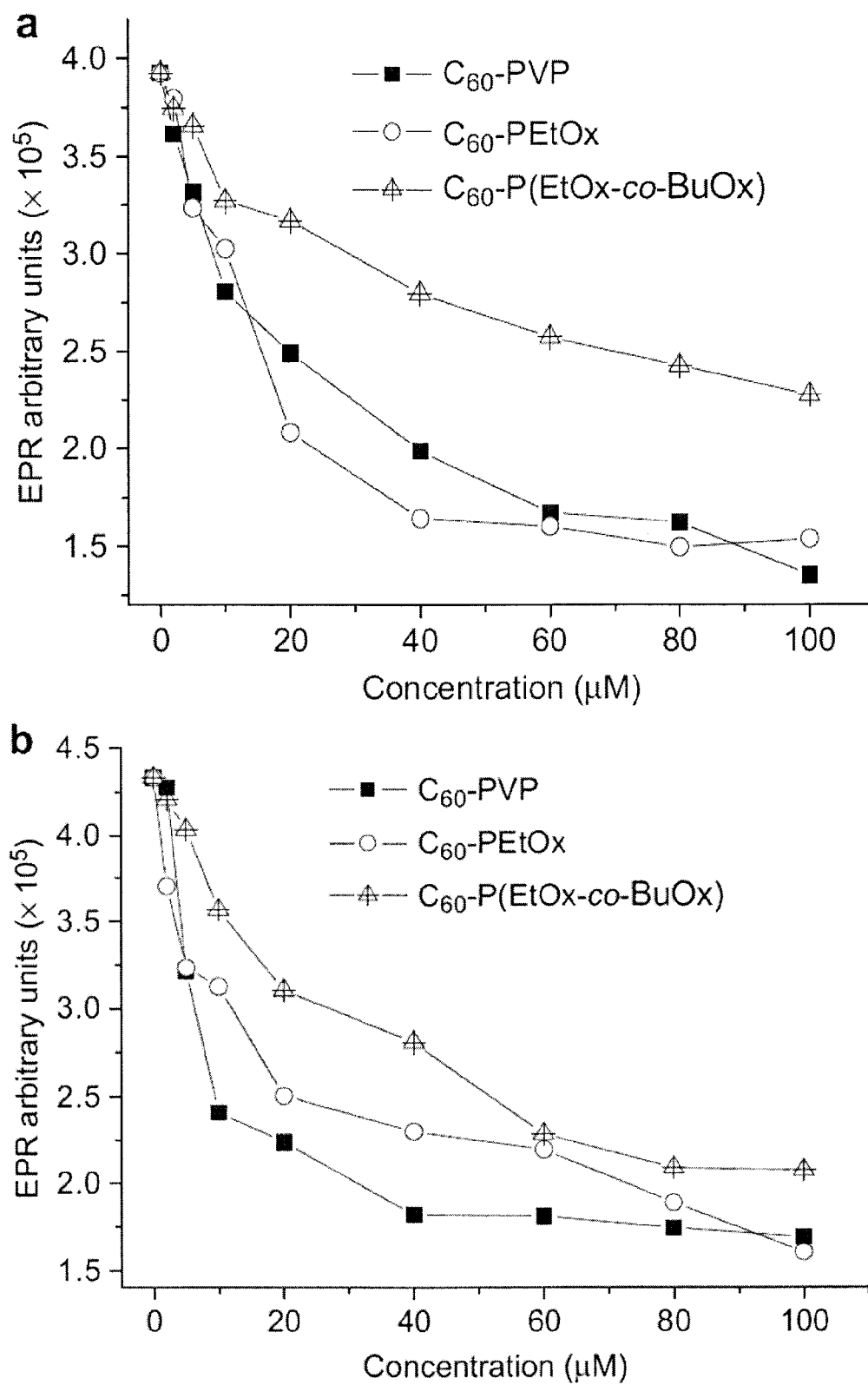


Figure 5

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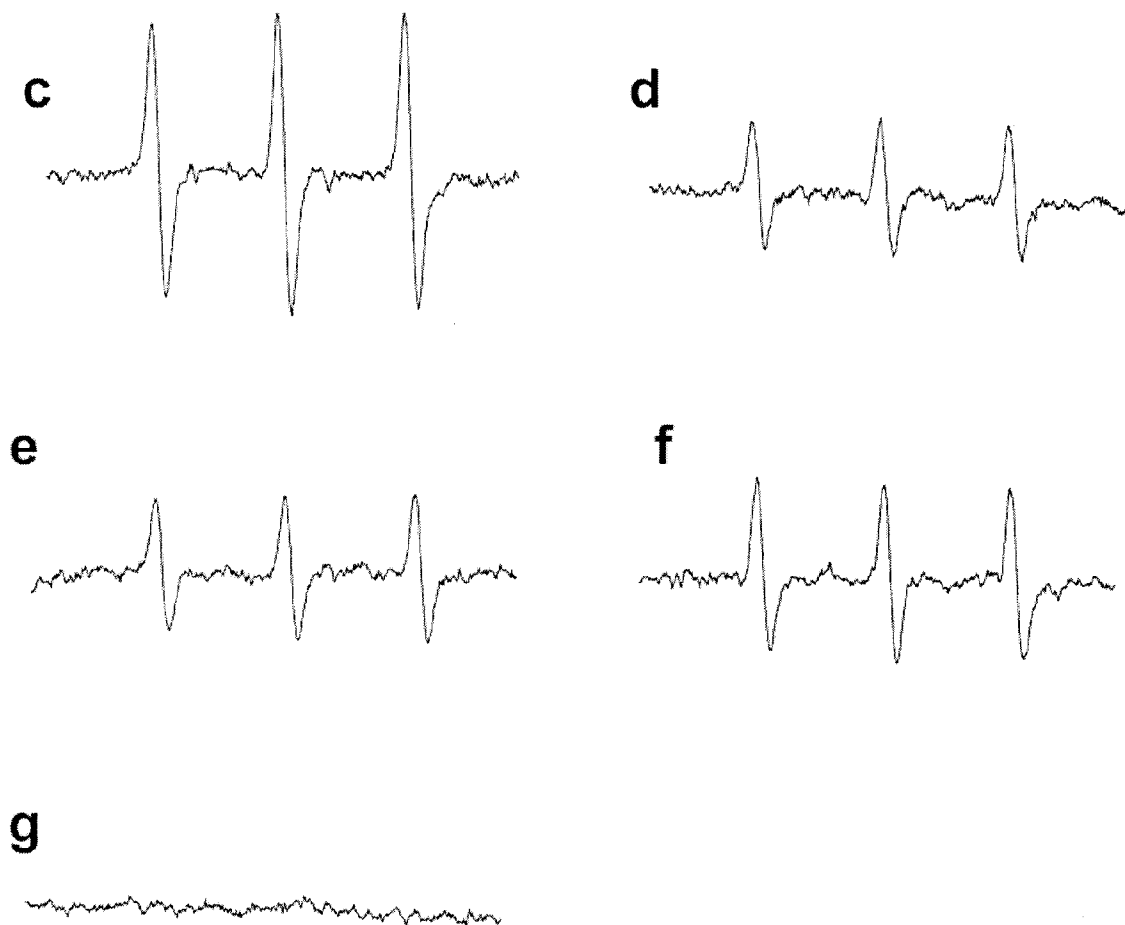


Figure 5

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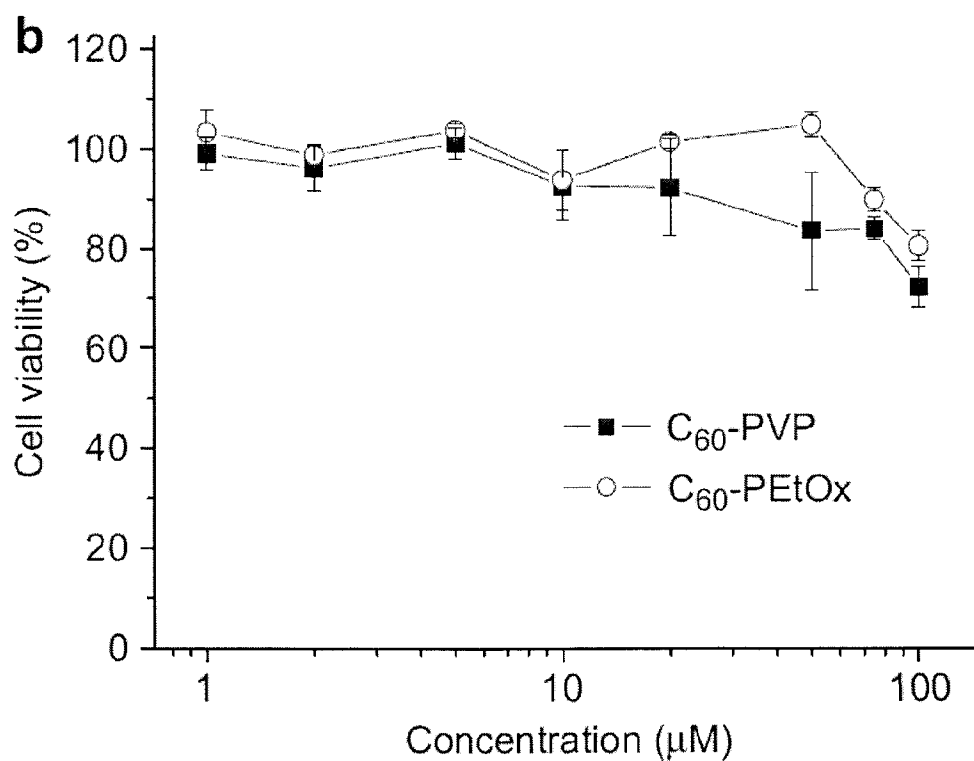
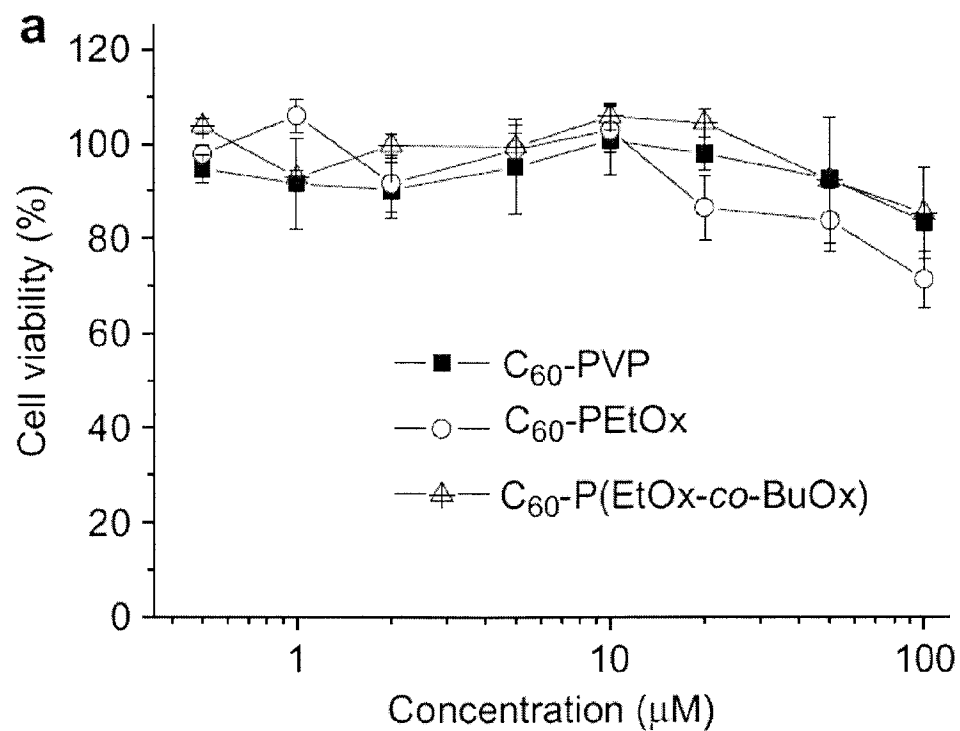


Figure 6

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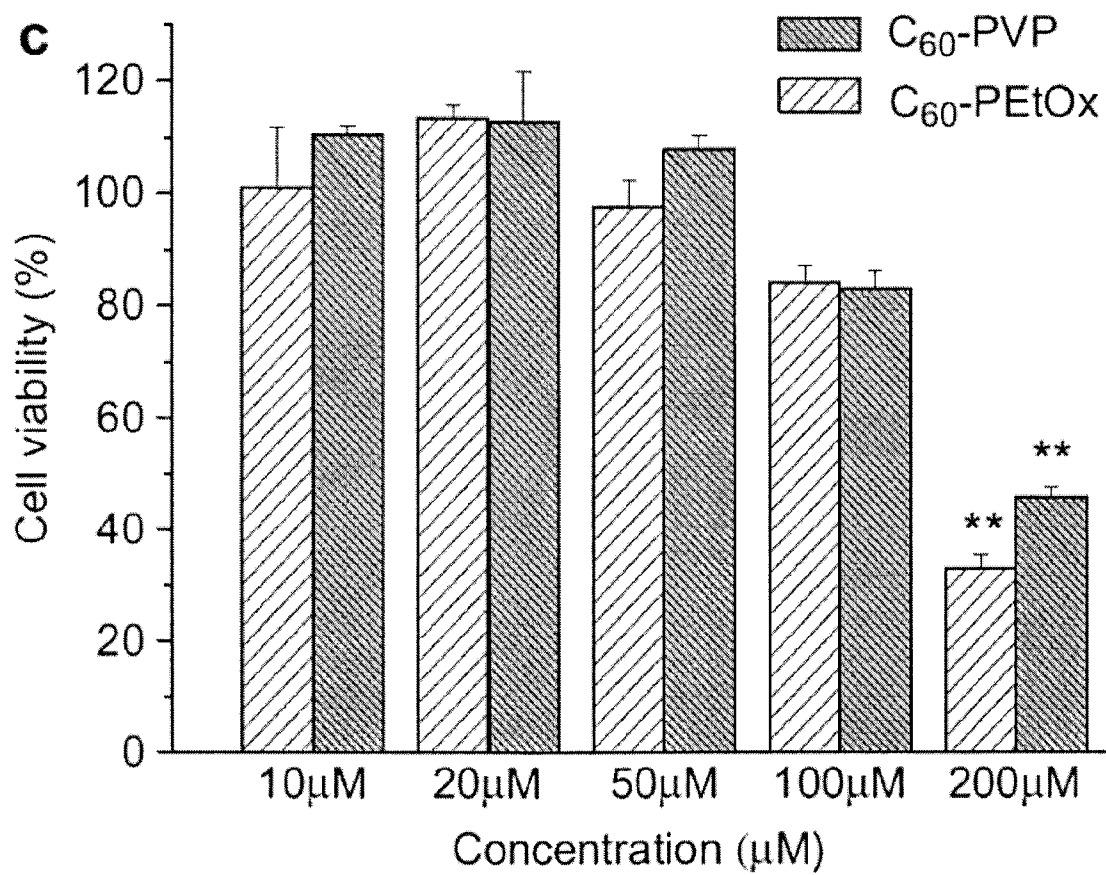


Figure 6C

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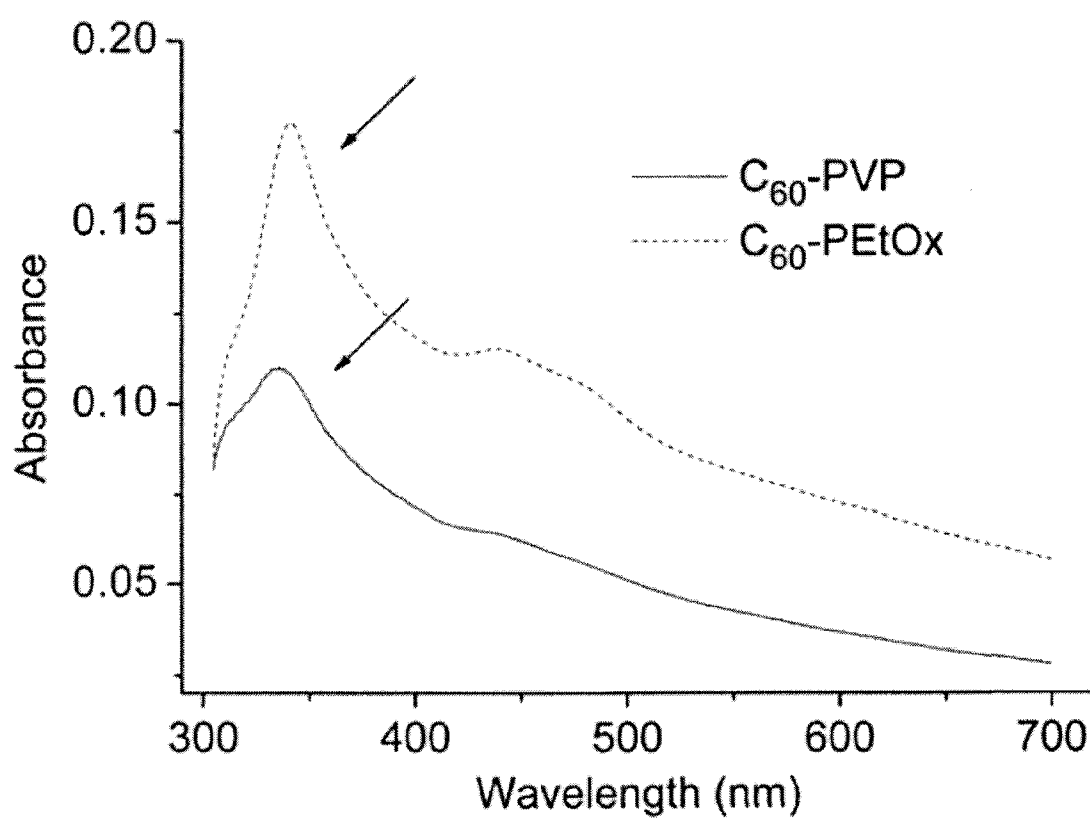


Figure 7A

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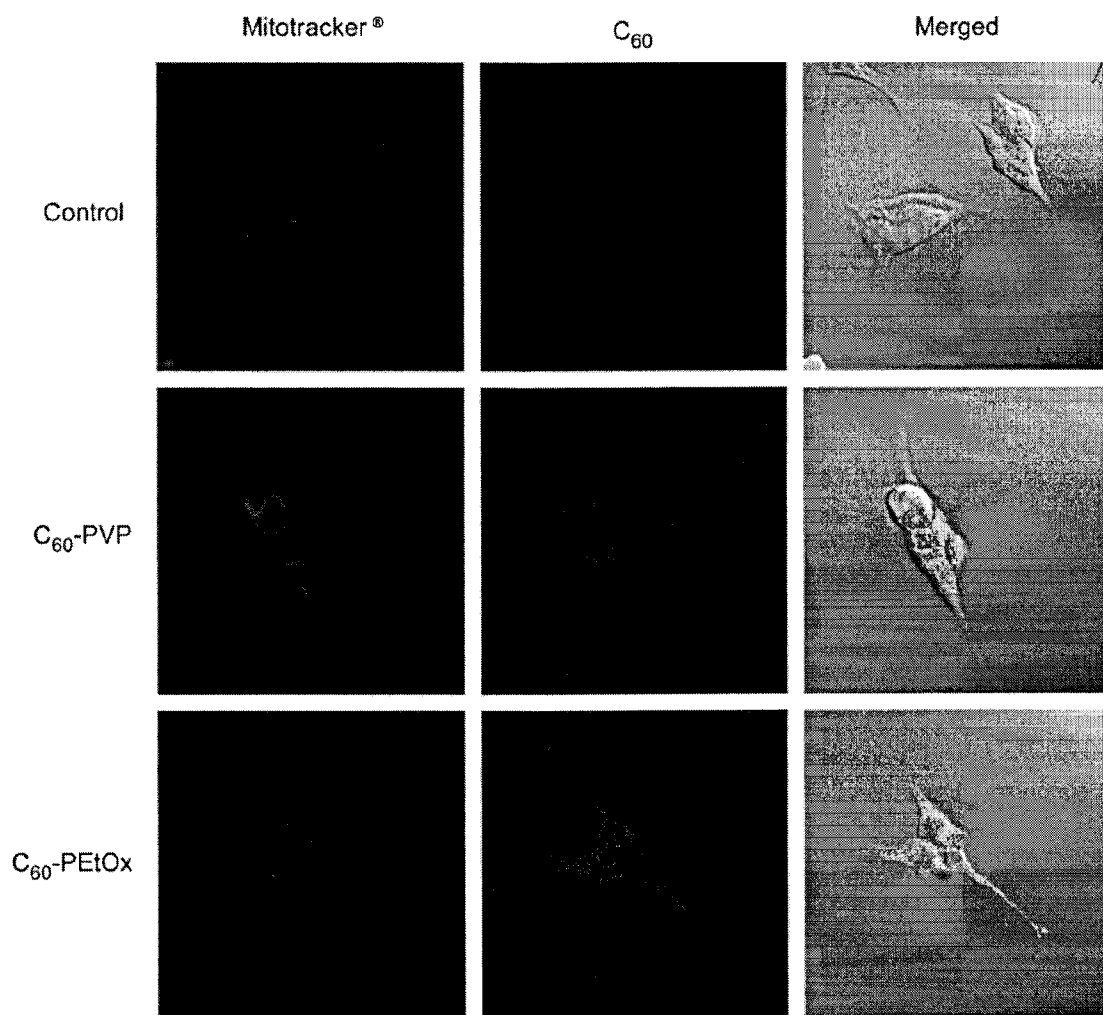


Figure 7B

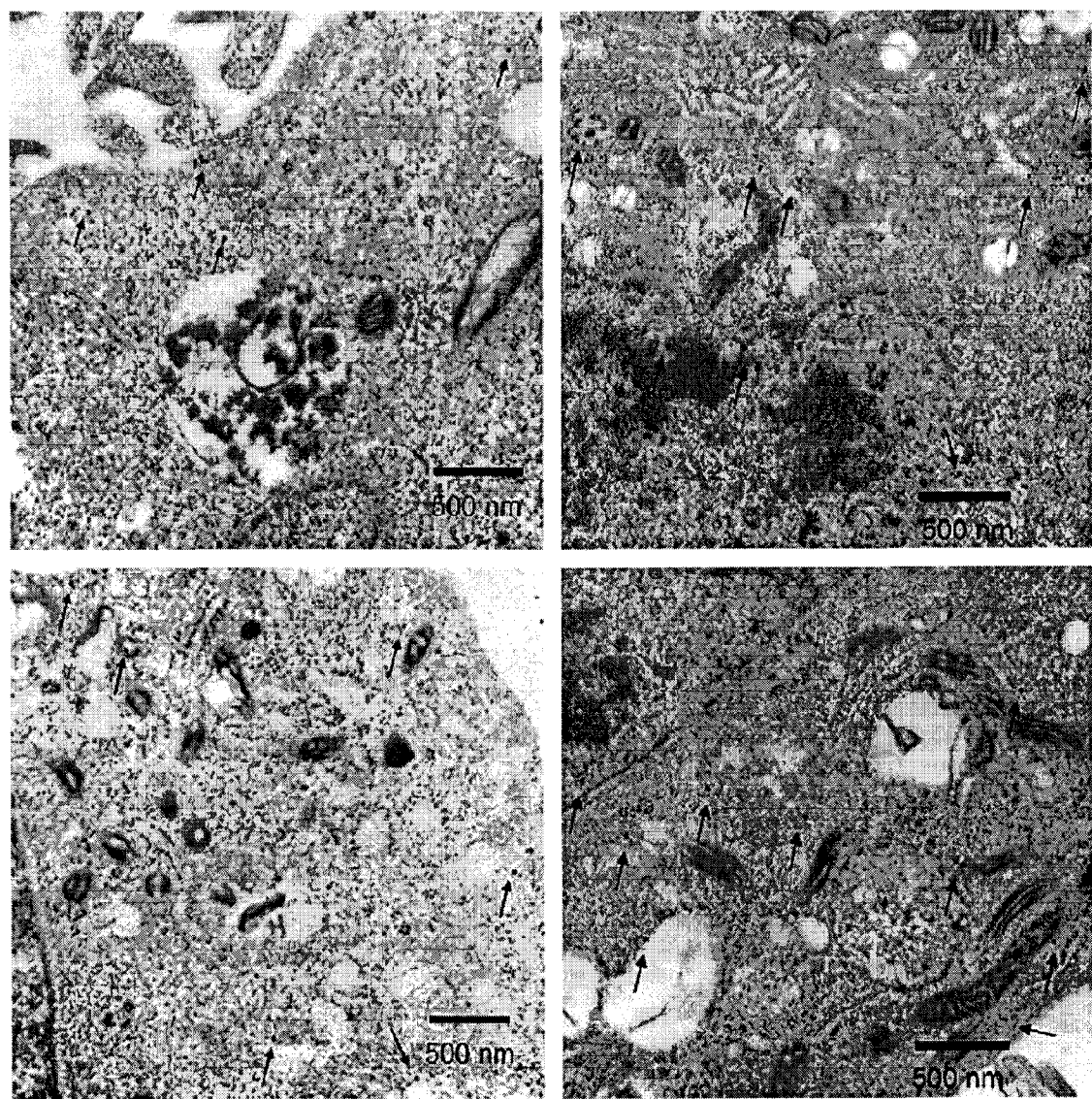
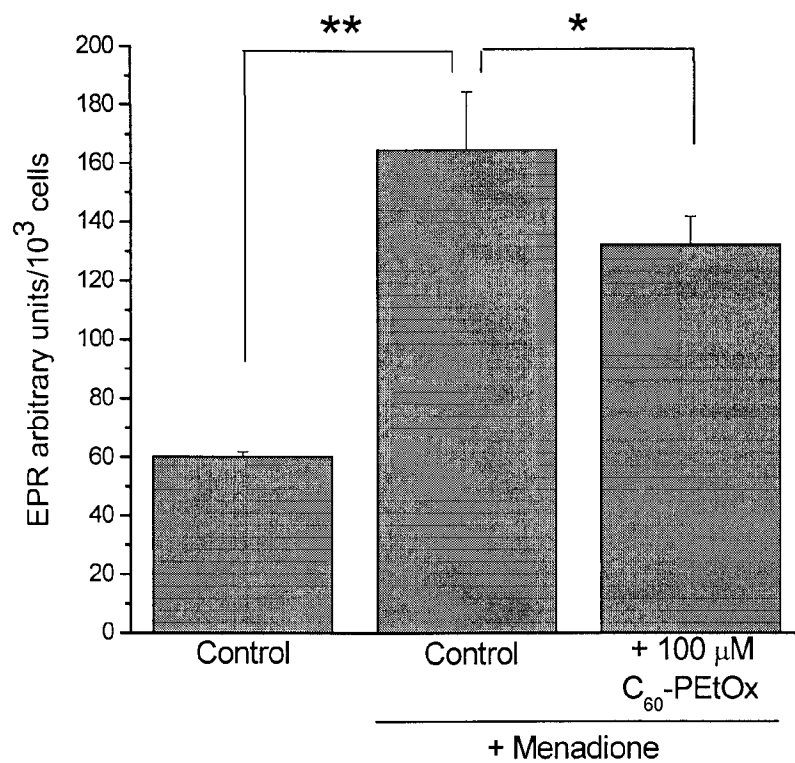
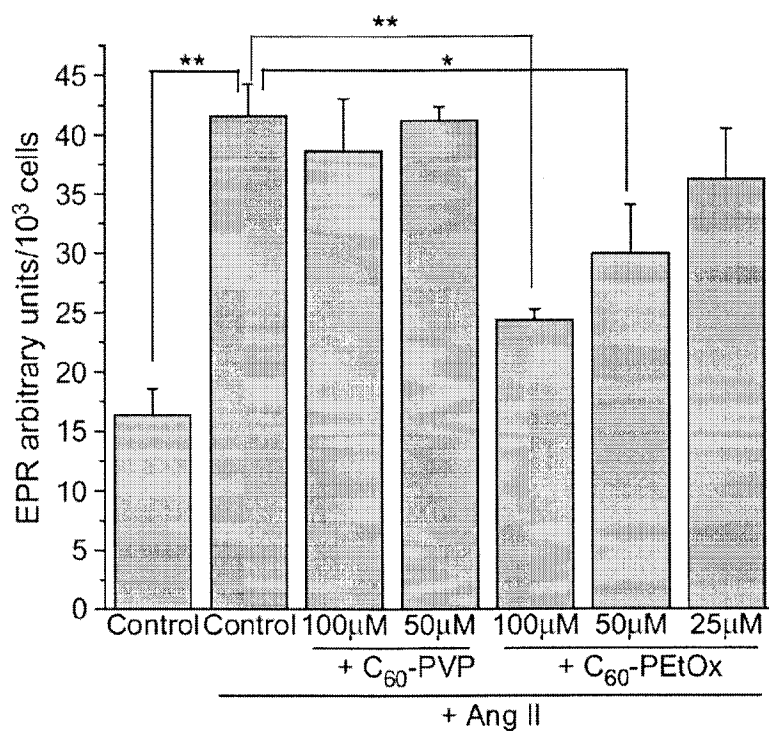


Figure 7C

18/18

**Figure 8A****Figure 8B**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/21234

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C01B 31/02; C08G 73/06 (2012.01)

USPC - 423/445B; 528/403, 423

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 423/445B; 528/403, 423

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWEST(USPT,PGPB,EPAB,JPAB); Google; Google Patents

Search Terms: Fullerene, complexed, oxazoline, copolymer, pharmaceutical, cosmetic, buckyball

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2010/0069536 A1 (Sau) 18 March 2010 (18.03.2010) para [0044]; [0060]; [0081]	1-3, 5-11 ----- 4, 12-14
Y	Tokuyama et al. 'Photoinduced Biochemical Activity of Fullerene Carboxylic Acid' Journal of American Chemical Society 1993, vol 115, No. 17, pg 7918-7919	4
Y	US 2009/0012033 A1 (DeMattei et al.) 08 January 2009 (08.01.2009) para [0136]; [0138]; [0195]	12-14
A	US 2007/0298006 A1 (Tomalia et al.) 27 December 2007 (27.12.2007) entire document	1-14
A	US 7,186,274 B2 (Vic et al.) 06 March 2007 (06.03.2007) entire document	1-14
A	US 2010/0028559 A1 (Yan et al.) 04 February 2010 (04.02.2010) entire document	1-14

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 April 2012 (13.04.2012)

Date of mailing of the international search report

08 MAY 2012

Name and mailing address of the ISA/US

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