
Young Investigator's Award, The Society for Biomaterials 2009 Annual Meeting, San Antonio, Texas, April 22–25, 2009

Chemiluminescent PEG-PCL micelles for imaging hydrogen peroxide

Madhuri Dasari, Dongwon Lee, Venkata Reddy Erigala, Niren Murthy

The Wallace H. Coulter Department of Biomedical Engineering and Parker H. Petit Institute of Bioengineering and Bioscience, Georgia Institute of Technology, 315 Ferst Drive, Atlanta, Georgia 30332

Received 24 December 2008; accepted 7 January 2009

Published online 27 March 2009 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.32430

Abstract: Hydrogen peroxide is one of the fundamental molecules of biology, regulating key cell signaling pathways and the development of numerous inflammatory diseases. There is therefore great interest in developing contrast agents that can detect hydrogen peroxide *in vitro* and *in vivo*. In this report, we present a new contrast agent for imaging hydrogen peroxide, termed the chemiluminescent poly(ethylene glycol)-*b*-poly(ϵ -caprolactone) (PEG-PCL) micelles (CPMs), which can detect hydrogen peroxide at nanomolar concentrations and chemiluminesce in the near IR range (676 nm) in the presence of hydrogen peroxide. The CPMs are composed of a PEG-PCL scaffold and have fluorescent dyes and peroxalate esters in

their hydrophobic PCL core. The CPMs image hydrogen peroxide by undergoing a three-component chemiluminescent reaction involving a peroxalate ester, a fluorescent dye, and hydrogen peroxide. The CPMs also have a stealth PEG corona to enhance their circulation half life. The CPMs should find numerous applications for imaging hydrogen peroxide because of their nanomolar sensitivity, small size, and stealth pegylated surface. © 2009 Wiley Periodicals, Inc. *J Biomed Mater Res* 89A: 561–566, 2009

Key words: hydrogen peroxide; chemiluminescence; PEG-PCL; micelles; imaging

INTRODUCTION

Hydrogen peroxide is a small molecule metabolite that regulates key cell signaling pathways and the development of numerous life-threatening diseases.^{1,2} Despite its significance, very little is known about the function of hydrogen peroxide *in vivo* because of a lack of imaging technologies, and there

is therefore great interest in developing new contrast agents that can image hydrogen peroxide.^{3–5} Contrast agents based on peroxalate chemiluminescence (PO-CL)^{6–8} have great potential for imaging hydrogen peroxide because of their excellent sensitivity and specificity. However, using peroxalate chemiluminescence for the *in vivo* imaging of hydrogen peroxide is challenging because it requires a methodology for sequestering peroxalate esters and fluorescent dyes within a close proximity. We previously demonstrated that nanoparticles composed of polymeric peroxalate esters and fluorescent dyes, termed the peroxalate nanoparticles, were capable of imaging hydrogen peroxide *in vivo* via peroxalate chemiluminescence, in the peritoneal cavity of mice after an intraperitoneal injection.⁹ Although the peroxalate nanoparticles have great promise for *in vivo* imaging of hydrogen peroxide, their large size will prevent their extravasation into the tissue from the vasculature, limiting their *in vivo* applications. Furthermore, the hydrophobic surface of the peroxalate nanoparticles will also cause them to be primarily phagocytosed by macrophages. Therefore, new peroxalate-based contrast

Correspondence to: N. Murthy; e-mail: niren.murthy@bme.gatech.edu

Contract grant sponsor: Georgia Tech/Emory Center for the Engineering of Living Tissues; contract grant number: NSF-EEC-9731643

Contract grant sponsor: Career Award; contract grant number: NSF-BES-0546962

Contract grant sponsor: NIH; contract grant numbers: UO1 HL80711-01, R21 EB006418

Contract grant sponsor: J&J/GT Health Care Innovation Seed Grant Proposal

agents for *in vivo* imaging of hydrogen peroxide are greatly needed.

In this report, we present a new peroxalate-based contrast agent, termed the chemiluminescent PEG-PCL micelles (CPMs), which are 50 nm in size and can detect nanomolar concentrations of hydrogen peroxide. The CPMs are composed of a PEG-PCL scaffold, and are generated by self-assembling PEG-PCL into micelles, in the presence of fluorescent dyes and peroxalate esters. PEG-PCL was chosen as the scaffold of the CPMs because it is a biodegradable and biocompatible amphiphilic polymer that has been extensively used for drug delivery.^{10–12} PEG-PCL micelles also have a hydrophilic PEG corona, which should enhance the circulation half-life of the CPMs and their extravasation into tissue.^{13,14} Finally, the hydrophobic and crystalline PCL core of the CPMs should stabilize encapsulated peroxalate esters from water hydrolysis and enhance their stability in physiologic environments.

MATERIALS AND METHODS

MPEG ($M_n = 2000$), ϵ -caprolactone, stannous octoate, 6,13-pentacenequinone, 4-ethynyl- α,α,α -trifluorotoluene, tin(II) chloride, and *n*-butyllithium were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. Diphenyl oxalate was purchased from TCI America (Portland, OR). Tetrahydrofuran and toluene were dried using standard methods.¹⁵ Fetal Bovine Serum (FBS) was purchased from the ATCC (Manassas, VA). DMEM (Dulbecco's Modified Essential Medium) and Mediatech, Inc. (Manassas, VA). ¹H NMR spectra were recorded using a Varian 400 spectrometer in CDCl₃ (7.26 ppm). Gel permeation chromatography measurements were made using a Shimadzu SCL-10A.

Synthesis of PEG-PCL copolymer (1)

The copolymer **1** was synthesized using a modified literature procedure.¹⁶ MPEG ($M_n = 2000$ g/mol) (3g, 4 mmol) was dissolved in 80 mL of toluene and was azeotropically distilled to a 50 mL final volume to remove water. The MPEG solution was allowed to cool to room temperature and ϵ -caprolactone (9.3 mL, 84 mmol) was added under a nitrogen atmosphere, followed by the addition of 2.4 mL of a 0.1M solution of stannous octoate in dry toluene. The mixture was heated to 130°C and allowed to reflux for 24 h. The reaction mixture was then cooled to room temperature and poured into a mixture of *n*-hexane and ethyl ether (v/v = 4/1), to precipitate the polymer. The resulting polymer was separated from the supernatant by filtration. The obtained polymer was redissolved in CH₂Cl₂ and filtered. The filtrate was concentrated with a rotary evaporator and dried *in vacuo* to yield **1** as a colorless solid.

¹H NMR (CDCl₃, 400 MHz): δ 4.05 (t, $J = 6.8$ Hz, 2H), 3.63 (s, 4H), 3.37 (s, 3H), 2.30 (t, $J = 7.2$ Hz, 2H), 1.64 (m,

4H), 1.37 (m, 2H). The polydispersity index (PDI = 2) of **1** was determined by GPC, based on polystyrene standards obtained from Polymer Laboratories (1060 Mw, 10.3 min; 2970 Mw, 9.4 min; 10 800 Mw, 8.2 min).

Synthesis of 6, 13-bis(4-trifluoromethylphenylethynyl)pentacene (2)

The compound **2** was synthesized according to a modified literature method.¹⁷ To a solution of 4-ethynyl trifluorotoluene (0.85 mL, 5.19 mmol) in 10 mL of anhydrous THF was added *n*-BuLi (3.24 mL, 5.19 mmol, 1.6 M in hexane) at -78°C. The reaction mixture was allowed to warm to room temperature and stirred for 30 mins. 6, 13-Pentacenequinone (0.4 g, 1.29 mmol) was then added and the resulting reaction mixture was stirred for 2 h until all the solids dissolved, generating a dark brown solution. A mixture of SnCl₂ (1.17 g, 5.19 mmol) in 10% HCl (10 mL) was added carefully and heated for an additional 30 min, allowed to cool to room temperature and poured rapidly into 160 mL of methanol, generating a deep blue precipitate, which was filtered, washed with methanol, and dried. The recovered solid was then dissolved in toluene (40 mL) and passed through a short silica gel column. The product was obtained by removing the solvent under *vacuo* and further purified by repeated (five times) recrystallizations from hexane/toluene (v/v, 1:2) (15 mL each) to give pure **2** as a deep blue solid.

¹H NMR (CDCl₃, 400 MHz): δ 9.29 (s, 4H), 8.07 (dd, $J_1 = 6.4$ Hz, $J_2 = 3.2$ Hz, 4H), 8.16 (d, $J = 8.4$ Hz, 4H), 7.94 (d, $J = 8.4$ Hz, 4H), 7.61 (dd, $J_1 = 6.4$ Hz, $J_2 = 3.2$ Hz, 4H).

Formulation of the CPMs

The CPMs were prepared using a solvent displacement formulation protocol.¹⁸ The copolymer **1** (50 mg), diphenyl oxalate (5 mg), and the fluorescent dye (either rubrene or pentacene) (1mg) were dissolved in 2 mL of acetone. The resulting solution was then added dropwise to an excess of deionized water (20 mL) to form micelles. The organic solvent was removed under vacuum while maintaining the temperature at 0°C and the resulting micelle solution was used for further experiments. The hydrodynamic diameter of the CPMs was determined by dynamic light scattering (Particle Size Analyzer 96 Plus, Brookhaven Instrument Corporation, NY) at a concentration of 1 mg/mL in deionized water.

Sensitivity of the CPMs to hydrogen peroxide

The CPMs were prepared by the solvent displacement method, as described above, and were diluted with phosphate buffer (pH 7.4, 0.1M) to generate a micelle concentration of 1.5 mg/mL. Various amounts of a hydrogen peroxide stock solution (10 μ M in phosphate buffer pH 7.4, 0.1M), was then added to the CPMs and the resulting chemiluminescence was measured using a luminometer (Fentomaster FB12, Zylux Corporation, TN) with a 10-s acquisition time. The chemiluminescence emission

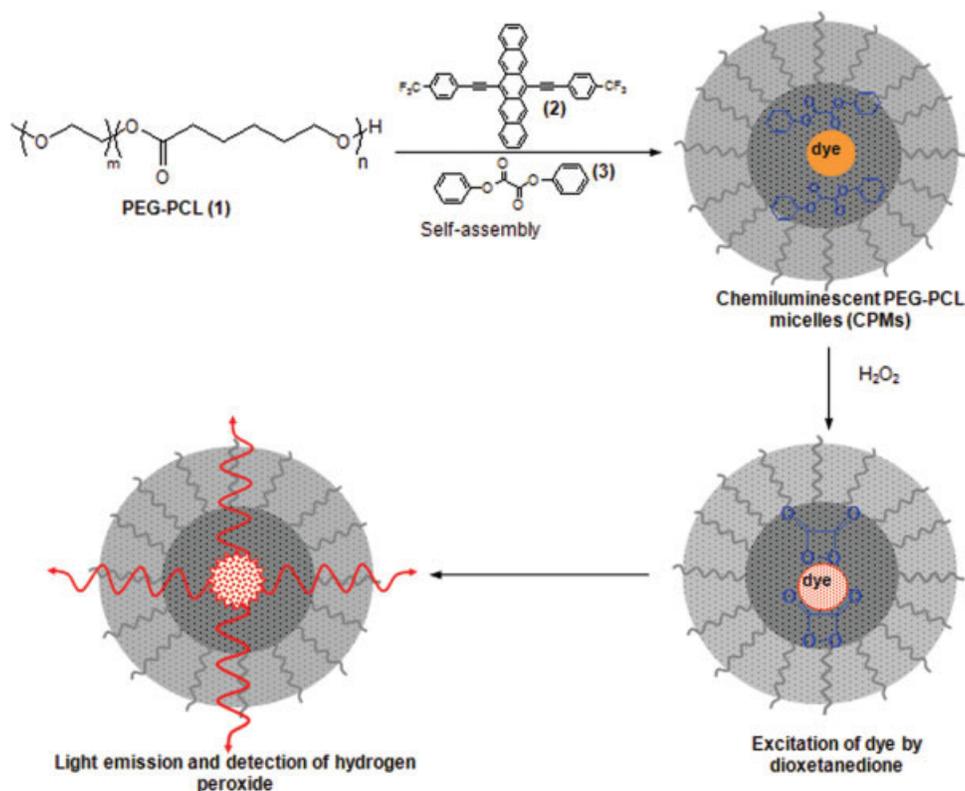


Figure 1. Chemiluminescent PEG-PCL micelles (CPMs) for imaging hydrogen peroxide. PEG-PCL copolymer (1) self-assembles into micelles in water and encapsulates 6, 13-Bis(4-trifluoromethylphenylethynyl)pentacene (2) and diphenyl oxalate (3) into their hydrophobic core, generating the CPMs. Hydrogen peroxide diffuses into the CPMs and reacts with its peroxalate esters to produce a high energy dioxetanedione intermediate. The dioxetanedione chemically excites encapsulated fluorescent dyes, leading to the emission of photons and the detection of hydrogen peroxide. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

spectrum of the CPMs was acquired in the presence of hydrogen peroxide (10 μ M) using a fluorometer (Photon Technology International, NJ).

Stability of the CPMs in deionized water

The CPMs were prepared by the solvent displacement method, as described above, and were incubated in deionized water for various time periods. The CPMs were then diluted with phosphate buffer (pH 7.4, 0.1M) to generate a micelle concentration of 1.5 mg/mL, and were subsequently mixed with a hydrogen peroxide stock solution (10 μ M in phosphate buffer pH 7.4, 0.1M) to generate a 1 μ M concentration. The chemiluminescence of this solution was measured using a luminometer (Femtomaster FB12, Zylux Corporation, TN) with a 10-s acquisition time.

Sensitivity of the CPMs to hydrogen peroxide in serum

The CPMs were prepared by the solvent displacement method, as described above, and were diluted with 10% FBS (supplemented with DMEM) to generate a micelle concentration of 1.5 mg/mL, containing 5% FBS. Various

amounts of a hydrogen peroxide stock solution (10 μ M in phosphate buffer pH 7.4, 0.1M) were then added to the CPMs and the resulting chemiluminescence was measured using a luminometer with a 10-s acquisition time.

RESULTS AND DISCUSSION

Molecular design of the CPMs

Peroxalate chemiluminescence has great potential for imaging hydrogen peroxide; however, its biological applications have been limited because it is a three-component reaction, which requires a methodology for sequestering peroxalate esters and fluorescent dyes in close proximity *in vivo*. In this report, we demonstrate that micelles composed of PEG-PCL can encapsulate peroxalate esters and fluorescent dyes in their hydrophobic core, and can detect hydrogen peroxide under physiologic conditions via peroxalate chemiluminescence. The molecular design of the CPMs is shown in Figure 1. The CPMs are formulated by self-assembling PEG-PCL (1) in the pres-

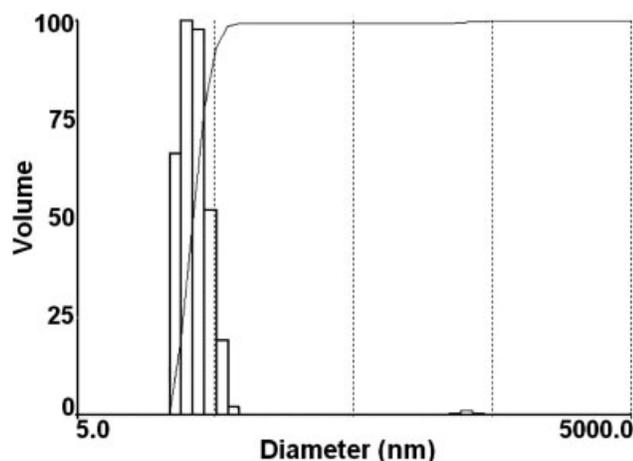


Figure 2. Dynamic light scattering of the CPMs.

ence of diphenyl oxalate (3) and a hydrophobic fluorescent dye (such as 2), generating micelles, which have a hydrophobic PCL core that contains peroxalate esters and fluorescent dyes. The CPMs image hydrogen peroxide through a two-step chemiluminescent process. First, hydrogen peroxide diffuses into the micelles and reacts with its peroxalate esters, generating a high-energy dioxetanedione intermediate within the micelles.^{6,8} This dioxetanedione then chemically excites encapsulated fluorescent dyes, through the chemically initiated electron-exchange luminescence (CIEEL) mechanism,^{19,20} leading to chemiluminescence from the micelles and the detection of hydrogen peroxide.

Synthesis and formulations of the CPMs

The copolymer 1, used to formulate the CPMs, was synthesized by ring opening polymerization of ϵ -caprolactone, using MPEG ($M_n = 2000$) as the initiator and stannous octoate as a catalyst. The composition and molar ratio of the PEG and PCL blocks in the copolymer 1 was determined by ^1H NMR, which indicated that the PCL block was 6600 (M_n) and that the PEG block was 2000 (M_n). The CPMs were prepared using a solvent displacement procedure generating micelles that have a mean diameter of 50 nm, with a majority of the micelles being in the 30-nm range (Fig. 2). Diphenyl oxalate and the fluorescent dye (rubrene or pentacene derivative 2) were encapsulated in the copolymer 1 during micelle preparation. The small size of the CPMs should make them suitable for imaging extracellular hydrogen peroxide produced in inflammatory tissues because micelles in this size range can extravasate into inflammatory tissues through their leaky endothelial junctions.^{13,14}

Sensitivity of the CPMs to hydrogen peroxide

The sensitivity of the CPMs to hydrogen peroxide was investigated by measuring their chemiluminescence intensity in the presence of hydrogen peroxide at various concentrations in the range of 0–1 μM . Figure 3 demonstrates that the CPMs have excellent sensitivity for hydrogen peroxide, and can easily detect hydrogen peroxide at concentrations as low as 50 nM. This is significantly better than the peroxalate nanoparticles, which could only detect hydrogen peroxide at concentrations above 250 nM. The improved sensitivity of the CPMs is possibly due to their smaller size, which provides a larger surface area and enhances the diffusion of hydrogen peroxide into the CPMs. The CPMs also contain aromatic peroxalates instead of mixed aliphatic/aromatic peroxalates, as in the peroxalate nanoparticles. Aromatic peroxalates are in general more efficient at performing peroxalate chemiluminescence than mixed aliphatic/aromatic peroxalates, because phenol is a better leaving group than an aliphatic alcohol, and this may also contribute to the enhanced sensitivity of the CPMs to hydrogen peroxide.

Stability of the CPMs in deionized water

A key issue with using peroxalate-based contrast agents in aqueous solutions is the instability of peroxalate esters to water hydrolysis. For example, diphenyl oxalate has a hydrolysis half-life of ~ 2 min in water,^{21,22} which makes it unsuitable for physiological applications. However, encapsulation of diphenyl oxalate in the CPMs should protect it from water hydrolysis by providing a hydrophobic environment that has low water permeability. We therefore investigated the stability of the CPMs in aqueous solutions, by incubating the CPMs in water for

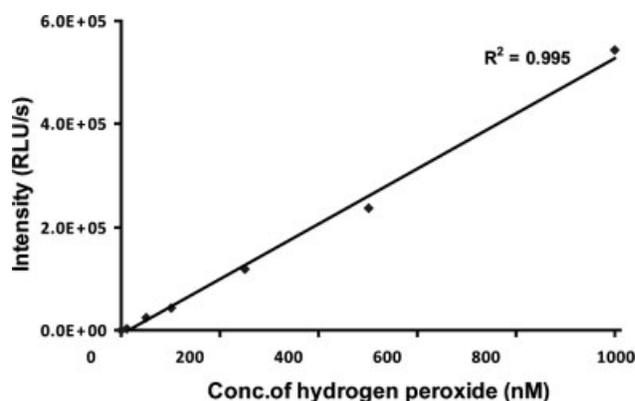


Figure 3. Sensitivity of diphenyl oxalate and rubrene encapsulated CPMs to hydrogen peroxide in phosphate buffer (pH 7.4).

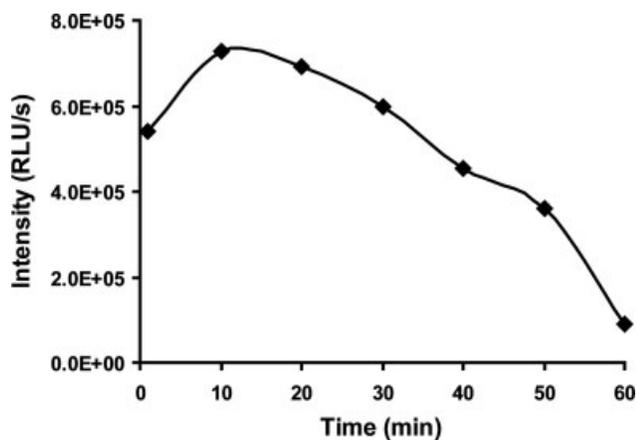


Figure 4. Stability of diphenyl oxalate and rubrene encapsulated CPMs in deionized water.

various time periods and then measuring their chemiluminescence in response to hydrogen peroxide ($1 \mu\text{M}$). Figure 4 demonstrates that the CPMs have a half-life of ~ 30 min in water, suggesting that the CPMs stabilize diphenyl oxalate from water hydrolysis. The high stability of the CPMs should allow them to image hydrogen peroxide after an intravenous injection.

Sensitivity of the CPMs to hydrogen peroxide in serum

Serum contains amphiphilic molecules that frequently partition into and disrupt micellar aggregates; serum, therefore, has the potential to lower the efficacy of the CPMs. The sensitivity of the CPMs to hydrogen peroxide, in the presence of serum, was therefore investigated. CPMs were mixed with 10% FBS (supplemented with DMEM), incubated with various concentrations of hydrogen peroxide ($0\text{--}1 \mu\text{M}$), and the resulting chemiluminescence was then measured. Figure 5 demonstrates that the

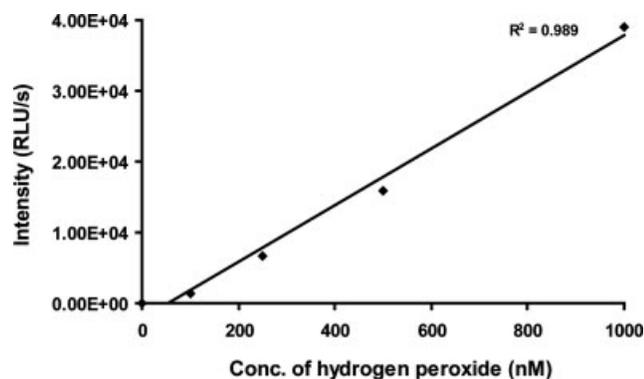


Figure 5. Sensitivity of the CPMs to hydrogen peroxide in 5% FBS (supplemented with DMEM).

chemiluminescence efficiency of the CPMs is dramatically reduced in the presence of serum proteins, and is in general one order magnitude lower than serum free containing CPM solutions. However, the CPMs still have a linear correlation between hydrogen peroxide and chemiluminescence intensity, in the $0.1\text{--}1 \mu\text{M}$ concentration range in serum, and therefore can still be used for numerous physiologic applications.

The CPMs have tunable emission wavelengths

A key benefit of using the CPMs for hydrogen peroxide imaging is that the dioxetanedione intermediate formed by their reaction with hydrogen peroxide has the potential to excite a wide variety of fluorescent dyes, allowing the emission wavelengths of the CPMs to be easily engineered for a particular application. For example, a major application of the CPMs is for the *in vivo* imaging of hydrogen peroxide, which requires CPMs that have emission wavelengths greater than 600 nm because of the low tissue scattering at these wavelengths.^{23,24} We therefore investigated the chemiluminescence emission wavelengths of CPMs with different encapsulated dyes. Figure 6 demonstrates that CPMs encapsulating rubrene emit at 560 nm in the presence of hydrogen peroxide, which is similar to the fluorescence emission spectrum of rubrene. Importantly, CPMs encapsulating the pentacene derivative 2 emit at 676 nm in the presence of hydrogen peroxide, which is ideal for deep tissue imaging of hydrogen peroxide *in vivo* because of the high tissue penetration at this wavelength. In

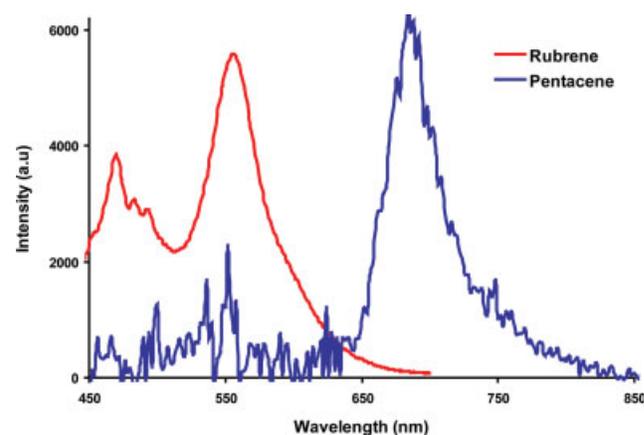


Figure 6. Chemiluminescence emission spectra of rubrene and 6, 13-Bis(4-trifluoromethylphenylethynyl)pentacene encapsulated CPMs in the presence of hydrogen peroxide. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

summary, the CPMs have tunable emission wavelengths and can be engineered to chemiluminesce in the near IR range, making them suitable for *in vivo* applications.

CONCLUSION

In this report, we present a new contrast agent for imaging hydrogen peroxide termed the CPMs, which have a mean diameter of 50 nm and can detect nanomolar concentrations of hydrogen peroxide. The CPMs are micelles composed of PEG-PCL, which encapsulate fluorescent dyes and peroxyoxalate esters. The CPMs image hydrogen peroxide by chemiluminescence imaging, hydrogen peroxide initiates a three-component reaction within the core of the CPMs, which results in the emission of photons. The CPMs have tunable emission wavelengths and also a stealth PEG corona. On the basis of these properties, we anticipate numerous applications of the CPMs for the *in vivo* imaging of hydrogen peroxide.

References

- Lim SD, Sun C, Lambeth JD, Marshall F, Amin M, Chung L, Petros JA, Arnold RS. Increased noxl and hydrogen peroxide in prostate cancer. *Prostate* 2005;62:200–207.
- Reth M. Hydrogen peroxide as second messenger in lymphocyte activation. *Nature Immunol* 2002;3:1129–1134.
- Chang MCY, Pralle A, Isacoff EY, Chang CJ. A selective, cell-permeable optical probe for hydrogen peroxide in living cells. *J Am Chem Soc* 2004;126:15392–15393.
- Miller EW, Albers AE, Pralle A, Isacoff EY, Chang CJ. Boronate-based fluorescent probes for imaging cellular hydrogen peroxide. *J Am Chem Soc* 2005;127:16652–16659.
- Wu M, Lin ZH, Schaferling M, Durkop A, Wolfbeis OS. Fluorescence imaging of the activity of glucose oxidase using a hydrogen-peroxide-sensitive europium probe. *Analytical Biochemistry* 2005;340:66–73.
- Arnous A, Petrakis C, Makris DP, Kefalas P. A peroxyoxalate chemiluminescence-based assay for the evaluation of hydrogen peroxide scavenging activity employing 9,10-diphenylanthracene as the fluorophore. *J Pharmacol Toxicol Methods* 2002;48:171–177.
- Grayesi ML, Seitz WR. Determination of fluorophore-labeled compounds based on peroxyoxalate chemiluminescence. *Anal Biochem* 1984;136:277–284.
- Rauhut MM, Roberts BG, Maulding DR, Bergmark W, Coleman R. Infrared liquid-phase chemiluminescence from reactions of bis(2,4,6-trichlorophenyl) oxalate, hydrogen-peroxide, and infrared fluorescent compounds. *J Organ Chem* 1975;40:330–335.
- Lee D, Khaja S, Velasquez-Castano JC, Dasari M, Sun C, Petros J, Taylor WR, Murthy N. In vivo imaging of hydrogen peroxide with chemiluminescent nanoparticles. *Nat Mater* 2007;6:765–769.
- Meier MAR, Aerts SNH, Staal BBP, Rasa M, Schubert US. PEO-b-PCL block copolymers: Synthesis, detailed characterization, and selected micellar drug encapsulation behavior. *Macromol Rapid Commun* 2005;26:1918–1924.
- Forrest ML, Won CY, Malick AW, Kwon GS. In vitro release of the mTOR inhibitor rapamycin from poly(ethylene glycol)-b-poly(epsilon-caprolactone) micelles. *J Control Release* 2006;110:370–377.
- Zhang Y, Zhuo RX. Synthesis and in vitro drug release behavior of amphiphilic triblock copolymer nanoparticles based on poly(ethylene glycol) and polycaprolactone. *Biomaterials* 2005;26:6736–6742.
- Allen TM, Cullis PR. Drug delivery systems: Entering the mainstream. *Science* 2004;303(5665):1818–1822.
- Torchilin VP. PE. G-based micelles as carriers of contrast agents for different imaging modalities. *Adv Drug Deliv Rev* 2002;54:235–252.
- Armarego WLF, Chai CLL. Purification of Laboratory Chemicals. Burlington, MA: Butterworth-Heinemann; 2003.
- Hyun H, Kim MS, Jeong SC, Kim YH, Lee SY, Lee HB, Khang G. Preparation of diblock copolymers consisting of methoxy poly(ethylene glycol) and poly(epsilon-caprolactone)/poly(L-lactide) and their degradation property. *Polym Eng Sci* 2006;46:1242–1249.
- Li YN, Wu YL, Liu P, Prostran Z, Gardner S, Ong BS. Stable solution-processed high-mobility substituted pentacene semiconductors. *Chem Mater* 2007;19:418–423.
- Aliabadi HM, Mahmud A, Sharifabadi AD, Lavasanifar A. Micelles of methoxy poly(ethylene oxide)-b-poly(epsilon-caprolactone) as vehicles for the solubilization and controlled delivery of cyclosporine A. *J Control Release* 2005;104:301–311.
- Schuster GB. Chemi-luminescence of organic peroxides—Conversion of ground-state reactants to excited-state products by the chemically-initiated electron-exchange luminescence mechanism. *Acc Chem Res* 1979;12:366–373.
- Stevani CV, Silva SM, Baader WJ. Studies on the mechanism of the excitation step in peroxyoxalate chemiluminescence. *Eur J Organ Chem* 2000;24:4037–4046.
- Orosz G. The role of diaryl oxalates in peroxyoxalate chemiluminescence. *Tetrahedron* 1989;45:3493–3506.
- Neuvonen H. Neutral hydrolysis and imidazole-catalyzed decomposition of bis(4 nitrophenyl) oxalate—1,1'-oxalyldiimidazole as an intermediate. *J Chem Soc-Perkin Trans* 1995;2:945–949.
- Rice BW, Cable MD, Nelson MB. In vivo imaging of light-emitting probes. *J Biomed Opt* 2001;6:432–440.
- Hilderbrand SA, Kelly KA, Weissleder R, Tung CH. Mono-functional near-infrared fluorochromes for imaging applications. *Bioconjug Chem* 2005;16:1275–1281.