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Evaluation of the Catalytic Activity and Cytotoxicity of Palladium Nanocubes. The Role of Oxygen

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Abstract

Recently it has been reported that palladium nanocubes (PdNC) are capable of generating singlet oxygen without photo-excitation simply via chemisorption of molecular oxygen on its surface. Such a trait would make PdNC a highly versatile catalyst suitable in organic synthesis and a Reactive Oxygen Species (ROS) inducing cancer treatment reagent. Here we thoroughly investigated the catalytic activity of PdNC with respect to their ability to produce singlet oxygen and to oxidize 3,5,3',5'-tetramethyl-benzidine (TMB), as well as, analyzed the cytotoxic properties of PdNC on HeLa cells. Our findings showed no evidence of singlet oxygen production by PdNC. The nanocubes' activity is not necessarily linked to activation of oxygen. The oxidation of substrate on PdNC can be a first step followed by PdNC regeneration with oxygen or other oxidant. The catalytic activity of PdNC towards oxidation of TMB is very high and shows direct two-electrons oxidation when the surface of PdNC is clean and the ratio of TMB/PdNC is not very high. Sequential one electron oxidation is observed when the pristine quality of PdNC surface is compromised by serum or uncontrolled impurities and/or the ratio of TMB/PdNC is high. Clean PdNC in serum-free media efficiently induce apoptosis of HeLa cells. It is the primary route of cell death and is associated with hyperpolarization of mitochondria, contrary to a common mitochondrial depolarization initiated by ROS. Again, the effects are very sensitive to how well the pristine surface of PdNC is preserved, suggesting that PdNC can be used as an apoptosis inducing agent but only with appropriate drug delivery system.

Keywords

Palladium nanocubes; catalytic activity; cytotoxicity; ROS; cancer treatment

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Supporting Information contains FTIR of PdNC at different stages of cleaning, detailed analysis of HRTEM, additional kinetics of TMB oxidation, detailed flow cytometry data for DNA fragmentation and mitochondrial membrane potential assays, confocal microscopy images, SEM and TEM of PdNC@SWCNT, estimation of the oxidation turnover number for PdNC. It is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Singlet oxygen is a highly reactive species that plays an important role in various biologically relevant oxidation processes¹⁻³ and in cancer therapy.⁴⁻⁶ However, generating singlet oxygen efficiently is challenging because promoting the triplet ground state of molecular oxygen to a singlet-excited state is a spin forbidden and energetically demanding process. The lowest energy ¹g singlet state is ~1 eV above the ground state. Generally, photosensitizers are used to produce singlet oxygen.^{4, 6} Recently, metal nanoparticles of different morphology were shown to sensitize formation of singlet oxygen by photo-excitation via their surface plasmon resonance (SPR) bands. Spherical metal nanoparticles such as Au, Ag, and Pt can promote sensitization of singlet oxygen upon exposure to visible light,⁷ whereas Au nanorods and decahedral Ag nanoparticles can produce singlet oxygen by near infrared (NIR) irradiation.⁸

Metallic nanoparticles have shown promising results as versatile catalysts for peroxidation of olefins and photodynamic therapy reagents for cancer treatment.⁷⁻¹⁰ Wilkinson et al. showed that spherical palladium nanoparticles could induce apoptosis in human primary bronchial epithelial cells (PBEC), but not in the human alveolar carcinoma cell line A549.¹⁰ In a recent paper, Long et al.⁹ reported that palladium nanocubes (PdNC) were capable of efficiently generating singlet oxygen without any assistance of light, simply via chemisorption of molecular oxygen on their surface. Stronger binding of oxygen to (100) surface of nanocubes apparently had a greater effect than (111) surface of octahedrons. Electron transfer from the Pd (100) surface to the adsorbed molecular oxygen was believed to facilitate the spin flip process.⁹ In the proposed electron transfer mechanism it was speculated that the decrease in the magnetic moment of chemisorbed O₂ spontaneously enabled the change in the spin state of O₂ from triplet to singlet, despite the higher energy of the latter. Such a peculiar trait, where no photo-irradiation is required to form singlet oxygen, if possible, would make PdNC a highly versatile catalyst in organic synthesis and a convenient Reactive Oxygen Species (ROS) inducing cancer treatment reagent. Therefore, in this paper we first thoroughly investigated the capability of PdNC in producing singlet oxygen and then assessed their catalytic activity towards oxidation of 3,5,3',5'-tetramethylbenzidine (TMB) along with the detailed analysis of their cytotoxicity on HeLa cells.

MATERIALS AND METHODS

Synthesis of palladium nanocubes (PdNC)

PdNC were synthesized following the published protocol, by reducing Pd from K₂PdCl₄ (Sigma Aldrich) using ascorbic acid in the presence of poly(vinyl pyrrolidone) and KBr.^{9, 11} The size of PdNC was controlled by reaction time and typically was ~10 nm. In a typical synthesis, 105 mg of poly(vinyl pyrrolidone) (PVP, Sigma Aldrich), 300 mg of KBr (Sigma Aldrich) and 60 mg of ascorbic acid (Sigma Aldrich) were mixed in 8 mL of deionized water and heated in 50 mL 3-neck flask (with a stir bar, 320 rpm) at 80 °C for five minutes. Subsequently, a solution of 65 mg of K₂PdCl₄ in 3 mL of deionized water was added into the flask. The reaction mixture was heated for 3 hours. The resulting product was collected and washed first with acetone and then consecutively with ethanol and water multiple times with collecting PdNC by centrifugation. Washing multiple times in EtOH and water is

crucial and affects the properties of PdNC significantly. Each washing step involved 5 minute sonication of 1 mg PdNC suspended in 1mL of solvent (ethanol or DI water) followed by centrifuging to collect PdNC.

TEM, (including high-resolution TEM) images were taken on a JEOL 2010 by dropcasting samples of suspended in water PdNC onto carbon-coated copper grids.

Detection of singlet oxygen

Invitrogen's Singlet Oxygen Sensor Green (SOSG) was employed for direct detection of singlet oxygen.^{7,8,12} SOSG becomes fluorescent upon oxidation of its anthracene moiety by $^1\text{O}_2^*$, which makes it very specific for the latter. 25 mg/L of PdNC was added to 5 μM SOSG in D_2O and fluorescence spectra were recorded upon excitation at 480 nm as a function of time using PerkinElmer LS 55 Luminescence Spectrometer.

Oxidation of TMB

In a typical experiment, suspension of PdNC (typically to the final concentration between 10 or 25 mg/L) was added to 2 mL of TMB (3,5,3',5'-tetramethyl-benzidine) solution (typically between 2.5 and 8.9 μM) in acetic buffer solution (HAc:NaAc 1:1, 0.2 M) at 10 °C and UV-Vis absorption spectra (Agilent 8453) of TMB oxidation products were recorded as a function of time. Because the solubility of TMB is fairly small, its solutions were centrifuged to remove the undissolved particles and the concentration was calculated by absorbance at 285 nm ($\epsilon = 2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).²⁰ In experiments with H_2O_2 , the TMB solution was bubbled for 5 min with Ar, again for 5 min after adding PdNC before collecting the spectra, and another 5 min after adding H_2O_2 .

In an alternative geometry, either 20 μg or 200 μg of purified PdNC were immobilized on the surface of a PTFE membrane (Sigma Aldrich S7062-50EA Cameo Syringe Filter, pore size 0.22 μm , 17 mm diameter) with and without 25 μg of semiconducting single wall carbon nanotubes (SWCNT) with a broad distribution of diameters near $\sim 1.3 \text{ nm}$, from OCSiAl (TuballTM). The adhesion of PdNC on PTFE membrane is so strong that withstands even sonication. The adhesion did not compromise when SWCNT were first deposited on the membrane. SEM and TEM images of the PdNC@SWCNT are shown in Figure S6 in Supplemental. Solution of TMB in the acetic buffer with concentration of either 15 μM or 30 μM was passed through the membrane using a syringe pump at a constant rate of 0.1 mL/min. Every 6 min the collected 0.6 mL of filtrate was used to take UV-Vis spectrum. For experiments in argon, the solution was bubbled in the syringe for 15 min by the corresponding high purity gas prior to measurements.

Cell Culture

Immortalized human epithelial HeLa cells (ATCC®, CCL-2TM) were cultured in T-25 flasks in Dulbecco's modified Eagles Medium (DMEM, Santa Cruz Biotechnology) supplemented with 10% fetal bovine serum. Cells were maintained in the incubator at 37 °C under 5% CO_2 and 95% relative humidity. After reaching 80% confluency, the cells were split using trypsin (0.25%) and reseeded with appropriate seeding density either into a T-25 flask to continue to grow them or in 12 well plates for different cytotoxicity assays. Typically, in 12-

well plates, 100,000 of viable HeLa cells were seeded per well in 1 mL of cell culture medium for 24 hours prior to experiments.

Mitochondrial Membrane Potential and Cell Viability Assays

Dual staining by rhodamine 123 (Rh 123, Sigma Aldrich) and propidium iodide (PI, Sigma Aldrich) was performed on the live HeLa cells treated with either PdNC or hydrogen peroxide. The staining was performed following the published protocol¹³ with employing flow cytometry for simultaneous assessment of the mitochondrial membrane potential and late apoptotic/necrotic cells (cell viability). Briefly, PdNC (425 µg/mL) and H₂O₂ (5.6 mM) were incubated separately with HeLa cells in 12-well plates in the cell culture medium with and without serum. Cells without PdNC acted as a negative control, whereas cells with H₂O₂ are considered as a positive control. After 24 hours of incubation, cells were trypsinized and suspended (including floating cells) in the 1 mL of culture cell culture medium with 20 µL of 10 µM Rh123. Following incubation for 20 min at 37°C, cells were centrifuged at 300 g for 5 min at room temperature and resuspended in 1 mL PBS. After that, 10 µL of 1 mg/mL PI was added to the resuspended cells and incubated for 5 min at room temperature in the dark. Fluorescence intensity statistics (from 10,000 cells) were immediately analyzed by flow cytometry (BD Accuri™ C6, Becton Dickinson) with 488-nm laser excitation. Forward scattered light and side scattered light intensities were collected (488-nm) in addition to the fluorescence from Rh123 at 530 ± 30 nm and PI above 600 nm.

DNA Fragmentation Assay

Cells were incubated with PdNC or H₂O₂ as described above in the mitochondrial membrane potential and the cell viability assays. After 24 hours of incubation, cells were trypsinized and suspended (including floating cells) in 1 mL of PBS and centrifuged at 200 g for 5 min at room temperature. The literature protocol for DNA fragmentation assay¹⁴ was exactly followed. Cell pellets were resuspended in 500 µL PBS and fixed with 4.5 mL of 70% (v/v) cold ethanol. Fixed cells were first washed in PBS and then resuspended in 0.5 mL of PBS and 0.5 mL of DNA extraction buffer (192 µL of 0.2 M Na₂HPO₄ with 8 µL of 0.1% Triton X-100). The suspension was kept at room temperature for 5 min and then centrifuged at 400 g to remove the supernatant. Cells were then resuspended in 1 mL of DNA staining solution (200 µg of PI in 100 µL of PBS plus 2 µg of DNase free RNase) and incubated for 30 min at room temperature in the dark before flow cytometry analysis. 10,000 Cells were analyzed by flow cytometry (BD Accuri™ C6, Becton Dickinson) with 488-nm laser excitation and collection of red fluorescence (>600 nm) as well as forward and side scattered light (488-nm). Cells displaying hypodiploid (sub-G1) DNA content in the cytometry data represent the apoptotic cells.

RESULTS AND DISCUSSION

To thoroughly investigate the capability of PdNC in producing singlet oxygen, we first synthesized high quality PdNC following the procedure of Long et al.⁹ who claimed that PdNC could produce singlet oxygen. PdNC were synthesized by reducing Pd from K₂PdCl₄ using ascorbic acid in the presence of PVP and KBr. Figure 1 shows the TEM and HRTEM images of PdNC resulting from such synthesis. The size of PdNC was controlled by reaction

time and was found to be typically around 10 nm. Morphology of PdNC was similar to that published in the literature with the typical 1.9 Å spacing between fringes, characteristic of Pd (200).^{9,11} See also Figure S2 in Supplemental for details.

The capability of PdNC in producing singlet oxygen without photoirradiation can be directly tested by using Singlet Oxygen Sensor Green (SOSG), a singlet oxygen-specific fluorescent sensor composed of fluorescein and anthracene moieties.^{12,15} SOSG becomes strongly fluorescent with an emission maximum at 525 nm upon oxidation of its anthracene moiety by $^1\text{O}_2^*$.¹⁵ Because of its high sensitivity and specificity towards singlet oxygen¹², SOSG has been already used in direct detection of singlet oxygen produced by metal nanoparticles^{7, 8} and by porous silicon nanoparticles.¹⁶ It has even been used in detection of singlet oxygen inside mammalian cells¹⁷ and in quantitative measurements of singlet oxygen generation.¹⁸ In our experiment, green fluorescence of SOSG in D_2O was recorded upon excitation at 480 nm as a function of time after addition of 25 mg/L PdNC. No increase in fluorescence of SOSG was observed in the time interval from 10 s to 6 hours. Figure 2 shows representative fluorescence spectra of SOSG before and after adding PdNC. No increase in the fluorescence of SOSG was observed irrespective of bubbling the solution with oxygen and/or increasing the concentration of PdNC. Overall, our findings clearly show no evidence of direct free singlet oxygen production by PdNC.

At the time this paper was under preparation for publication, another report from the same group appeared¹⁹ in which the authors have tried to be more specific and, instead of referring to active molecules as singlet oxygen, they called them “species that behaves like singlet O_2 both chemically and physically”.¹⁹ It remains unclear to what that analogy pertains, but now the authors imply that the activated oxygen molecules do not leave the surface of PdNC until the reaction with the substrate. Thus, the catalytic activity of PdNC cannot be simply reduced to activation of oxygen and needs to be evaluated for oxidation of a substrate.

One of the convenient substrates reported in ref.9, 3,5,3',5'-tetramethyl-benzidine (TMB), can be used for evaluating the catalytic activity of PdNC in more details. TMB is often used in spectrophotometric²⁰ and electrochemical²¹⁻²³ immunosorbent assays. It can be oxidized electrochemically²¹⁻²³ or by a strong oxidant, such as hydrogen peroxide^{19,24} or oxygen⁹ but typically needs a catalyst to achieve a reasonable rate. The oxidation products can be easily distinguished (see Scheme 1): the one-electron oxidation intermediate TMB^+ gives a blue color due to charge transfer absorption peaks at 370 and 652 nm, while the two-electron oxidation final product diimine TMB^{2+} has the absorption peak at 450 nm.²⁰

UV-Vis spectra of TMB solutions at different times after adding PdNC are shown in Figure 3. The spectroscopic changes are not exactly the same as in ref.9. Notably, Figure 3A illustrates that TMB can be oxidized directly into two-electron oxidation final product TMB^{2+} immediately from the beginning of the reaction. It bypasses the one-electron oxidation intermediate TMB^+ (reaction pathway “a” in Scheme 1) or, at least, passes very quickly through it. Sequential oxidation of TMB (reaction pathway “b” in Scheme 1) reported by Long et al. and attributed to oxidation by singlet oxygen or its analogue⁹ is observed when PdNC are insufficiently clean and/or the relative amount of TMB is too high.

In Figure 3B such a situation is illustrated for the same concentrations of PdNC and TMB as in Figure 3A but with insufficiently cleaned PdNC. Washing PdNC after synthesis with ethanol and water multiple times and using them fresh is vital to observation of direct two-electron oxidation. As a matter of fact, the suggested protocol of washing 3 times in ethanol⁹ is insufficient to achieve 'pristine' quality PdNC. FTIR analysis indicates residual hydrocarbon CH bonds even after additional 5 washings in water. By the 9th washing in water almost no residual methylene bands were observed (Figure S1). Note that singlet oxygen production was not detected for either 'poorly cleaned' PdNC or for the highest quality of purification. When the PdNC surface is insufficiently clean or is compromised by serum or uncontrolled impurities after long storage, sequential one electron oxidation as in Figure 3B is observed. In this case, both, the intermediate TMB⁺ and the final TMB²⁺ oxidized products appear immediately after adding PdNC but majority of the TMB molecules eventually converts into TMB²⁺ gradually with time (see also Figure S3).

The catalytic role of PdNC, as of any other catalyst in a redox reaction, can be crudely divided into the two options: activation (oxidation) of a reductant, TMB in this case, or activation (reduction) of an oxidant. The latter can be oxygen, hydrogen peroxide, or other reagents with sufficient reduction potential. In the assay for H₂O₂ with horseradish peroxidase (HRP) and TMB it has been shown that the initial reaction of peroxide with HRP is followed by formation of TMB⁺.²⁴ The case of PdNC catalyzed oxidation of TMB or other substrates does not have to be the same. Opposite to Long et al.^{9,19} who believe that it originates exclusively by oxygen activation to produce 'singlet oxygen like species', we suggest considering both routes and believe that direct oxidation of a substrate by PdNC is likely to happen as well and, in some cases like with TMB, can be just as important if not more. Oxygen is a very strong oxidant with the redox potential sufficient for oxidizing TMB to TMB²⁺ while H₂O₂ seizes at the first step, *i.e.*, production of TMB⁺. Nevertheless, PdNC catalyzes both reactions; as seen in Figure S5 for H₂O₂. Oxidation of TMB by hydrogen peroxide can be also achieved with other metal or metal oxide nanoparticles,²⁵⁻²⁸ as well as electrochemically.²¹⁻²³ All these suggests that PdNC activated O₂ is not unique in oxidizing TMB and it does not have to be exclusively via oxygen activation.

There are other indications of a possibility that this reaction can start with direct oxidation of TMB on the surface of PdNC. One notices that the absorption peak of TMB monomer at 285 nm gets strongly suppressed upon addition of PdNC before any oxidation products are observed. It correlates with the previously reported higher binding affinity of TMB²⁺ on metal surfaces in electrochemical oxidation of TMB.²³ Optical absorption of TMB (or its oxidized forms) on the metal surface is significantly suppressed as it is well known for molecules strongly coupled to a metal surface with the transition moment parallel to it.²⁹⁻³⁰

One can speculate that PdNC is a strong enough oxidizer to oxidize TMB to TMB²⁺ on its surface similar to electrochemical oxidation easily seen for TMB at high enough potential.²¹⁻²³ Because PdNC is not kept at a constant potential, its Fermi energy shifts upon accepting electrons and eventually making PdNC incapable to oxidize further. The overall oxidative capacitance of PdNC (the maximum amount of stored negative charge) is limited by the PdNC concentration and presence of oxygen or other oxidant in the solution that helps to recuperate their catalytic activity. Indeed, oxidation of TMB does not completely

cease in Ar, in Fig.1C of ref. 9 it only slowed down. In our Figure S5 under similar conditions it shows suppression of TMB^{2+} production but one electron oxidation to TMB^+ is still present. Long et al. also had demonstrated¹⁹ the importance of the charge state on PdNC surface and were able to enhance the oxidation yields by photoinduced charge injection from TiO_2 in the corresponding PdNC/ TiO_2 hybrid. Unfortunately, they did not confirm the unique role of oxygen by using oxygen free conditions. Since other oxidizers, such as hydrogen peroxide, also can oxidize TMB with catalytic ‘help’ (see Fig. S5), *i.e.*, by capturing charge from PdNC (‘reoxidizing’ it), the role of oxygen does not appear unique and can be replaced.

One can extend the idea of charge injection or controlling the Fermi energy without photoexcitation. To this goal, we have conducted experiments with the high purity PdNC bound to the surface of a PTFE membrane while solution of TMB is pushed through at a constant rate (0.1 mL/min). In this pseudo continuous approach, we can manipulate separately with the states of PdNC and TMB in solution while measure kinetics of the reaction by collecting products in the filtrate solution for analysis of small aliquots (0.6 mL). Figure 4 illustrates the results for the two conditions of high (A-C) and low (D-F) ratios between the PdNC and TMB amounts: 200 μg PdNC/ 15 μM TMB (A-C) and 20 μg PdNC/ 30 μM TMB (D-F). The kinetic behaviors observed in air and in Ar are dramatically different and depend on the PdNC/TMB ratio. First of all, lowering the ratio causes almost disappearance of the TMB^{2+} peak at 450 nm and leads to significant survival of unoxidized TMB at 285 nm, as seen in comparing Figures 4A and 4D. At low PdNC/TMB ratio, unoxidized TMB comes off at the amount slightly smaller than that in the original solution only at the beginning. At higher PdNC/TMB ratio, almost no unoxidized TMB comes through at first. Its amount builds up very slowly and does not reach even 20% of the original amount in solution within the course of 30 min. At the same time, the amount of products significantly increases with time (Figure 4A), similar to that in Figure S3. The TMB^{2+} is favored at first but gradually gives way to the one electron oxidized products. As shown in Figure 4B, deoxygenating the solution by bubbling with argon instantaneously releases unoxidized TMB (data for Figure 4B were collected in the same run right after 4A) and suppresses production of the TMB^{2+} diimine. The latter is not surprising because the oxidation potential of TMB increases between the one- and two-electron products and thus production of TMB^{2+} should cease first. The effect of ‘oxygen free’ atmosphere is particularly pronounced for low PdNC/ TMB ratio (Figure 4E), where not only production of TMB^{2+} is suppressed but the yield of TMB^+ declines with time as well. All of these can be rationalized by PdNC being a rate limiting component that has a finite oxidizing (charge) capacity and oxygen needing only to reoxidize PdNC. From the total amount of oxidized product in the latter case one can approximate that each PdNC performed on the order of $\sim 3 \times 10^3$ oxidations in Ar (see Supplemental), which is the upper limit estimate because some residual oxygen might be still present in the solution due to its traces in Ar and imperfections in the procedure. A crude estimate based on the double layer capacitance gives the maximum turnover number for each PdNC without reoxidation $\sim 10^3$ which is not such a dramatic discrepancy given the above mentioned possibility of oxygen traces (see Supplemental).

Regeneration of PdNC can be achieved by other means, for example by hydrogen peroxide or by carbon nanotubes (CNT), instead of oxygen. SWCNT are more convenient for our experiment with membrane. The Fermi energy of SWCNT depends on their diameter and doping³¹ but is typically higher than that of Pd. For small diameter *p*-doped SWCNT the difference in Fermi levels becomes quite small so that one can anticipate insignificant charge transfer from SWCNT to Pd and, at the same time, sufficient to oxidize TMB at least to TMB⁺.³¹ There is a significant distribution of redox potentials for SWCNT even of the same diameter,³² which ensures that there is noticeable population of SWCNT with redox potentials capable to support TMB oxidation to TMB²⁺.

We placed the same amounts of PdNC (20 µg and 200 µg) on the surface of single walled CNT (SWCNT). Even though the amount of SWCNT was only 25 µg, its addition significantly increased the charge capacitance and allowed charge collection from PdNC, even when the amount of TMB is quite high (Figure 4C). Note that for PdNC@SWCNT in Ar the TMB²⁺ product is clearly visible at 450 nm. Although, the kinetics of TMB oxidation with PdNC@SWCNT in Ar is different from that for PdNC in air (compare Figure 4A with 4C and 4D with 4F, respectively) primarily due to capturing of TMB directly on SWCNT. Notably, SWCNT without PdNC do not oxidize TMB at all (Figure S7), while elution of TMB is clearly delayed due to capturing of TMB on SWCNT.

The suggested mechanism of PdNC catalytic activity bypassing the oxygen involvement cannot be uniformly applied, especially for reactions where oxygen ends up in the product, but we believe that the situation with TMB is not unique. In any case, we see no indication of free ROS that get produced by PdNC. Besides, as we saw above, hydrogen peroxide gets reduced on PdNC via oxidation of TMB and thus is also unlikely produced by PdNC in ambient conditions. The issue of the mechanism of oxidation with PdNCs becomes particularly important when it comes to their possible medical applications. The remaining part of the study deals with evaluation of the effects of PdNCs in cancer treatment, in particular, the detailed analysis of their cytotoxic properties on HeLa cells.

It was previously shown through MTT assay that PdNCs significantly reduced the viability of HeLa cells.⁹ So far, the mechanism of cytotoxicity induced by PdNCs is not well understood and requires further study. Herein we offer some insight by differentiating apoptosis and necrosis in the PdNC treated HeLa cells and by analyzing the change in the mitochondrial membrane potential in them.

DNA fragmentation is an established marker of apoptosis.¹⁴ We performed DNA fragmentation assay with flow cytometry to verify whether PdNC induces apoptosis in HeLa cells. As shown in Figure 5 (see also Figure S8 in supplemental for details), 425 µg/mL of PdNCs induce DNA fragmentation in HeLa cells associated with apoptosis, but most effectively in the serum free cell culture media. There is roughly 3.5-fold increase in the number of apoptotic cells after treatment with PdNC in the absence of serum. Cells treated with 5.6 mM of H₂O₂ (positive control) also effectively induced apoptosis in the serum free media, whereas untreated cells (negative control) showed no significant increase in apoptotic behavior without serum. The effect of serum on compromising the efficacy of PdNC to induce apoptosis is likely of two causes. On one hand, different proteins, antibodies

and antigens present in serum can ‘heal’ the cytotoxic effect similar to that in the case of H_2O_2 .³³ On the other hand, these serum components can also wrap around PdNC to coat them and thus decrease their surface activity. Formation of protein corona around nanoparticles in biological milieu is well known.³⁴ It suggests that PdNC can be used as apoptosis inducing drug but only with appropriate drug delivery system that preserves its pristine surface before contact with the cancer cells.

The apoptotic mechanism of PdNC cytotoxicity confirmed by the DNA fragmentation assay does not clarify whether it is the same or different from the effect of hydrogen peroxide or other reactive oxygen species (ROS). Additional information is required to elucidate the mechanism of the PdNC cytotoxicity effect. The mitochondrial membrane potential has been extensively used in studies of such phenomena.^{35,36} Mitochondria play important role in mediating apoptosis in most of the apoptotic pathways.³⁷ In particular, reactive oxygen species, like singlet oxygen and hydrogen peroxide, damage mitochondria and induce the loss of the mitochondria membrane potential in the process of triggering apoptosis.³⁸⁻⁴¹ This collapse of mitochondrial potential, an early sign of mitochondrial-mediated apoptosis, can be tracked using Rhodamine 123 (Rh123). Rh123 is a mitochondrial specific cationic fluorescent dye commonly used to assess mitochondrial membrane potential.^{13,35,36} The uptake of Rh123 in mitochondria is directly proportional to the mitochondrial membrane potential.³⁶ We employed this technique to further investigate the mechanism of PdNC induced apoptosis.

As seen in Figure 6 (see also Figure S9 in supplemental for details), HeLa cells treated with H_2O_2 (positive control) show a decrease in the fluorescence intensity of Rh123 as compared to the untreated cells. As expected from literature,³⁸⁻⁴¹ ROS like H_2O_2 depolarized the mitochondria (decrease of the mitochondria membrane potential). However, HeLa cells treated with PdNC showed the opposite effect – hyperpolarization of the mitochondria membrane potential (see also confocal images in Figure S11 in supplemental). Such a behavior is not uncommon; mitochondrial membrane potential in HeLa cells can be increased by treating with manganese(II)⁴² or nigericin⁴³, but it is quite opposite to typical effects of various ROS. Note that, again, the increase in the mitochondrial membrane potential is more pronounced when the cells were treated with PdNC in the cell culture medium without serum, while depolarization by H_2O_2 was also more pronounced without serum. The effect of serum is in alignment with the DNA fragmentation but the opposite signs for hydrogen peroxide and PdNC.

PI staining can be used for discriminating cells undergoing late apoptosis or necrosis (nonviable cells). PI can only permeate into nonviable cells because of their compromised plasma membrane integrity.¹³ Figure 7 shows that only 6.6% with serum and 14.7% without serum of cells treated with 425 $\mu\text{g}/\text{mL}$ of PdNC were nonviable (see also Figure S9 in supplemental for details), which is slightly less than the number of apoptotic cells from DNA fragmentation assay (Figure 5). The effect of hydrogen peroxide is clearly different – the number of nonviable cells was greater than the apoptotic cells: 40.9 % with serum and 46.3 % without serum. Untreated cells (negative control) showed no significant amount of nonviable cells, which was also smaller than the number of apoptotic cells. Lower yield of nonviable cells for PdNC treatment in serum again emphasizes the effectiveness of PdNC in

the serum free biological environment. The dramatic difference for hydrogen peroxide treatment, where amount of nonviable cells significantly exceeds the apoptotic cells, suggests that the necrotic pathway is dominant in that case. PdNC treatment, on the other hand, with 23% of apoptotic cells from DNA fragmentation assay being much greater than 14.7% of total nonviable cells, indicates that necrosis is a minor route of cell death induced by PdNC. It also implies that PdNC do not produce hydrogen peroxide or singlet oxygen as cytotoxic ROS. Thus the mechanism of PdNC toxicity is different from ROS, mainly induces apoptosis, at least in HeLa cells, and is accompanied by hyperpolarization of mitochondrial membrane potential.

CONCLUSIONS

In conclusion, Pd nanocubes provide efficient catalytic surfaces for oxidation of multiple substrates but do not produce singlet oxygen as reported previously.⁹ Nevertheless, direct two-electron oxidation of TMB is observed when surface of PdNC is clean and the ratio of TMB/PdNC is not very high, which indicates strong oxidative ability of PdNC and sensitivity to surface fouling. The nanocubes' activity is not necessarily linked to oxygen which only serves as a reoxidant of PdNC, at least in oxidizing TMB, and can be alternatively substituted by SWCNT. PdNC also efficiently induce apoptosis of HeLa cells but by a mechanism different from that of ROS and associated with hyperpolarization of mitochondria. The effects are very sensitive to how well the pristine surface of PdNC is preserved, suggesting that PdNC can be used as apoptosis inducing drug but only with appropriate drug delivery system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TEM images of PdNC. The high resolution TEM on the right shows the spacing of 0.19 nm between (200) planes. See Supplemental for details.

Figure 2.

Fluorescence signal of 5 μ M SOSG after 10 min with 25 mg/L PdNC (red) and without it (blue). No increase in the fluorescence intensity with PdNC indicates lack of free singlet oxygen production.

Figure 3.

Time dependent UV-Vis spectra of TMB solutions upon addition of PdNC: a) with PdNC washed more than 20 times in ethanol and water and b) with PdNC washed five times in ethanol and water. The 0 min curve in the latter corresponds to PdNC only. Concentration of PdNC used was ~25 mg/L and [TMB] concentration 8.9 μ M. The absorbance peaks for one-electron oxidation intermediate are seen at 370 and 652 nm and the absorption peak for the two electron-oxidation final diimine product is seen at 450 nm.

Figure 4. Kinetics of TMB oxidation (concentrations 15 μM in (A-C) and 30 μM in (D-F)) in the pseudo continuous geometry with PdNC immobilized on filter membrane at the amount of 200 μg (A-C) and 20 μg (D-F). A and D are in air, B and E – argon, while C and F in argon but PdNC were also supported by 25 μg of SWCNT. The insets show time-evolution of the absorbance for unoxidized TMB (285 nm, black squares), single-electron oxidized (370 nm, red circles) and two-electron oxidized (450 nm, green rhombs) TMB peaks. See text for details.

Figure 5.

Percent of the apoptotic HeLa cells after treatment with PdNC and H₂O₂ in the cell culture medium with and without serum measured as the mean percentage of cells displaying hypodiploid (sub-G1) DNA content using flow cytometric analyses for two independent experiments.

Figure 6.

Analysis of the mitochondrial membrane potential by Rh123 uptake in HeLa cells treated with PdNC and H₂O₂ in the cell culture medium with and without serum. Data represents the mean of fluorescence intensity of cells analyzed using flow cytometry from three independent experiments.

Figure 7.

Percent of nonviable cells (necrotic or late apoptotic) by PI uptake in the HeLa cells treated with PdNC and H₂O₂ in the cell culture medium with and without serum. Data represents the mean fluorescence intensity of PI coming from cells analyzed using flow cytometry from three independent experiments.

Scheme 1.

Reaction pathways for oxidation of TMB, partially adopted from ref. 20. Pathway a) represents the oxidation of TMB into direct two-electron oxidation final product and pathway b) represents sequential one electron oxidation of TMB.