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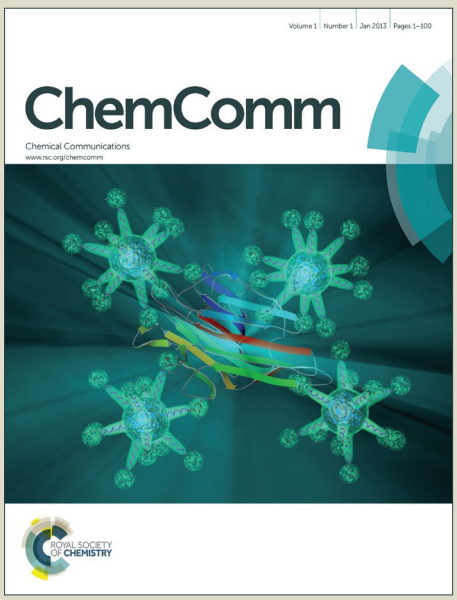

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Nanoparticles Based on Quantum Dots and a Luminol Derivative: Implications for *in vivo* Imaging of Hydrogen Peroxide by Chemiluminescence Resonance Energy Transfer

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Eun Sook Lee,[a,b] V. G. Deepagan,[c] Dong Gil You,[d] Jueun Jeon,[d] Gi-Ra Yi,[d] Jung Young Lee,[d] Doo Sung Lee,[d] Yung Doug Suh,[*,b,d] and Jae Hyung Park[*,a,b,d]

Overproduction of hydrogen peroxide is involved in the pathogenesis of inflammatory diseases such as cancer and arthritis. To image hydrogen peroxide via chemiluminescence resonance energy transfer in the near-infrared wavelength range, we prepared quantum dots functionalized with luminol derivative.

Reaction oxygen species (ROS), produced by incomplete reduction of oxygen, play a role in functions ranging from cell homeostasis to cell death as signaling molecules.[1-4] In recent years, overproduction of hydrogen peroxide, the abundant form of ROS, under an abnormal redox balance has been shown to be a main etiological cause of numerous diseases such as cancer, cardiovascular disease and diabetes mellitus.[5-7] Therefore, hydrogen peroxide is emerging as an important diagnostic marker. Nevertheless, there are difficulties with *in vivo* imaging of hydrogen peroxide because of its low level and low reactivity, compared to other ROS molecules including superoxide, hydroxyl radical and singlet oxygen.[8]

For *in vivo* optical imaging of ROS, fluorescence probes such as hydroethidium and dichlorodihydrofluorescein diacetate have been widely investigated.[9] However, fluorescence probes often fail to detect low concentrations of ROS because an external energy source is necessary for excitation of fluorophores. The fluorescence, generated by an analyte, can be also disturbed by high background emission from cellular auto-fluorescence, leading to low tissue penetrance of the signal. Chemiluminescence (CL), the emission of light as a consequence of a chemical reaction, can be an alternative to overcome the aforementioned defects of fluorescence probes because no excitation source is required. In fact, CL has exhibited the potential useful for ROS detection.[10] CL enables precise and sensitive analysis, since the intensity of light emission is related to the analyte concentration.[11] Of chemiluminescent molecules, luminol has been extensively used in bioanalyses to determine ROS, enzyme and cytokines.[10, 12] However, the CL reaction of luminol showed the highest reactivity under basic conditions and should be catalyzed by enzymes, indicating that luminol is not appropriate for use *in vivo*.[13, 14] Furthermore, the emission maximum at a 425-nm wavelength suggests that single-handed application of luminol is unavailable for noninvasive *in vivo* near-infrared (NIR) imaging.[15] To facilitate the *in vivo* NIR imaging of hydrogen peroxide, development of a chemiluminescent agent that can produce the NIR light in the presence of hydrogen peroxide is required.

Although the organic dyes have been widely investigated as emitters which produce the NIR, they have limitations for extended applications such as photobleaching and low photostability.[16, 17] In comparison to organic dyes, quantum dots (QDs) exhibit resistance to photobleaching and high quantum yield especially in the NIR wavelength region, allowing for a strong fluorescence signal.[18, 19] Even though QDs possess good photoluminescence properties, the practical use of QDs as an *in vivo* imaging agent was a highly controversial issue in the past due to their cytotoxicity. In recent years, polymer shell-covered and surface-modified QDs have been prepared to improve their biocompatibility.[20-22] For example, chemical conjugation of poly(ethylene glycol) (PEG) onto the surface of QDs have been demonstrated to significantly reduce cytotoxicity of QDs.[21, 22] As a result, these less toxic QDs have received attention as luminescence and fluorescence imaging probes. In this study, we designed hydrogen peroxide-responsive hybrid nanoparticles (HNPs) composed of the PEGylated QDs (PEG-QDs), which generate emission at a wavelength around 800 nm, and a luminol derivative (L012) as the CL agent. L012 exhibits 100-fold higher CL intensity than luminol in physiological conditions, implying that L012 can compensate for the above-mentioned drawbacks of luminol.[14, 23] In addition to hydrogen peroxide, L012 chosen as the

[a] Department of Health Sciences and Technology, SAIHST, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[b] Research Center for Convergence Nanobiotechnology (RC2NT), Korea Research Institute of Chemical Technology (KRICT), Daejeon 305-600, Republic of Korea
[c] Department of Polymer Science and Engineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[d] School of Chemical Engineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea

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luminol derivative is sensitive to the superoxide anion and peroxynitrite radicals.[24] We hypothesized that the L012-hydrogen peroxide system would acts as a chemiluminescence resonance energy transfer (CRET) donor while the PEG-QD acts as an acceptor. The resulting HNPs may allow for *in vivo* detection of ROS by generating the NIR, which implies that they have potential as the diagnostic agent for diseases involving overproduction of hydrogen oxide such as cancer, stroke, and rheumatoid arthritis (Fig. 1).

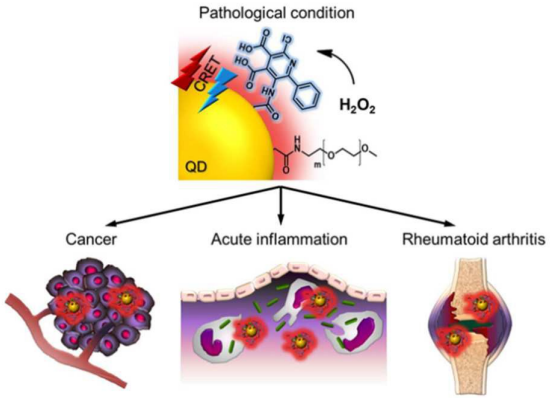

Fig. 1 Schematic illustration of HNPs as the diagnostic agent for diseases involving overproduction of hydrogen oxide.

HNPs were prepared by conjugating PEG and L012 onto the surface of commercial QDs through the amide bond in the presence of *N*-(3-dimethylaminopropyl)-*N*'-ethylcarboiimde (EDC) and *N*-hydroxysuccimide (NHS). The commercial QDs that have CdSeTe core with amphiphilic polymer coating showed minimal toxicity in other studies.[17, 25] The particle size of PEG-QDs (37 nm) was higher than that of bare QDs before PEGylation, implying that the presence of hydrophilic shell of PEG on QDs.[26] Meanwhile, the size of HNPs (36 nm) is similar to PEG-QDs, indicating that L012 might be contribute negligibly to size. (Fig. S1)

The chemical structures of PEG-QD and HNP were characterized using a FT-IR (Fig. S2). Both PEG-QD and HNP showed a peak in the region of 1640-1550 cm^{-1} (N-H bending) appearing from the amide bond between PEG and QD. Owing to the PEG chain, an aliphatic ether peak is also shown at 1150-1070 cm^{-1}.[27, 28] For HNPs, the characteristic peaks of L012 appeared at 690 cm^{-1} (C-H in benzene), 800-600 cm^{-1} (chloride vibration), 930 cm^{-1} (C-O stretching) and 1720 cm^{-1} (cyclic 6-membered C=O). The presence of L012 on HNPs was also confirmed using an elemental analyzer. The weight percentages of PEG-QD and HNP were 0.01 and 0.06, respectively. Since L012 has three nitrogen atoms in its structure, the weight percentages of nitrogen in HNP might be a bit higher.

The CL spectrum of L012 and fluorescence spectra of PEG-QD were monitored to verify the feasibility of energy transfer between L012 and PEG-QD (Fig. S3). L012 generated PL around 460-nm wavelength after reaction with hydrogen peroxide and PEG-QD showed maximum excitation wavelength at around 400-nm, emission wavelength range of NIR, 780-nm. The overlap between L012 CL spectrum and PEG-QD excitation spectrum could elucidate that the L012 would be adequate excitation energy source for NIR emission of PEG-QD.

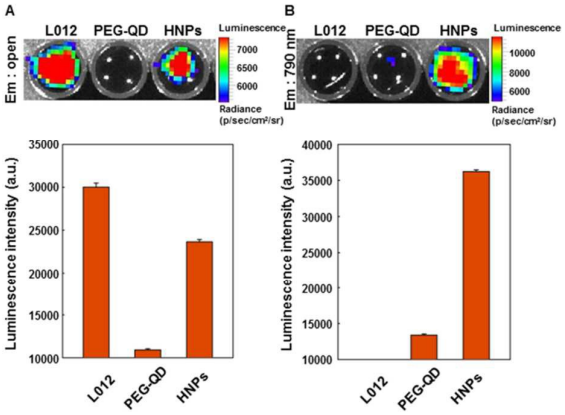

Fig. 2 CL images of L012, PEG-QD and HNPs in the presence of hydrogen peroxide: (A) open filter and (B) a 790-nm emission filter.

To estimate CRET between PEG-QD and L012, CL was observed using L012, PEG-QD, and HNPs in the presence of hydrogen peroxide. As shown in Fig. 2, for the case using an open filter, a CL signal was observed in all groups after treatment with hydrogen peroxide. L012 showed strong CL emission under an open filter, since luminol has emission at a 425-nm wavelength.[15] PEG-QD showed very weak CL signals because of their intrinsic properties: i.e., QDs can either catalyze the redox CL reaction or act as emitter species after direct oxidation.[29] Therefore, the weak signals might be due to its properties as an emitter. Since the image using an open filter was not enough to demonstrate CRET phenomena, we took an additional CL image at a wavelength of 790 nm. Interestingly, strong signals were observed in HNPs, whereas no signals were obtained in L012 itself (Fig. 2B), implying that CL energy from L012 of HNPs was transferred to PEG-QDs.

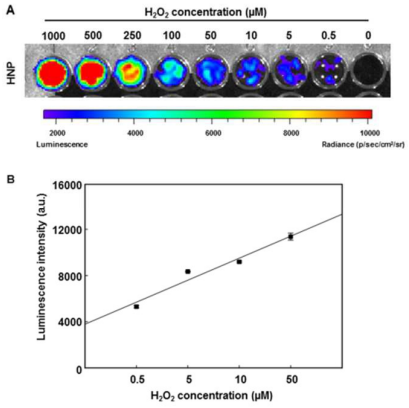

Fig. 3 (A) CL image and (B) the luminescence intensity at various concentrations of hydrogen peroxide.

We next examined the sensitivity of HNPs under various concentrations of hydrogen peroxide. As shown in Fig. 3A, the CL signals of HNPs are strong under a high level of hydrogen peroxide and become weaker as hydrogen peroxide concentration decreases. Especially at concentrations below 50 μ*M*, the light emission intensity exhibited linearity with the concentration of hydrogen peroxide (Fig. 3B and Fig. S4). This linear correlation indicates that HNPs could be used for quantification analysis of hydrogen peroxide under the pathological condition. Furthermore, since the detection limit of HNPs is contiguous to 0.5 μ*M*, it is expected that HNPs have sufficient sensitivity for detection of abnormal increase in the level of hydrogen peroxide.[30]

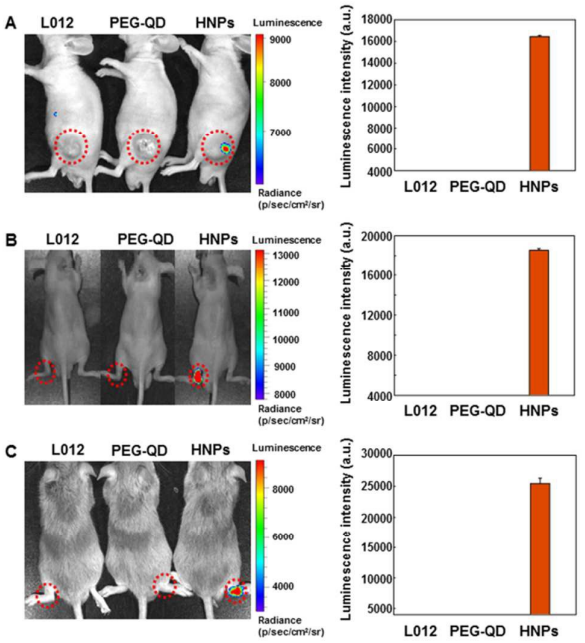

Fig. 4 Luminescent signals in (A) PC3 tumor-bearing mice, (B) LPS-induced inflammation mice and (C) CIA mice. The error bars represent standard deviation (*n*=3).

To validate the potential of HNPs as an *in vivo* diagnostic agent, we prepared three disease models associated with hydrogen peroxide including a tumor model, an acute inflammation model and an arthritis model.[31-34] For the tumor model, we used a human prostate cancer cell line (PC3). Prostate cancer is known for high levels of intracellular ROS and PC3 has an innate high production rate of hydrogen peroxide, compared to other prostate cancer cell lines.[31] Lipopolysaccharide (LPS)-induced inflammation was used to prepare the acute inflammation model,[32] and a collagen-induced arthritis (CIA) model was prepared for chronic inflammation.[33, 34] In the tumor model, CL images were obtained after intra-tumoral administration. As shown in Fig. 4A, HNPs exhibited a distinct and strong luminescent signal, while L012 and PEG-QD showed no signals. A strong luminescent signal may have been generated as HNPs reacted with hydrogen peroxide in the tumor site. The results demonstrated that only a luminescent signal from HNPs penetrated the skin and was then captured through a NIR wavelength filter. The quantified data corroborated the difference observed in the signals. Fig. 4B showed the CL images of early stage inflammation induced by LPS in a mouse ankle. The observed modality was the same as the result of the tumor model. To verify whether this aspect monitored late stage inflammation, a rheumatoid arthritis model was used. The CL imaging was captured right after the intra-articular administration of L012, PEG-QD, or HNPs. In the same manner, the signal was only detected in the mouse that was treated with HNPs (Fig. 4C). Comparing the quantified luminescent intensities of acute and chronic inflammation, those of late-stage inflammation are higher than those of the early stage. This might be due to the fact that chronic inflammation has more abundant and long-lasting ROS than acute inflammation.[35] The control group was prepared by subcutaneous injection of HNPs into normal mice. As anticipated, none of the mice showed the fluorescent signal from the injected site (Fig. S5.). This result suggests that HNPs are useful to detect abnormal levels of hydrogen peroxide in the disease site. Overall, specific and high CL signals from *in vitro* and *in vivo* conditions reveal that HNPs have promising potential as a diagnostic agent.

In summary, we prepared nanoparticles based on PEG-QD and L012 as the potential imaging agent to detect hydrogen peroxide. L012 and PEG were readily conjugated onto the QD surface in the presence of EDC and NHS. It was confirmed from an *in vitro* study that the HNPs could generate light as a consequence of energy transfer from a reaction between L012 and hydrogen peroxide. Furthermore, HNPs facilitated the visualization of hydrogen peroxide in the NIR wavelength region by a red shift in the CL under a low hydrogen peroxide concentration, corresponding to the level of indiscriminate cell growth. In addition, we demonstrated clear and distinct CL signals of HNPs from three different disease models, allowing for noninvasive *in vivo* NIR imaging of hydrogen peroxide in disease lesions without background noise. Our results indicate that the HNP is a promising diagnostic agent for hydrogen peroxide-associated diseases.

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