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Luminescent CeO₂:Eu³⁺ nanocrystals for robust *in situ* H₂O₂ real-time detection in bacterial cell cultures

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Abstract

Hydrogen peroxide (H₂O₂) quantification in biomedicine is valuable as inflammation biomarker but also in assays employing enzymes that generate or consume H₂O₂ linked to a specific biomarker. Optical H₂O₂ detection is typically performed through peroxidase-coupled reactions utilizing organic dyes that suffer, however, from poor stability/reproducibility and also cannot be employed *in situ* in dynamic complex cell cultures to monitor H₂O₂ levels in real-time. Here, we utilize enzyme-mimetic CeO₂ nanocrystals that are sensitive to H₂O₂ and study the effect of H₂O₂ presence on their electronic and luminescent properties. We produce and dope with Eu³⁺ these particles in a single-step by flame synthesis and directly deposit them on Si and glass substrates to fabricate nanoparticle layers to monitor in real-time and *in situ* the H₂O₂ concentrations generated by *Streptococcus pneumoniae* clinical isolates. Furthermore, the small CeO₂:Eu³⁺ nanocrystals are combined in a single-step with larger, non-responsive Y₂O₃:Tb³⁺ nanoparticles during their double-nozzle flame synthesis to engineer hybrid luminescent nanoaggregates as ratiometric robust biosensors. We demonstrate the functionality of these biosensors by monitoring their response in the presence of a broad range of H₂O₂ concentrations *in vitro* from *S. pneumoniae*, highlighting their potential for facile real-time H₂O₂ detection *in vitro* in cell cultures.

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Keywords

Nanozymes; rare-earth doped nanoparticles; hydrogen peroxide; flame nanoparticle synthesis

1 Introduction

Inorganic materials with enzyme-mimetic properties, the so-called nanozymes, are an emerging type of materials with decisive applications in healthcare (Wei and Wang, 2013). Among them perhaps the most well-known are CeO₂ nanoparticles (Walkey et al., 2015; Gupta et al., 2016) that exhibit both catalase and superoxide dismutase mimetic properties depending on the oxidation state of the Ce atoms on their surface (Lee et al., 2013; Li et al., 2015; Wang et al., 2015). Such CeO₂ crystalline nanoparticles (nanocrystals) readily interact with hydrogen peroxide (H₂O₂) in solution due to their redox potential (Lee et al., 2013; Wang et al., 2015). This type of interaction has led towards the development of H₂O₂ sensors, for example by their optical absorption (Lee et al., 2013), but also recently by doping these nanocrystals with a rare-earth ion (Eu³⁺) that renders them luminescent and by monitoring their luminescence quenching (Pratsinis et al., 2017). The small size of such CeO₂:Eu³⁺ nanocrystals enables them as highly-sensitive H₂O₂ biosensors with limit-of-detection (LOD) down to the nM levels (Pratsinis et al., 2017).

H₂O₂ plays a major role in biomedicine; for example high H₂O₂ concentrations are released as host defense mechanism during infection or inflammation *in vivo* (Halliwell et al., 2000) while its presence is correlated with several diseases (Yang et al., 2015; Yang et al., 2016). Furthermore, H₂O₂ quantification is important for *in vitro* enzyme-linked assays (*e.g.* ELISA) that generate or consume H₂O₂ rendering it also a potential intermediary biomolecule for detection of other disease-specific biomarkers (de la Rica and Stevens, 2012). Low LODs are attractive, however, robustness and stability of biosensors becomes increasingly more important due to the complexity of biological systems that typically contain biomolecules, organisms and metabolic byproducts (Duee et al., 2015; Liu et al., 2015). Even though several electrochemical methods to detect H₂O₂ at low concentrations have been reported (Gupta et al., 2017), commercially-available assays for the optical detection typically utilize enzyme-based dyes (colori-/fluorimetric detection) through peroxidase-coupled reactions. These assays suffer from low optical and chemical stability, precision and detection speed and cannot be employed to monitor in real-time H₂O₂ concentrations *in situ* during *in vitro* biological experiments with cells (Duee et al., 2015). These drawbacks have prompted several groups to develop alternative materials for H₂O₂ sensing such as lanthanide coordination polymer-based ones (Zeng et al., 2016; Zeng et al., 2017) but also sensors from inorganic materials, and thus more stable than organic dyes, luminescent nanomaterials that are sensitive to H₂O₂ for its optical detection, such as CeO₂:Eu³⁺ nanocrystals (Yuan et al., 2009; Lv et al., 2014; Zheng et al., 2014; Duee et al., 2015; Pratsinis et al., 2017).

Here, we rationally design and produce small (~5 nm) CeO₂:Eu³⁺ nanocrystals by flame spray pyrolysis, a nanomanufacturing process famous for its scalability and reproducibility (Pratsinis, 2010). We perform detailed morphological characterization and further study their

interaction with H_2O_2 with electron paramagnetic resonance spectroscopy to identify the electronic changes in the $\text{CeO}_2\text{:Eu}^{3+}$ nanocrystals. Furthermore, we fabricate functional nanoparticle films on Si and glass substrates by flame aerosol deposition and *in situ* flame annealing (Tricoli et al., 2008) of the freshly-formed $\text{CeO}_2\text{:Eu}^{3+}$ nanocrystals and monitor in real-time the effect of H_2O_2 on their luminescence in bacterial cell cultures *in situ* with *Streptococcus pneumoniae* (the pneumococcus) clinical isolates. *S. pneumoniae* is a main cause of pneumonia, sepsis and meningitis and one of the most common cause of death worldwide (Henriques-Normark and Tuomanen, 2013; Iovino et al., 2016). Some clinically-relevant *S. pneumoniae* strains generate high levels of H_2O_2 as part of their aerobic metabolism. The produced H_2O_2 can inhibit the growth of other nasopharyngeal flora members such as *Haemophilus influenzae* and *Neisseria meningitidis* and can have cytotoxic effect both *in vivo* and *in vitro* on human epithelial and endothelial cells (Andisi et al., 2012). Pneumococci convert pyruvate to acetyl phosphate and H_2O_2 via a pyruvate oxidase enzyme encoded by the *spxB* gene (Spellerberg et al., 1996; Syk et al., 2014).

Finally, we further explore the suitability of flame nanoparticle synthesis towards the development of a ratiometric H_2O_2 biosensor based on a single-step double-nozzle flame spray pyrolysis (Strobel et al., 2006) of multicomponent nanoparticles. Double-nozzle flame synthesis is one of the latest developments of this nanomanufacture process (Pratsinis, 2010) that allows for the production of multicomponent nanoparticle systems with independent control of the product particle size and morphology from each nozzle (Grossmann et al., 2015). This technology has been used so far only for production of nanoparticles tailored for catalysis (Buchel et al., 2009). Here, we extend this process for the fabrication of bioresponsive materials and we explore the synthesis of enzyme-mimetic luminescent and H_2O_2 -responsive $\text{CeO}_2\text{:Eu}^{3+}$ nanoparticles that are mixed in the nanoscale within the same agglomerates/aggregates with larger luminescent non-responsive (~ 30 nm) $\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ nanoparticles. Both components from this nanoparticle system are excited at the same near-UV wavelength and their emission wavelengths are monitored as a function of H_2O_2 concentrations. The robustness of these biosensors is further explored under realistic conditions by detecting H_2O_2 concentrations *in vitro* from *S. pneumoniae* cultures.

2 Materials and Methods

2.1 Particle synthesis and characterization

CeO_2 and Y_2O_3 nanoparticles were produced by flame spray pyrolysis (FSP), by dispersing a liquid precursor solution containing 0.3 M cerium 2-ethylhexanoate (49 % in 2-ethylhexanoic acid, Alfa Aesar) and 5 at% europium nitrate hexahydrate (99.9 %, Alfa Aesar), or 0.4 M yttrium nitrate hexahydrate (99.8 %, Aldrich) and 2.5 at% terbium nitrate hydrate (99.9 %, Alfa Aesar) dissolved in a 1:1 ratio of 2-ethylhexanoic acid (99 %, Alfa Aesar) and ethanol (99.96 %, Alfa Aesar). The dual nozzle nanoparticles were synthesized using the previously described precursors and the same process with an alternative set-up, described by Strobel et al. with a flame angle of 60 degrees. The solution was fed through a capillary from 100 mL syringes (SGE Analytical Science) by syringe pumps (New Era Pump Systems, Inc.) at rates of 3 or 5 mL/min and 12 mL/min, respectively. The precursor was subsequently dispersed by an oxygen (>99.5 %, AGA Gas AB) flow at a rate of 8 or 5

L/min and 3 L/min (EL-FLOW Select, Bronkhorst), respectively. The spray was ignited by a premixed support flame of methane/oxygen (>99.5 %, AGA Gas AB) at flow rates of 1.5 L/min and 3.2 L/min respectively. The particles were collected on a glass fiber filter (Albet LabScience) with the aid of a Mink MM 1144 BV vacuum pump (Busch). Nanoparticle films were deposited on Si and glass substrates by mounting them on a water-cooled substrate holder and placing them 18 cm above the nozzle for 60 s. The films were subsequently flame annealed by an impinging particle-free (ethanol) flame by placing the substrates 15 cm above the nozzle for 15 s. The Si-substrates were mounted on a custom-made 3D printed (Wanhao Duplicator 4S) flow cell (ABS) and placed inside the spectrophotometer. For deposition on glass substrates, deposition time was 45 s at 18 cm above the nozzle and annealed for 10 s at 15 cm above the nozzle.

The powder X-ray diffraction of the particles was performed with a Rigaku MiniFlex (1.5406 Å Cu K α 1 radiation; step size 0.01°). Rietveld refinement was performed using the Rigaku software. The specific surface area (SSA) was determined using nitrogen absorption performed on a Gemini VII 2390 (Micromeritics) after degassing at 150°C for at least 3 hours. Scanning electron microscopy was performed on a Zeiss Ultra 55 equipped with field emission gun (FESEM). Scanning transmission electron microscopy (STEM) and EDX analysis was performed with an aberration-corrected TEM (Titan Themis) operated with an acceleration voltage of 200 kV. The chemical analysis is performed by STEM-EDX and the beam current was set to 24 nA for an acquisition time of 30 minutes. Quantification was performed on the Esprit (Bruker), after background subtraction and deconvolution on different X-ray lines. For the TEM, nanoparticles were suspended in ethanol by bath ultrasonication and one drop of the suspension was deposited onto a 3 mm carbon coated copper grid. The thickness of the carbon film is about 10 nm. The TEM grid was dried in the air for 14 hours. Before the TEM measurements, 2 minutes plasma cleaning was applied on the samples in order to remove hydrocarbon contamination. The size of nanoparticles is measured by manually drawing an intensity profile across one nanoparticle and 90% threshold value is chosen to measure the diameter of nanoparticles. The measurement error is in a range of ± 0.14 nm. EPR spectra were recorded with a Bruker ER200D spectrometer at liquid N₂ temperature 77K. EPR spectra were recorded for 10mg of nanoparticles in quartz tubes (Suprasil, 5mm, Wilmad Glass). The estimated concentration of the various Ce³⁺ centers per mass of material, the EPR signal intensity of each species was quantified based on numerical simulation and double integration of the signal and spin calibration using DPPH. EPR conditions: modulation amplitude 6 Gpp, modulation frequency 100 KHz. The evolution of the Ce³⁺ and Eu²⁺ centers before and addition of H₂O₂ were monitored *in situ* by freeze quenching the sample at 77 K after a predefined incubation time. X-ray photoelectron spectroscopy (XPS) spectra were recorded using SPECS spectrometer equipped with a twin Al-Mg anode X-ray source and a multichannel hemispherical sector electron analyzer (HSA-Phoibos 100). A monochromatized Mg K α line at 1253.6 eV and analyzer pass energy of 20 eV were used in all XPS measurements. The binding energies were calculated with reference to the energy of C1s carbon peak at 284.5 eV. The surface composition of all samples in terms of atomic ratios was calculated, using a Shirley background and empirical cross section factors for XPS. Dynamic light scattering was

performed with a Malvern Zetasizer upon the nanoparticle dispersion in aqueous solutions by ultrasonication.

2.2 Phosphorescence characterization and H₂O₂ sensing

Emission and excitation spectra, as well as sensor response measurements were performed using a LS 55 spectrophotometer (Perkin Elmer) with the well plate reader accessory and black, flat bottom, 96-well plates (Perkin Elmer). All measurements were performed in solution, either MilliQ DI water (Advantage A10, Millipore), or C+Y bacteria medium with varying nanoparticle and H₂O₂ concentrations. Different concentrations of H₂O₂ were titrated from a standard solution (Alfa Aesar, 35% w/w aq. sol.) and stored for maximum 24 hours at 4°C. Slit width was held constant throughout the measurements at 15 nm for excitation and 20 nm for emission. The single read measurements were averaged over 1 second for each measured wavelength. The photomultiplier voltage was set to 900 V for all measurements and neither excitation nor emission filter were used. Excitation light bulb settings were held constant throughout the measurements with gate time of 5 ms and delay of 0.1 ms. Ratiometric sensor response S_R was calculated by $S_R = 1 - (R_S/R_0)$, where R_S is the ratiometric (I_{590}/I_{545}) signal of samples with various H₂O₂ concentrations, and R_0 is the ratiometric signal of pure H₂O.

2.3 H₂O₂ production by *S. pneumoniae* clinical isolates and *in vitro* biosensing

All pneumococcal strains were cultured on blood agar plates for 24 hours and in semisynthetic C+Y medium and incubated overnight at 37 °C and 5 % CO₂. The strains were grown until mid-log phase (OD₆₂₀ 0.3-0.4) in liquid culture. For H₂O₂ measurements, the strains were lysed using penicillin (Sigma Aldrich) and stored at 4°C. Nanoparticles in C +Y solution (2 g/L) were added to the bacterial cultures to a final concentration of 1 g/L. After 30 minutes of incubation time at 4°C and vortex mixing every 5 minutes, the solutions were measured under the same conditions as the H₂O₂ calibration curve. Calculation of the H₂O₂ concentration was performed by logarithmic interpolation between two measurement points from the calibration curve.

2.4 High-resolution fluorescence microscopy

Nanoparticle films were deposited on Si and glass substrates that were placed at the bottom of multi-well plates. Imaging was performed with DV Elite microscope (Applied Precision) using a scientific complementary metal-oxide-semiconductor (sCMOS) camera. Images were acquired using Softworx (Applied Precision). Luminescence quenching was analyzed performing continuous imaging for 90 minutes (one snapshot every 15 seconds), using 100X magnification objective, excitation at 350 nm wavelength and emission at 594 nm wavelength.

3 Results and Discussion

3.1 Particle morphology and interaction with hydrogen peroxide

Enzyme-mimetic CeO₂ nanocrystals are made by flame spray pyrolysis and doped in a single step with Eu³⁺ ions (5 at%) (Pratsinis et al., 2017). The crystal structure of the as-prepared nanoparticles is evaluated by X-ray powder diffraction (XRD) as shown in Figure

1a, in which the characteristic cubic CeO₂ (solid circles, ICSD: 61595) crystal phases are present. There are no separate XRD peaks corresponding to any Eu-oxide crystal phases, indicating the incorporation of Eu³⁺ in the CeO₂ crystal lattice. The average crystallite size of the CeO₂:Eu³⁺ nanoparticles is calculated by Rietveld refinement analysis and corresponds to dXRD = 4.1 nm, in agreement to the literature for such nanoparticles made by identical process conditions (Pratsinis et al., 2017). The specific surface area of the CeO₂:Eu³⁺ nanoparticles as determined by N₂ adsorption is 185 m²/g that would correspond to an average primary particle diameter (Sauter mean diameter) d_{BET} = 4.4 nm, assuming monodispersed solid spherical particles with homogeneous density.

The primary particle size is also evaluated by particle counting in high-angle annular dark-field scanning transmission electron microscopy (STEM-HAADF) images and their elemental composition is further validated with energy-dispersive X-ray spectroscopy (EDX). The average size of the CeO₂:Eu³⁺ determined by electron microscopy is d_{TEM} = 5.3 nm (Supplementary material, Figures S1-S2) and is in good agreement with the dXRD and dBET. Importantly, the STEM-EDX elemental mapping (Supplementary material, Figure S3) reveals the homogeneous distribution of Eu within the nanoparticles, validating that the Eu³⁺ ions are incorporated within the CeO₂ crystal matrix and do not form segregated domains. The elemental composition as determined by the EDX analysis is also in good agreement with the nominal one (5.76 at% Eu-content vs 5 at%) highlighting the efficiency of flame nanoparticle synthesis.

When Eu³⁺ ions are located inside the CeO₂ crystalline host matrix these nanocrystals become luminescent (Pratsinis et al., 2017). Figure 1b shows the excitation (broken line, emission λ = 590 nm) and emission spectra (solid line, excitation λ = 330 nm) of the CeO₂:Eu³⁺ nanocrystals. The emission peaks of the nanocrystals due to Eu³⁺ are attributed to the ⁵D₀-⁷F_J transitions (Kumar et al., 2009; Li et al., 2009), with the peak at ~590 nm from the magnetic dipole transition ⁵D₀-⁷F₁ (Kumar et al., 2009). The excitation spectrum (Figure 1b, broken line) has a broad band originating from the charge transfer between Ce-O (Li et al., 2009), that extends, however, to the near-UV region enabling the detection of such nanocrystals with conventional fluorescence microscopes (Spyrogianni et al., 2018), as it will be demonstrated later on.

Figure 2a shows the emission spectra before (solid line) and after (broken line) H₂O₂ addition in an aqueous suspension containing the CeO₂:Eu³⁺ nanocrystals. The catalase-mimetic activity of these nanocrystals induces a drastic quenching of the main emission peak at λ = 590 nm upon addition of H₂O₂ (Pratsinis et al., 2017). This has been attributed to the interaction of oxygen species with the CeO₂:Eu³⁺ surface that may affect the charge distribution (Cafun et al., 2013), Ce-O coordination number (Wang et al., 2015) and the oxidation state of Ce (Lee et al., 2013).

To better understand this interaction, we perform electron paramagnetic resonance (EPR) spectroscopy on these CeO₂:Eu³⁺ nanocrystals. Figure 2b shows the EPR spectrum of the CeO₂:Eu³⁺ nanocrystals. Several characteristic species are detected: (i) bulk Ce³⁺ ($s = 5/2$), (ii) Ce³⁺ - O - Ce⁴⁺, (iii) Ce³⁺ ($s = 5/2$) with an E/D ratio of 0.1 and (iv) several Eu²⁺ centers, presumably on the surface of the nanocrystals that have been reduced from Eu³⁺.

The Ce^{3+} species with $E/D = 0.1$ are observed for the first time, to the best of our knowledge, in CeO_2 -based nanoparticles. From these EPR spectra it is possible to quantify the concentration of each species, and each concentration is presented in Figure 2c before and after H_2O_2 addition. In the absence of H_2O_2 the Ce^{3+} with $E/D = 0.1$ have the highest concentration in these samples while the remaining species have lower concentrations (Figure 2c). Upon the addition of H_2O_2 there are some differences observed. The concentration of most species is significantly reduced indicating its strong interaction with the $\text{CeO}_2:\text{Eu}^{3+}$ nanocrystals. To get a clearer image of the changes, the concentration decrease of each species can be calculated as a percentage (%) in Figure 2d. From this decrease (%) it is concluded that the bulk Ce^{3+} ions are not significantly affected by the presence H_2O_2 , while all other species are affected. More specifically, the largest decrease (~70%) is obtained from the newly-discovered Ce^{3+} with $E/D = 0.1$ that are oxidized to Ce^{4+} , while both the Eu^{2+} surface ions and the $\text{Ce}^{3+} - \text{O} - \text{Ce}^{4+}$ ions are oxidized to Eu^{3+} and Ce^{4+} , respectively. The surface compositions and valence states of the individual $\text{CeO}_2:\text{Eu}^{3+}$ and $\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ nanoparticles as well as of their hybrid nanoaggregates $\text{CeO}_2:\text{Eu}^{3+}/\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ have also been measured by X-ray photoelectron spectroscopy corroborating the EPR results (Supplementary material, Figure S4).

3.2 Robust *in situ* biosensing in bacterial cell cultures

The strong luminescence quenching in the presence of H_2O_2 renders the $\text{CeO}_2:\text{Eu}^{3+}$ nanocrystals powerful biosensors with limit-of-detection (LOD) down to the nM range (Pratsinis et al., 2017) outperforming most particle-based H_2O_2 biosensors in the literature, however, in this study we focus not only their sensitivity but also on their superior robustness to detect H_2O_2 levels *in situ* and in real-time during biological experiments. Therefore, and perhaps more importantly, the inorganic nature of the material enables it to be rather robust and stable in dynamic environments. To further evaluate the potential of the developed $\text{CeO}_2:\text{Eu}^{3+}$ H_2O_2 biosensors, we directly *in situ* deposit the freshly-formed $\text{CeO}_2:\text{Eu}^{3+}$ during nanoparticle synthesis by inserting a water-cooled substrate above the hot aerosol (Figure 3a). The nanoparticles stochastically self-assemble due to thermophoresis on that substrate covering large areas homogeneously (Madler et al., 2006). The structural stability of the self-assembled nanoparticle film can be enhanced by employing an *in situ* flame annealing step (Tricoli et al., 2008). Figure 3b shows an SEM image of such a film deposited on a Si substrate highlighting the homogeneity and porous structure of this aerosol-deposited particle film. In fact, *in situ* flame annealing makes it possible for such porous nanoparticle films to be immersed in liquids without any particle film restructuring and/or loss (Sotiriou et al., 2013). Figure 3c shows an SEM image of such a particle film after its immersion in aqueous solution in the presence of H_2O_2 . The morphology is practically identical validating that flame annealing renders such particles films stable in liquids.

Upon mounting such a functional substrate in a custom-made flow cell made by additive manufacturing (Figure 3d, inset), we can read-out the luminescence signal of the particle film and the sensor response can be monitored in real-time under flow of various H_2O_2 concentrations. Figure 3d shows the sensor response (luminescence intensity decrease) of such a nanoparticle film deposited on a Si substrate. The sensor response gradually changes

for increasing H_2O_2 concentrations and reaches a plateau for each one only after a few minutes. Interestingly, upon flowing pure water after the sensing experiment, the sensor response returns to the initial value, indicating that the effect of the H_2O_2 species on the $\text{CeO}_2\text{:Eu}^{3+}$ nanoparticle surface is reversible (Supplementary Information, Figure S5), but also highlighting that the developed biosensors here may be re-used several times.

To further evaluate the robustness of the developed biosensing particle films in realistic biological experiments, we utilize a rather complex biological system consisting of *S. pneumoniae* that generate H_2O_2 by converting pyruvate to acetyl phosphate and hydrogen peroxide via a pyruvate oxidase enzyme encoded by the *spxB* gene (Spellerberg et al., 1996; Syk et al., 2014). The excitation band of the $\text{CeO}_2\text{:Eu}^{3+}$ nanocrystals extends to the near-UV (Figure 1b, broken line) which renders it possible to utilize existing filter sets (Spyrogianni et al., 2018) in conventional fluorescence microscopes to detect their luminescence. So, upon placing the nanoparticle films (this time deposited on transparent glass coverslips) on the bottom of the microplate wells of bacterial cell cultures with *S. pneumoniae* (serotype 1 BHN32 strain (Syk et al., 2014)) and incubating them at 37 °C (Figure 3e) in casitone and yeast solution (C+Y medium), we can monitor the luminescence intensity *in situ* with a fluorescence microscope. Figure 3f show the characteristic red color emission from the $\text{CeO}_2\text{:Eu}^{3+}$ nanoparticle film just at the beginning of the incubation period ($t = 0$ min). However, the luminescence intensity drastically decreases over time (Figure 3g, $t = 45$ min) becoming almost completely diminished after $t = 90$ min (Figure 3h), after which time the *S. pneumoniae* population has grown generating large amounts of H_2O_2 . Therefore, the robustness of the developed biosensing nanocrystals here is validated at realistic conditions by monitoring H_2O_2 concentrations *in situ* in bacterial cell cultures.

3.3 Ratiometric hydrogen peroxide biosensing

The versatility of flame nanoparticle synthesis allows also for the production of multicomponent systems. Thus, we engineer a ratiometric H_2O_2 biosensor that allows for the rationing of the sensor signal providing a way for built-in correction and, thus, is advantageous over biosensors that utilize a single emission wavelength (Liu et al., 2011; Feng et al., 2012). That way, a non-responsive and rather stable luminescent material is employed to provide the reference signal and is combined with the $\text{CeO}_2\text{:Eu}^{3+}$ H_2O_2 -sensitive nanocrystals. For this, a double-nozzle setup is employed for the single-step production of nano-mixed $\text{CeO}_2\text{:Eu}^{3+}/\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ (2.5 at% Tb^{3+}) (Sotiriou et al., 2012) hybrid nanoaggregates (Figure 4a). The precursor molarity and flame conditions (liquid precursor feed rate, O_2 dispersion gas, please see also Experimental section) are adjusted to keep the small size of $\text{CeO}_2\text{:Eu}^{3+}$ but yield ~30 nm $\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ nanoparticles (Sotiriou et al., 2011; Pratsinis et al., 2017). The primary particle sizes correlate with the short and long flame heights (Supplementary material, Figure S6) of the $\text{CeO}_2\text{:Eu}^{3+}$ and $\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ flames, respectively. Long flame heights correspond to longer residence times at high temperatures of the particles within the flame yielding, thus, larger particle sizes (Madler et al., 2002). In the double-nozzle setup, the angle of the two nozzles is adjusted at 60° to ensure that the product nanoparticles are only mixed in the nanoscale and do not form solid solutions or mixed crystal phases that would occur in higher angles (Strobel et al., 2006). One of the main advantages of the double nozzle flame synthesis here over the single nozzle

synthesis of the two individual components and their simple mechanical mixing is that the hybrid nanoaggregates are produced in a single-step, essentially halving the required nanoparticle production time and at the same time all components are present within the same nanoaggregate enabling also their utilization as particle-based sensors in fluorescence microscopes. Furthermore, pure $\text{CeO}_2\text{:Eu}^{3+}$ nanoparticles are known to form rather large agglomerates of several hundred nanometers (Pratsinis et al., 2017) upon their dispersion in aqueous and biological buffer solutions due to their isoelectric point at pH 7.6 (Gulicovski et al., 2014). However, a further advantage of the hybrid $\text{CeO}_2\text{:Eu}^{3+}/\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ nanoaggregates here is the tuning of their hydrodynamic diameter to significantly lower sizes (Supplementary Information, Table S1 and Figure S7).

The crystallinity of the nano-mixed $\text{CeO}_2\text{:Eu}^{3+}/\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ nanoparticles is also examined by XRD in Figure 4b. For these nanoparticles, the cubic phase Y_2O_3 peaks (inverse triangles, ICSD: 26190) dominate the pattern, however, the CeO_2 peaks are also present (solid circles, ICSD: 61595). Rietveld refinement analysis reveals the average crystal size as well as mass fraction wt% of each crystal phase from the $\text{CeO}_2\text{:Eu}^{3+}$ and $\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ nanoparticles that is 6 and 32.5 nm, respectively. Furthermore, the calculated mass fractions of $\text{CeO}_2\text{:Eu}^{3+}$ and $\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ crystal phases from the XRD pattern is in excellent agreement with the nominal ones at 22 and 78 wt%, respectively (Supplementary material, Table S2). The nominal mass fractions of each individual component were tuned by adjusting the precursor molarity and liquid precursor feed rate and were selected after optimization to ensure sufficient emission intensity from the non-responsive $\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ along with the responsive $\text{CeO}_2\text{:Eu}^{3+}$ nanoparticles (Supplementary material, Figure S8). These results indicate that both the $\text{CeO}_2\text{:Eu}^{3+}$ and $\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ nanoparticles are present in the nano-mixed aggregates and this is further corroborated by the specific surface area (SSA) of the $\text{CeO}_2\text{:Eu}^{3+}/\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ nanoparticles that falls in between the SSA values of the individual samples (Supplementary material, Table S2).

The morphology and composition of the $\text{CeO}_2\text{:Eu}^{3+}/\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ nanoaggregates is further studied by STEM-HAADF along with EDX. Figure 5a shows an STEM-HAADF image of the $\text{CeO}_2\text{:Eu}^{3+}/\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ nanoaggregates in which both large and small nanoparticles can be detected. Figure 5a also shows the elemental mapping of this image tracking the presence of Y (green signal), Ce (red signal) and both Y and Ce (composite image), validating that both small $\text{CeO}_2\text{:Eu}^{3+}$ and large $\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ nanoparticles are present in the $\text{CeO}_2\text{:Eu}^{3+}/\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ nanoaggregates and that they are segregated (for EDX spectrum see Supplementary material, Figure S9). These nanoaggregates exhibit a bimodal particle size distribution (Figure 5b) with a mode around 5 nm and another one around 30 nm in excellent agreement with the particle size distributions of the individual samples (Supplementary material, Figures S2 and S10,S11), additionally verifying that both the small $\text{CeO}_2\text{:Eu}^{3+}$ and large $\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ nanoparticles are present in the nanoaggregates.

Both $\text{CeO}_2\text{:Eu}^{3+}$ and $\text{Y}_2\text{O}_3\text{:Tb}^{3+}$ are luminescent upon the same UV excitation wavelength $\lambda = 330$ nm. The main emission wavelength due to the Tb^{3+} ions in Y_2O_3 crystal host matrix is at 545 nm ($^5\text{D}_4\text{-}^7\text{F}_5$ transition) (Mukherjee et al., 2008) distinctly different that the one due to the Eu^{3+} ions in CeO_2 crystal host matrix around 590 nm ($^5\text{D}_0\text{-}^7\text{F}_1$ transition) (Kumar et al., 2009; Li et al., 2009). Figure 6a indeed shows the emission spectra of aqueous

suspension from the $\text{CeO}_2:\text{Eu}^{3+}/\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ nanoaggregates upon excitation at 330 nm, before (solid lines) and after 1mM H_2O_2 addition (broken lines). The luminescence intensity of the $\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ is not affected by the addition of H_2O_2 , however, the one from $\text{CeO}_2:\text{Eu}^{3+}$ is significantly quenched. Thus, monitoring the ratio of the two emission intensities at wavelengths $\lambda = 590$ and 545 nm (I_{590}/I_{545}) allows for ratiometric H_2O_2 detection.

The potential of such a ratiometric H_2O_2 biosensor is further examined by monitoring the sensor response R as a function of a wide range of H_2O_2 concentration in C+Y medium. Figure 6b shows this sensor response curve (yellow circles) calculated ratiometrically. Sensor response S_R is calculated by $S_R = 1 - (R_S/R_0)$, where R_S is the ratiometric (I_{590}/I_{545}) signal of samples with various H_2O_2 concentrations, and R_0 is the ratiometric signal of pure H_2O . The H_2O_2 response concentration range spans several orders of magnitude, rendering this H_2O_2 biosensor suitable for studies where H_2O_2 concentrations are unknown. It should be noted that the $\text{CeO}_2:\text{Eu}^{3+}$ nanoparticles that are responsive to H_2O_2 exhibit high selectivity over a wide range of other analytes such as ions (K^+ , Na^+ , PO_4^{3-} , Cl^- , Fe^{3+} , NO_3^-), proteins (serum) and negatively charged and neutral amino acids, but there is some interference from antioxidants (e.g. uric acid, ascorbic acid) and positively charged amino acids (Pratsinis et al., 2017). Nonetheless, the response curve in Figure 6b indicates that our biosensor is functional in biological buffers over a rather broad (>4 orders of magnitude) range. Furthermore, it should be noted that the developed nanoaggregates here exhibit high optical and chemical stability due to their inorganic nature (Supplementary material, Figure S12) in agreement with the literature for the single $\text{CeO}_2:\text{Eu}^{3+}$ nanoparticles (Pratsinis et al., 2017).

Furthermore, we test two pneumococcal serotype 1 clinical isolates that generate high amounts of H_2O_2 (BHN31 and BHN32) as well as one genetically-modified strain that lacks the *spxB* gene (*spxB* mutant strain, BHN123) and, thus, does not generate H_2O_2 (Syk et al., 2014). After incubation of the strains in C+Y medium to reach mid-log phase (OD_{620} : 0.3-0.4) we neutralize the growth by adding penicillin and measure the produced H_2O_2 concentrations using our ratiometric biosensors. Figure 6b also shows the sensor response of the BHN31 (blue square), BHN32 (green triangle) and BHN123 (red diamond) that is fitted onto the sensor response curve by logarithmic interpolation between the two adjacent data points. The resulting H_2O_2 concentrations of the three individual strains is also plotted in Figure 6c (red bars) and directly compared with the values reported in the literature acquired by conventional peroxidase-based assays (blue bars) (Syk et al., 2014). The H_2O_2 determination from our biosensors is in excellent agreement with the reported values in the literature, with lower standard deviation, further highlighting the functionality of the developed all inorganic nanoparticle-based H_2O_2 biosensors here and verifies their suitability as a robust H_2O_2 sensing tool.

4 Conclusions

We present here the rational design and flame spray synthesis of $\text{CeO}_2:\text{Eu}^{3+}$ nanocrystals and evaluate their performance as a luminescent robust H_2O_2 biosensor. The small size of the developed $\text{CeO}_2:\text{Eu}^{3+}$ nanocrystals facilitates their interactions with H_2O_2 in solution drastically affecting their electronic and luminescent properties. Upon the direct nanoparticle

aerosol deposition on Si and glass substrates during flame synthesis, functional nanoparticle films can be produced that may be employed in bacterial cell cultures to detect *in situ* in real-time H_2O_2 concentrations with a fluorescence microscope. Furthermore, double-nozzle flame synthesis is employed to develop nanoaggregates that consist of H_2O_2 -sensitive $\text{CeO}_2:\text{Eu}^{3+}$ nanoparticles, and non-responsive larger $\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ nanoparticles. Even though the luminescence intensity of the $\text{CeO}_2:\text{Eu}^{3+}$ nanocrystals is drastically quenched in the presence of H_2O_2 , the luminescence of the $\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ nanoparticles is unaffected rendering these hybrid nanoaggregates robust optical-based ratiometric H_2O_2 biosensors. Both components are luminescent upon the same UV excitation wavelength simultaneously, avoiding the need to perform two separate measurements and correlate them afterwards in a second step. Furthermore, the developed biosensor here consists of purely inorganic materials, and thus, it is highly robust with long shelf-life in ambient conditions and it is further suitable for autoclave processes without the risk of degradation. The biosensor response is examined here by monitoring the luminescence intensity of the two emission wavelengths corresponding to the $\text{CeO}_2:\text{Eu}^{3+}$ and $\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ nanoparticles, respectively, in a broad range of H_2O_2 concentrations in biological-relevant solutions. In fact, the biosensor performance is explored beyond simple biosensing in buffer solutions towards measurements under realistic conditions in complex bacterial cell cultures *in vitro* by detecting the generated H_2O_2 concentration from clinically-relevant *S. pneumoniae* strains. The luminescent $\text{CeO}_2:\text{Eu}^{3+}$ developed here could set the basis for facile and robust H_2O_2 detection using *in vitro* assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) X-ray diffraction patterns of the $\text{CeO}_2:\text{Eu}^{3+}$ nanocrystals, along with the diffraction peak positions for the cubic CeO_2 (standard ICSD card shown in red lines). The average crystallite size d_{XRD} is also shown as determined by Rietveld refinement analysis. (b) The excitation (broken line, emission wavelength $\lambda = 590$ nm) and emission spectra (solid line, excitation wavelength $\lambda = 330$ nm) of the $\text{CeO}_2:\text{Eu}^{3+}$ nanocrystals. The excitation bands are attributed to Ce-O charge transfer (Ce-O CT), while the emission peaks to the Eu^{3+} ions $^5\text{D}_0$ - $^7\text{F}_j$ transitions.

Figure 2.

(a) Emission spectra of aqueous suspensions upon excitation at $\lambda = 330$ nm before (solid lines) and after H_2O_2 (1 mM) addition (broken lines) for the $\text{CeO}_2:\text{Eu}^{3+}$ nanocrystals exhibiting the drastic luminescence quenching in the presence of H_2O_2 . (b) EPR spectrum of the $\text{CeO}_2:\text{Eu}^{3+}$ nanocrystals. (c) Concentrations of different species detected by the EPR spectra before and after H_2O_2 addition. (d) Decrease (%) of each species after the addition of H_2O_2 .

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Figure 4.

(a) Schematic of double-nozzle flame reactor for the synthesis of the nano-mixed hybrid $\text{CeO}_2:\text{Eu}^{3+}/\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ nanoaggregates. The small $\text{CeO}_2:\text{Eu}^{3+}$ nanoparticles are made in the short flame while the large $\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ nanoparticles by the long flame. The angle between the nozzle is kept at 60° to facilitate the nano-mixing of the as-prepared individual nanoparticles within the same nanoaggregates. (b) X-ray diffraction patterns of the nano-mixed $\text{CeO}_2:\text{Eu}^{3+}/\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ nanoaggregates, along with the diffraction peak positions for both the cubic Y_2O_3 (inverse triangles) and cubic CeO_2 (circles). The average crystallite sizes for each component are also displayed.

Figure 5.

(a) High-angle annular dark-field scanning transmission electron microscopy (STEM-HAADF) of the nano-mixed $\text{CeO}_2:\text{Eu}^{3+}/\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ and its elemental mapping for Ce in red, for Y in green, Ce and Y in a composite image verifying the presence of small $\text{CeO}_2:\text{Eu}^{3+}$ and large $\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ nanoparticles in these nanoaggregates. (b) The measured particle size d_{TEM} distribution for $n = 92$. There are both large and small crystalline nanoparticles observed with a bimodal particle size distribution.

Figure 6.

(a) Emission spectra of aqueous suspensions upon excitation at $\lambda = 330$ nm before (solid lines) and after H_2O_2 (1 mM) addition (broken lines) for nano-mixed $\text{CeO}_2:\text{Eu}^{3+}/\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ nanoaggregates. The $\text{CeO}_2:\text{Eu}^{3+}/\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ nanoaggregates exhibit the emission from both components attributed to $\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ ($\lambda = 545$ nm) and $\text{CeO}_2:\text{Eu}^{3+}$ ($\lambda = 590$ nm) enabling the ratiometric determination of H_2O_2 concentrations. (b) H_2O_2 sensor response determined ratiometrically from the $\text{CeO}_2:\text{Eu}^{3+}/\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ nanoaggregates in the presence of a broad range of H_2O_2 concentrations in C+Y cell culture medium (n = 3). The sensor signal from

bacterial cultures with three *S. pneumoniae* strains, two strains that generate high amounts of H_2O_2 , BHN31 (blue square) and BHN32 (green triangle) as well as one genetically-modified strain that does not generate H_2O_2 , BHN123 (red diamond) are also shown. (c) The generated H_2O_2 concentrations from the three *S. pneumoniae* strains as determined from this study with the ratiometric $\text{CeO}_2:\text{Eu}^{3+}/\text{Y}_2\text{O}_3:\text{Tb}^{3+}$ nanoaggregates (blue bars, $n = 3$) and reported in the literature by commercial peroxidase-based assays (red bars). For strain BHN123 the H_2O_2 concentration reported in the literature was <0.025 mM that is the limit of detection of the used assay.