

Durable Single-Atom Catalysts Enable Precise Chemiluminescence Tracing

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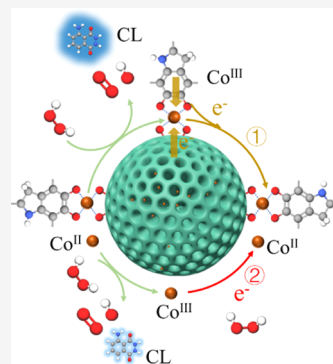


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ABSTRACT: Single-atom catalysts (SACs) have displayed remarkable merits in bioassays due to their superior catalytic performance. Nevertheless, the durability of both their catalytic activity and detection signal remains a great challenge due to the self-limited behavior of single-atom centers. Herein, Co SACs were synthesized via a template-assisted polymerization using dopamine as a ligand precursor, which provided electron-rich anchoring sites for single-atom Co centers. The designed Co SACs exhibited high activity in catalyzing typical luminol–H₂O₂ chemiluminescence (CL) reaction under both alkaline and neutral conditions and thus triggered intensive signal for CL detection. Experimental results and density functional theory calculations uncovered the electron transfer from phenolic hydroxyls with delocalized π -electrons to single-atom Co centers, which recovered their catalytic activity and could ensure the durability of the CL signal. The persistent CL signal supplied precise imaging protocols for in situ visualizing the molecular information under working conditions, which was demonstrated by real-time tracing the process of alkaline phosphatase-triggered dephosphorylation for revealing the inhibition of self-limited reaction to enzymatic activity, and monitoring the molecular changes on cell membrane during drug treatment. This work opens an avenue to design durable SACs via an internal electron-driven pathway for improving the accuracy of in situ CL tracing technologies.



INTRODUCTION

Owing to the special luminous mechanism of homogeneous chemiexcitation, chemiluminescence (CL) imaging presents remarkable advantages and has been applied in clinic diagnosis, inflammation imaging,¹ tumor treatment,² and superoxide anion (O₂^{•-}) tracing.³ These imaging methods are mainly based on glow-type CL systems involving peroxyoxalate¹ and Schaap's adamantylene-1,2-dioxetane.^{2,3} The former needs suitable fluorescein acceptors and a local hydrophobic environment, while the latter suffers from complex synthetic procedures and thermal instability.⁴ Thus, luminol as a luminophore has been used for CL imaging due to its low cost and ultrafast response to highly active intermediates, such as reactive oxygen species (ROS). Great efforts have been made to prolong its CL emission by slowing the contact of CL reagents⁵ and restraining the diffusion of H₂O₂ with hydrogel⁶ or supramolecular⁷ structures, which provide relatively repeatable CL images and thus achieve CL imaging applications of luminol. Recently, our group also designed a medium-entropy nanozyme for continuous generation of ROS via quick electron transfer among metal sites within framework to achieve CL imaging.⁸ However, the relatively short persistent period with nonnegligible change of the CL signal^{7,8} limits the practical application of the CL imaging technique in precise analysis, particularly real-time tracing of biomolecules, which highlights the urgency to further develop new catalysts for persistent CL emission with high luminous efficiency.

Single-atom catalysts (SACs)⁹ with well-defined atomic centers have attracted considerable interest in the catalytic field due to their extreme atom utilization and prominent activity.¹⁰ Unfortunately, the enhancement of activity generally means the loss of stability or selectivity,¹¹ which severely limits the potential of SACs in persistent catalysis. Although recent advances have demonstrated the superiority of SACs in enhancing CL emission, including 10-fold enhancement by Zn SACs,¹² 13.4-fold enhancement by Fe SACs,¹³ and even 3700-fold enhancement by Co SACs,¹⁴ the durability of their catalytic activity is rather poor, thus greatly restricting their applications in CL imaging. To improve the durability of SACs, several strategies have been proposed by using external light sources¹⁵ or electricity¹⁶ to boost the valence cycle of single-atom centers through continuous energy conversion, which not only increases the complexity of CL system but also brings severe interference signals, are thus unfavorable to analytical applications. Another effective way to enhance the durability of SACs is structural or functional modulation of single-atom centers. For example, ZnO nanoparticles have been used as a

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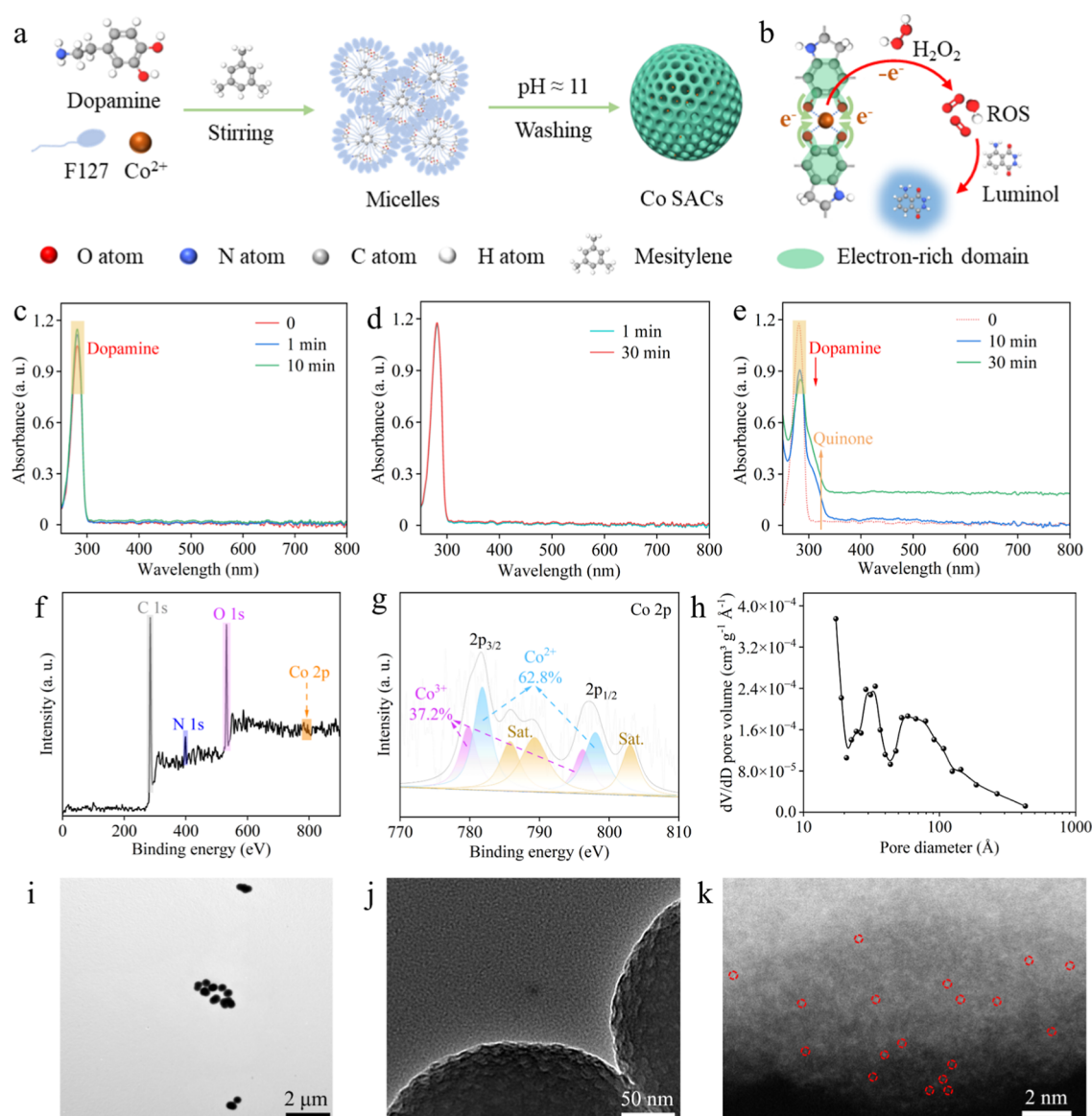


Figure 1. (a) Schematic illustration of the synthesis of Co SACs. (b) Internal electron-driven catalysis mechanism of Co SACs for producing intensive and durable CL signals. (c–e) UV–vis absorption spectra of the mixtures of (c) dopamine and Co^{2+} , (d) c + mesitylene, and (e) d + $\text{NH}_3 \cdot \text{H}_2\text{O}$ at different reaction times. (f) Full scan X-ray photoelectron spectroscopy (XPS) of Co SACs. (g) Co 2p XPS of Co SACs. (h) Pore size distribution of Co SACs. (i,j) TEM images of Co SACs at scale bars of 2 μm (i) and 50 nm (j). (k) AC-HAADF-STEM image of Co SACs.

carrier of Co SACs to enhance the activity and stability for persistent pollutant removal by maintaining a stable pH environment of single-atom Co centers with ZnO as the sacrificial agent.¹⁷ Ta–TiOx nanoparticles have been loaded on Fe–N–C catalysts to act as a scavenger of highly active radicals in the single-atom Fe center-catalyzed oxygen reduction reaction.¹⁸ Inspired by these strategies, here we designed an internal electron-driven catalysis mechanism by using electron-rich anchoring sites to continuously provide electrons to single-atom catalytic centers for functional modulation of SACs, which greatly enhanced both the activity and the durability of SACs.

The functional modulation of SACs has been achieved by directly regulating their relevant coordination environment, involving coordination numbers,¹⁹ coordination atoms,^{20,21} additional atoms²² or atomic clusters,²³ and carriers.²⁴ The proposed mechanism was performed using dopamine as the ligand²⁵ due to the presence of delocalized π -electrons around

its benzene ring structure and two phenolic hydroxyls. Moreover, dopamine could be easily polymerized²⁶ to achieve structural regulation.^{27,28} In the presence of F127 as a soft template and mesitylene as a pore mediator, this work synthesized autonomous Co SACs (Figure 1a), in which single-atom Co centers were posited at electron-rich anchoring sites to directly obtain electrons from phenolic hydroxyls for recovering their activity (Figure 1b). The internal electron-driven catalysis mechanism led to about 1000-fold enhanced CL signal of the luminol– H_2O_2 system with a persistent period of more than 25 min, providing an exciting avenue for the design of reliable CL imaging protocols. Moreover, Co SACs could overcome the pH limitations to initiate persistent CL imaging signal under the physiological environment. As a proof-of-concept, a real-time tracing protocol was constructed for probing the dephosphorylation process with ascorbic acid (AA) 2-phosphate (AAP) as the model and real-time visualizing the molecular information on cells.

Dephosphorylation is pivotal to metabolic regulation²⁹ and cellular signaling transduction³⁰ and can be triggered by alkaline phosphatase (ALP) as an essential biomarker for a clinical diagnosis. Unfortunately, the presence of a self-limited reaction,³¹ which refers to the retroaction effect of products on original reactions, generally results in inaccurate and even false measurement results of enzymatic activity. The constructed real-time tracing technology clarified the dynamic inhibition effect of the self-limited reaction on ALP activity. Moreover, this technology could be further used for efficacy evaluation of enzymatic inhibitors by visualizing the molecular information on the cell membrane, demonstrating the promising applications of the enhanced activity and durability of Co SACs in precise bioimaging under working conditions and biological process tracing.

■ EXPERIMENTAL SECTION

Preparation of mPDA

Mesoporous polydopamine (mPDA) was prepared via a template-assistant polymerization method according to the previous work with some modifications.^{28,32} Specifically, 50 mg of dopamine and 100 mg of F127 as the template were added to 10 mL of ethanol (60%) and kept stirring at 500 rpm. After fully dispersed, 115 μL of mesitylene as the pore mediator was added to the mixture with continuous stirring for 30 min to form nanomicelles. Whereafter, 500 μL of $\text{NH}_3\cdot\text{H}_2\text{O}$ was dropped to regulate the pH to ≈ 11 for initiating the self-polymerization of dopamine and kept stirring for 2 h. Afterward, the polymer was thoroughly washed with ethanol and water by centrifugation to remove F127 template and mesitylene for obtaining mPDA, which was redispersed in 10 mL of water.

Preparation of Co SACs

Co SACs were prepared with a procedure similar to that of mPDA, except adding 0.01 mmol $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$ as the metal source in the mixture of 50 mg of dopamine and 100 mg of F127 for previous coordination. The obtained Co SACs were also redispersed in 10 mL of water.

CL Kinetic Measurement of the Co SACs-Catalyzed Luminol– H_2O_2 Reaction

50 μL of Co SACs suspension at a Co content of 0.1 $\mu\text{g mL}^{-1}$ was mixed with 50 μL of luminol (1.0 mM) in a cuvette. In the dark box of CL analyzer, 50 μL of H_2O_2 (1.0 mM) was rapidly injected in the mixture to trigger the CL reaction. The voltage of photomultiplier was set at -400 V to obtain the kinetic curve.

CL Imaging Kinetic Curve of the Co SACs-Catalyzed Luminol– H_2O_2 Reaction

After orderly adding 50 μL of Co SACs suspension at a Co content of 0.1 $\mu\text{g mL}^{-1}$, 50 μL of luminol (1.0 mM), and 50 μL of H_2O_2 (1.0 mM) to a microplate well, the CL images were successively collected at an interval of 1 min using a cooled low-light CCD camera with an exposure time of 15 s.

ROS Elimination Experiments

To probe the species of ROS generated in Co SACs-participated luminol– H_2O_2 CL reaction, AA, thiourea, *p*-BQ, and tryptophan were adopted as specific scavengers of total ROS, $\cdot\text{OH}$, $\text{O}_2^{\cdot-}$, and $^1\text{O}_2$, respectively. Typically, 50 μL of Co SACs suspension at a Co content of 0.1 $\mu\text{g mL}^{-1}$, 50 μL of scavenger solution (1.0 or 10 μM), 50 μL of luminol (1.0 mM), and 50 μL of H_2O_2 (1.0 mM) were added in microplate wells. The CL images were immediately collected at an interval of 1 min using a cooled low-light CCD camera with an exposure time of 15 s.

Electron Paramagnetic Resonance (EPR) Tests

EPR tests using DMPO (10 μL , 50 mM) as a spin trapping reagent were carried out to probe $\cdot\text{OH}$ and $\text{O}_2^{\cdot-}$ produced in the mixture of Co SACs (50 μL at a Co content of 0.5 $\mu\text{g mL}^{-1}$) and H_2O_2 (50 μL , 5

mM in 0.05 M CBS). For $\text{O}_2^{\cdot-}$ measurement, the Co SACs suspension was diluted with methanol to a Co content of 0.5 $\mu\text{g mL}^{-1}$.

CL Tracing of Self-Limited Dephosphorylation

50 μL of Co SACs suspension at a Co content of 0.1 $\mu\text{g mL}^{-1}$, 50 μL of luminol (1.0 mM), and 50 μL of H_2O_2 (1.0 mM) were mixed in 18 microplate wells. After reaction for 35 min, three groups of the mixtures 25 μL of AAP (1.0 mM) and 25 μL of ALP (0.1, 0.5, 1.0, 5.0, or 10 mU mL^{-1}) or water as blank were added to the wells, respectively, to collect the CL images at an interval of 1 min using a cooled low-light CCD camera with an exposure time of 15 s.

Preparation of Co SACs@PEI and Co SACs@PEI-Aptamer

After Co SACs (1.0 mg) were dispersed into 1.0 mL of PEI solution (MW = 600 Da, 0.1 mg mL^{-1}) and kept continuous mixing for 10 min at room temperature, the suspension was washed three times by centrifugation to remove residual PEI. The obtained Co SACs@PEI were redispersed into 1.0 mL of water, which was further centrifuged and redispersed in 1.0 mL of PBS containing 10 mg of EDC, NHS, and 10 μL of aptamer (10 μM) to react for 12 h under continuous mixing at room temperature. Afterward, the suspension was washed three times by centrifugation. The obtained Co SACs@PEI-aptamer was redispersed into 1.0 mL of water and stored at 4 $^\circ\text{C}$ for further use.

Anchoring of the Co SACs@PEI-Aptamer on the Cell Membrane

After HeLa cells were cultured on a 6-well plate for 24 h, the cells were washed three times with PBS and cocultured with 100 μL of medium containing Co SACs@PEI-aptamer (0.1 mg mL^{-1}) for 0, 0.5, 1, 1.5, and 2 h. Afterward, the cells were washed three times with PBS.

Real-Time Visualization of Dephosphorylation on the Cell Membrane

For in situ tracing, the dephosphorylation triggered by ALP on the cell membrane, HeLa cells were cultured for 12 h on a white 96-well cell culture plate with transparent flat bottom and washed three times with PBS to coculture with 100 μL of medium containing Co SACs@PEI-aptamer (0.1 mg mL^{-1}) for 1 h. Afterward, 25 μL of luminol (2.0 mM), 25 μL of AAP (2.0 mM), and 50 μL of H_2O_2 (1.0 mM) were added to initiate the CL reaction and constantly capture CL images at an interval of 1 min with an exposure time of 15 s and signal gain factor of 4×4 . As control, the same volume of PBS without AAP was added to the reaction system. To uncover the real-time inhibition effect of Na_3VO_4 on dephosphorylation on the cell membrane, 25 μL of AAP contained different concentrations of Na_3VO_4 (40, 100, and 200 μM), which was used for the CL reaction.

■ RESULTS AND DISCUSSION

Preparation and Characterization of Co SACs

To achieve persistent catalysis, dopamine was used as a ligand precursor to provide electron-rich anchoring sites for single-atom Co centers. In the presence of F127 as a soft template, and mesitylene as a pore mediator, autonomous Co SACs were successfully prepared with a template-assistant polymerization method (Figure 1a). In the Co SACs, each single-atom Co center coordinated with four phenolic hydroxyls of two dopamine units. Thus, the dosage of dopamine far exceeded that of Co^{2+} , and the optimal mole ratio was 26/1 (see the detailed discussion). Mesitylene promoted the formation of micelles in the mixture (ethanol/water = 3/2) due to its inherent hydrophobic feature, which produced nano micelles under the assistance of F127. After pH of the mixture was modulated to $\text{pH} \approx 11$ with $\text{NH}_3\cdot\text{H}_2\text{O}$, self-polymerization of dopamine was initiated to obtain Co SACs.

UV–vis absorption spectra demonstrated the multistep synthetic procedures of Co SACs. Both F127 and Co^{2+} did not

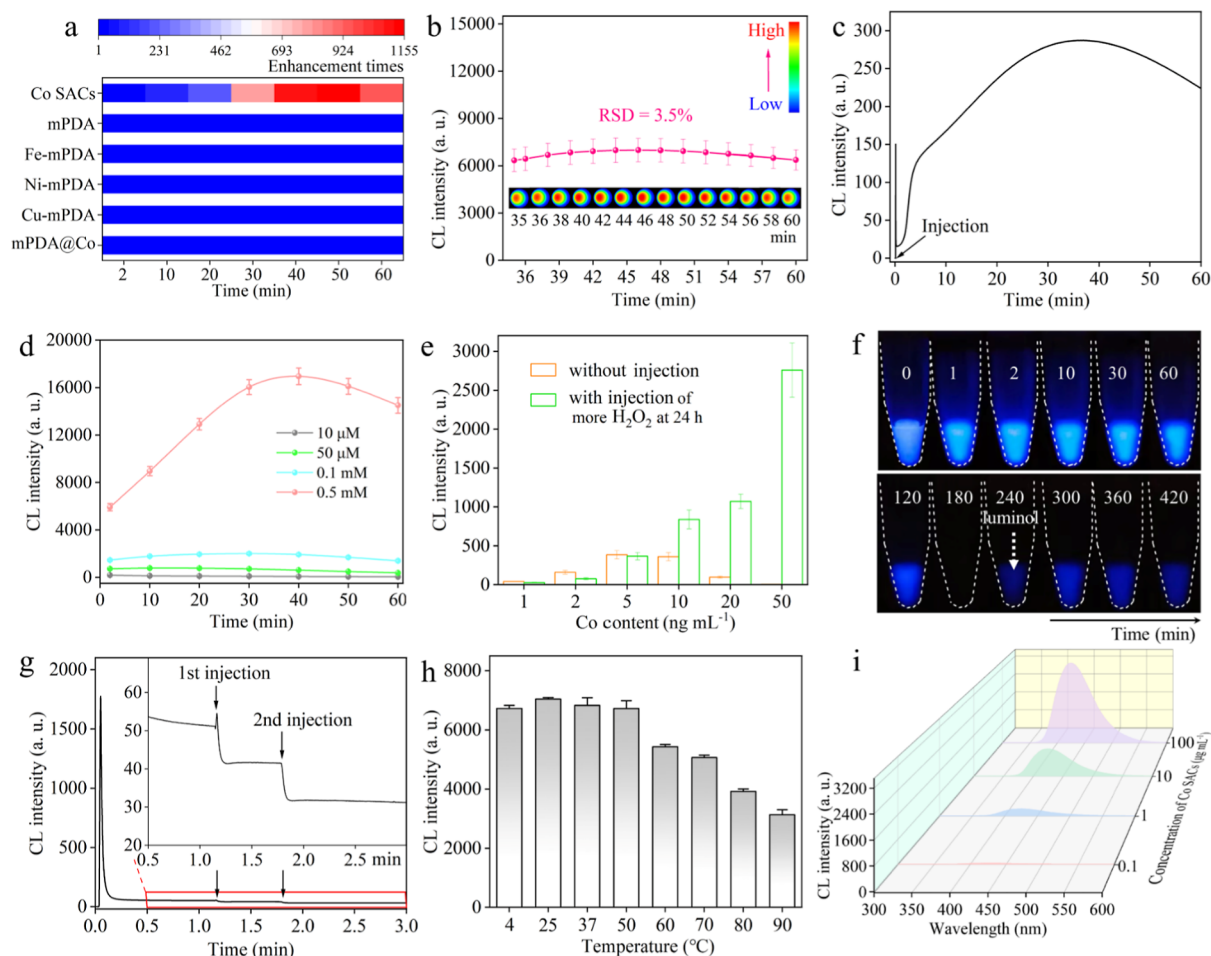


Figure 2. (a) Enhancement times of CL signals with various nanomaterials. (b,c) CL imaging (b) and CL (c) kinetic curves of the luminol–H₂O₂ reaction after injecting Co SACs. Inset in (b): corresponding CL images. (d) CL imaging kinetic curves of the Co SACs-catalyzed luminol–H₂O₂ reaction at various concentrations of added H₂O₂. (e) CL intensity of the luminol–H₂O₂ system at different Co SACs concentrations upon addition of more H₂O₂ at reaction for 24 h. (f) Photos of the Co SACs–luminol–H₂O₂ system after mixing 50 μ L of Co SACs (1.0 μ g mL^{−1} Co), 100 μ L of luminol (1.0 mM in pH 9.0 CBS), and 150 μ L of H₂O₂ (0.1 M) for different times and supplementing 100 μ L of luminol (1.0 mM in pH 9.0 CBS) at 240 min. (g) CL kinetic curve of Co²⁺–luminol–H₂O₂ system upon addition of the more luminol–H₂O₂ mixture. (h) CL intensity of the Co SACs–luminol–H₂O₂ system at different temperatures. (i) CL emission spectra of the Co SACs-catalyzed luminol–H₂O₂ reaction at different Co SACs concentrations. Exposure time: 15 s for a,b,h, and 60 s for d,e. Error bars: mean $\pm$ SD ($n = 3$).

show any absorption (Figure S1a). After Co²⁺ was added in dopamine solution, the absorption peak of dopamine at 280 nm increased and reached equilibrium (Figure 1c), indicating the coordination between dopamine and Co²⁺, while mixing F127 in dopamine solution did not change the absorption peak (Figure S1b). The addition of both mesitylene (Figure 1d) and the mixture of mesitylene and F127 (Figure S1c) in the balanced mixture of Co²⁺ and dopamine did not affect the coordination equilibrium, but the latter showed Tyndall effect (Figure S1c), demonstrating the necessity of mesitylene and F127 for forming nano micelles. After the addition of NH₃·H₂O in the mixture of mesitylene, dopamine, and Co²⁺ to adjust the pH to 11, the absorption peak of dopamine at 280 nm significantly decreased, while the absorption of quinone unit increased, illustrating that dopamine was oxidized to quinone radical for further polymerization (Figure 1e).^{33,34} Moreover, the absence of NH₃·H₂O failed to form any centrifugation product in the mixtures of mesitylene, F127 and Co²⁺ (Figures S2a,b) or mesitylene, F127, dopamine, and Co²⁺ (Figure S2c,d), demonstrating that the polymerization of dopamine was critical to the skeleton formation of Co SACs.

To reveal the component and structure of Co SACs, a series of characterizations were carried out. Fourier transform-infrared (FT-IR) spectra of mesoporous polydopamine (mPDA) and Co SACs showed a similar peak at 1112 cm^{−1} (Figure S3), which was ascribed to C–N stretching vibration of aromatic amine, further revealing the polymerization of dopamine. Inductively coupled plasma–optical emission spectrometric (ICP–OES) analysis of Co SACs showed a Co content of 1.5 wt %. X-ray diffraction analysis of Co SACs showed an amorphous crystal structure similar to mPDA without the presence of Co oxides or nanoparticles (Figure S4), illustrating excellent dispersion of Co species. The elemental composition of Co SACs and the valence state of Co were probed using XPS. The full-scan XPS indicated the existence of C, N, O, and Co elements (Figure 1f), and the Co 2p spectrum illustrated that Co element was +2 and +3, with a molar ratio of 0.63/0.37 (Figure 1g). To verify the porous structure of Co SACs, N₂ adsorption–desorption isotherms were recorded (Figure S5), which exhibited a Brunauer–Emmett–Teller surface area of 20.74 m² g^{−1} with various pore size distributions (Figure 1h).

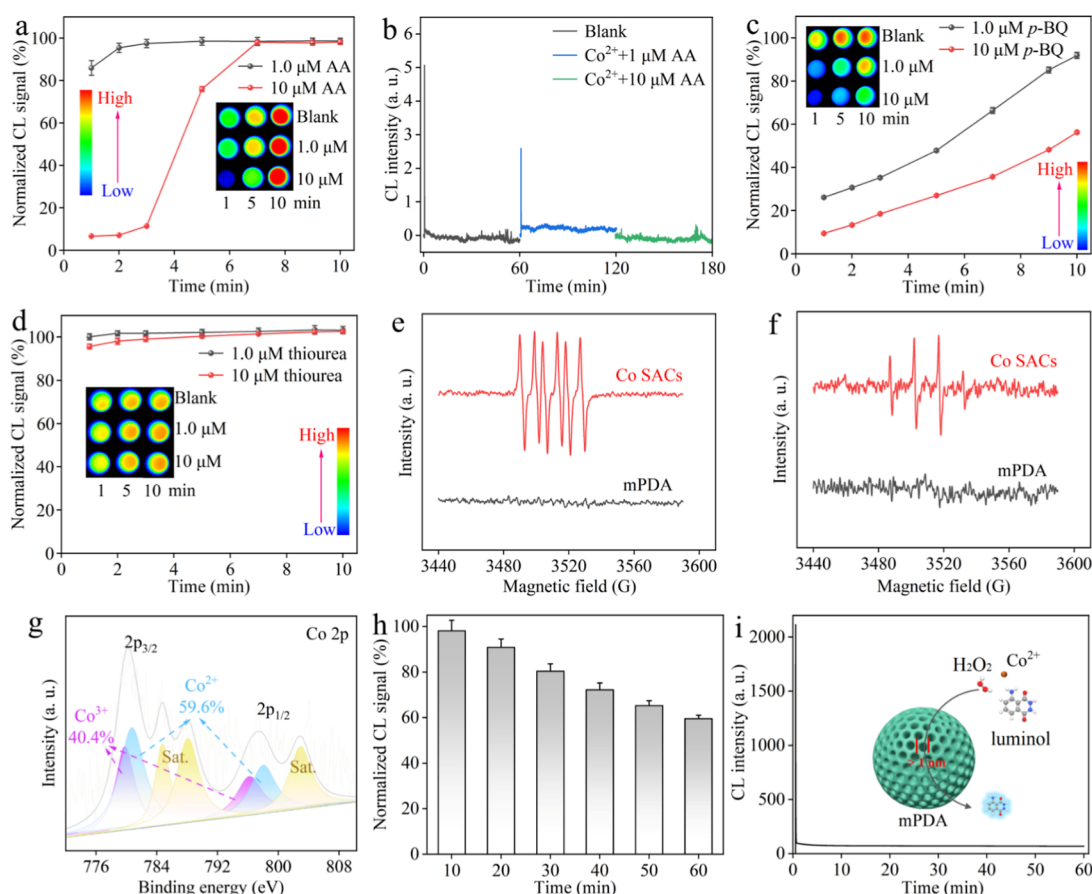


Figure 3. (a) Normalized CL imaging signals of the Co SACs–luminol–H₂O₂ system at different reaction times and (b) CL kinetic curve of the Co²⁺–luminol–H₂O₂ system in the absence as blank and presence of 1.0 and 10 μM AA. (c,d) Normalized CL imaging signals of the Co SACs–luminol–H₂O₂ system in the absence as blank and presence of 1.0 and 10 μM p-BQ (c) and thiourea (d). Insets in a,c,d: corresponding CL images. (e,f) EPR signals of O₂^{•−} (e) and •OH (f) captured from the mixtures of H₂O₂ with Co SACs or mPDA. (g) Co 2p XPS spectra of Co SACs after the CL reaction for 1 h. (h) Normalized CL imaging signals of the luminol–H₂O₂ reaction catalyzed by the Co SACs that were used for 1 h CL reaction. (i) CL kinetic curve of the Co²⁺–luminol–H₂O₂ system in the presence of mPDA. Error bars: mean ± SD (*n* = 3).

The transmission and scanning electron microscopy (TEM and SEM) images of Co SACs further demonstrated the porous structure and showed a spherical morphology with a size of about 300 nm (Figures 1i,j and S6). Their energy-dispersive spectra revealed the uniform distribution of C, O, N, and Co elements (Figure S7). The mPDA exhibited a similar morphology to Co SACs and a size of about 150 nm (Figures S8 and S9). An aberration-corrected high-angle annular dark-field scanning transmission electron microscopy (AC-HAADF-STEM) image further demonstrated excellent dispersion of Co atoms in Co SACs (Figure 1k). The massive bright points represented single-atom Co centers, and no obvious clusters or nanoparticles were observed, revealing the successful preparation of the Co SACs.

CL Properties of Co SACs-Catalyzed Luminol–H₂O₂ Reaction

Compared with Fe-mPDA, Ni-mPDA, and Cu-mPDA, which possessed similar coordination environments to that of Co SACs, as the coordination of phenolic hydroxyls and metal ions,²⁵ Co SACs prepared with the same procedures exhibited the superior enhancement on flash CL emission of the luminol–H₂O₂ system at the optimal pH (Figures S10 and S11), indicating the strongest catalytic activity of Co active centers. Different from Co SACs, Co²⁺-loaded mPDA (mPDA@Co) showed low activity, illustrating that the

previous coordination between Co²⁺ and dopamine was critical to the high activity of Co SACs. The catalytic durability of nanomaterials on the luminol–H₂O₂ CL reaction was evaluated with their enhancement times toward CL imaging signal of the luminol–H₂O₂ system. Fe-mPDA, Ni-mPDA, Cu-mPDA, and mPDA@Co all exhibited weak CL enhancement as that of mPDA, while Co SACs could persistently enhance the CL signal of the luminol–H₂O₂ system as blank by about 1000 times (Figure 2a). Thus, Co sites were critical to the high activity and durability. In the initial stage, the CL imaging signal of the mixture containing 50 μL of Co SACs suspension at a Co content of 0.1 μg mL^{−1}, 50 μL of luminol (1.0 mM), and 50 μL of H₂O₂ (1.0 mM) continuously increased (Figure S12). After the reaction was triggered for 30 min, the CL imaging signal trended a relatively stable value, which could persist for 25 min with a relative standard deviation of 3.5% (Figure 2b), showing a higher stability than that initiated by horseradish peroxidase (Figure S13). The duration of the CL imaging signal was better than that from the CL kinetic signal (Figure 2c). The catalytic durability was related to the dosage of Co source (Figure S14). At a dosage of 0.01 mmol, the Co SACs showed the best durability along with the strongest catalytic activity, indicating an optimal dopamine/Co mole ratio of 26/1. The excessive dopamine provided suitable

electron-rich anchoring sites for coordination with Co^{2+} and ensured the skeleton formation of Co SACs.

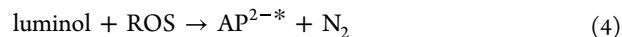
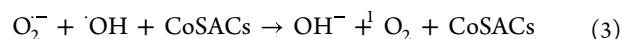
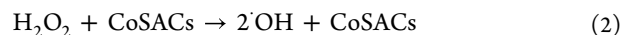
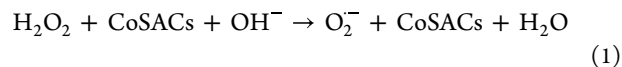
The catalytic durability of Co SACs depended on the concentration of H_2O_2 . With the lowering H_2O_2 concentration, the CL imaging signal decreased and reached the peak value in shorter time due to the fast consumption of H_2O_2 (Figure 2d), which also resulted in shorter duration of stable CL emission. Similarly, the increase of Co SACs concentration accelerated the catalytic oxidation, resulting in faster consumption of H_2O_2 and thus shorter duration of the CL signal (Figure S15). When the Co content was lower than 5 ng mL^{-1} , the CL signal was still observed after reaction for 24 h (Figure S15a–c). Interestingly, after H_2O_2 was consumed by the CL reaction, the injection of more H_2O_2 could recover the CL emission (Figure 2e), demonstrating the favorable catalytic durability of Co SACs. The high activity and durability of Co SACs enabled the CL imaging signal to be observed by naked eye for 2 h (Figure 2f). Moreover, the signal could also be recovered after supplementing more luminol. As a comparison, the flash CL signal of Co^{2+} –luminol– H_2O_2 could not be covered by adding more luminol– H_2O_2 mixture in the system. By contrary, the CL signal decreased due to Co^{2+} dilution (Figure 2g). These results highlighted the durability of Co SACs in catalyzing the CL reaction.

Co SACs showed good temperature stability for enhancing the CL signal of luminol– H_2O_2 reaction in $4 \sim 50^\circ\text{C}$ (Figure 2h). The excellent dispersibility in various solutions implied their favorable practicability under different conditions (Figure S16). In addition, the emission spectra of the Co SACs-catalyzed luminol– H_2O_2 CL system showed a peak at 425 nm (Figure 2i), and no CL emission was observed in the absence of luminol or H_2O_2 (Figure S17). The peak intensity increased with the increasing concentration of Co SACs. These phenomena were similar to those of the peroxidase-catalyzed luminol– H_2O_2 reaction, verifying the same CL reaction process.

Mechanism of Co SACs for Enhancing CL Imaging

To clarify the catalytic mechanism of the Co SACs-catalyzed luminol– H_2O_2 reaction, AA was adopted as a scavenger to eliminate total ROS generated in this system, which showed strong quenching effect on the CL imaging signal (Figure 3a), illustrating the generation of abundant ROS. In the presence of fixed amount of AA, the CL imaging signal could be recovered with the increasing reaction time, and the time to recover the signal became longer at higher AA concentration, which was attributed to the consumption of AA due to the persistent generation of ROS. As a comparison, after AA with the same amount was added into the Co^{2+} –luminol– H_2O_2 system, the quenched CL signal did not recover until 1 h (Figure 3b). The species of ROS in the Co SACs-catalyzed luminol– H_2O_2 reaction were probed using *p*-benzoquinone (*p*-BQ) and thiourea as the specific scavengers for eliminating O_2^- and $\cdot\text{OH}$ radicals,⁸ respectively. The presence of both *p*-BQ (Figure 3c) and thiourea (Figure 3d) decreased the CL imaging signal, indicating the coexistence of O_2^- and $\cdot\text{OH}$. The EPR test was further performed by adopting DMPO as the trapper to capture O_2^- (Figure 3e) and $\cdot\text{OH}$ (Figure 3f) in the presence or absence of methanol. The Co SACs– H_2O_2 system showed much greater signals of O_2^- and $\cdot\text{OH}$ than the mPDA– H_2O_2 system, suggesting that the single-atom Co centers were the active sites. The presence of singlet oxygen ($^1\text{O}_2$), another important kind of ROS, was also confirmed by

adding tryptophan as the specific scavenger in the CL system (Figure S18). Thus, the CL emission resulted from the generation of different ROS, which oxidized luminol to produce the excited species, as described in eqs 1–5.



To further illuminate the excellent durability of Co SACs, the valence state of Co element was probed by comparing the catalytic performance. After the CL reaction for 1 h, the composition of Co^{2+} and Co^{3+} was calculated to be about 0.60/0.40 (Figure 3g), which was slightly lower than the ratio (0.63/0.37) of freshly prepared Co SACs (Figure 1g), indicating the slow consumption of active Co^{2+} after the long-term catalytic process. The CL reaction did not change the full-scan and nonmetal XPS of Co SACs (Figure S19), though the durability of high catalytic activity for reused Co SACs was lowered (Figure 3h). To exclude the effect of porous structure of Co SACs, which restricted H_2O_2 diffusion for persistent CL emission,^{6,7} the CL signal of the mPDA– Co^{2+} –luminol– H_2O_2 system was tested, which showed a flash property (Figure 3i), illustrating that it was difficult to recover the activity of Co species,³⁵ and the slowed diffusion by porous structures in the Co SACs–luminol– H_2O_2 system was not the main factor to persist CL emission. On the contrary, the stable valence state equilibrium played a key role in persistent CL emission.

The stable valence state equilibrium of Co^{2+} and Co^{3+} in the CL reaction process could be achieved by continuously obtaining electrons from electron-rich anchoring sites. The electron transfer pathway was demonstrated by density functional theory calculations. To simulate the electron-rich environment with anchored single-atom Co centers, the structures of $[\text{Co}(\text{dopamine})_2]^{2+}$ and $[\text{Co}(\text{dopamine})_2]^{3+}$ were obtained by Gaussian calculations (Figure S20). Differential charge density (Figure 4a,b) and Hirshfeld atomic charge analysis (Tables S1,2) were then carried out to confirm the pathway and detailed electron transformation amount from phenolic hydroxyls to single-atom Co centers. Specifically, four phenolic hydroxyls of two dopamine units formed an electron-rich site for anchoring a Co atom (Figure 4a), resulting in the bivalent Co ion with only $0.532 e^-$ (Table S1), which indicated that four phenolic hydroxyls provided $1.468 e^-$ to a single-atom Co center. In the CL catalytic reaction, active bivalent Co lost an electron to form inactive trivalent Co, leading to gradual disappearance of catalytic activity. Specifically, the calculated results showed that a trivalent Co atom obtained $2.462 e^-$ (Table S2) from phenolic hydroxyls with delocalized π -electrons, showing $0.538 e^-$ (Figure 4b), which was similar to that before the catalytic reaction, illustrating that the electron-rich environment rapidly recovered the valence of single-atom Co centers to achieve persistent CL catalysis through an internal electron-driven pathway.

The mechanism of Co SACs with excellent activity and durability can be summarized in Figure 4c. First, single-atom Co centers fast reacted with H_2O_2 by a Fenton-like reaction

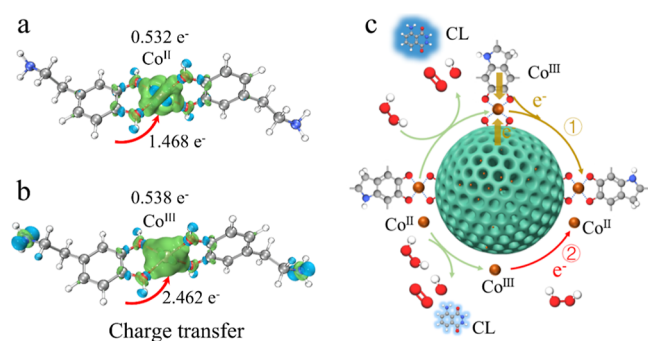


Figure 4. (a,b) Differential charge density analysis of $[\text{Co}(\text{dopamine})_2]^{2+}$ (a) and $[\text{Co}(\text{dopamine})_2]^{3+}$ (b). The blue and green in the electron-rich domain reveal charge depletion and accumulation, respectively. (c) Schematic illustration of the Co SACs and Co^{2+} -catalyzed CL reaction mechanism. Routes 1 and 2 represent the regeneration of active Co valence for Co SACs and Co^{2+} , respectively.

process to generate ROS (green arrows, eqs 1–3), which then oxidized luminol to produce the excited species for emitting the light (eqs 4 and (5)), leading to enhanced CL emission. For valence cycle in the persistent CL emission, single-atom Co centers could obtain electrons from the electron-rich domain via an internal electron-driven pathway (brown arrows) to recover the high catalytic activity (Route 1). However, the recovery of Co^{2+} in the Co^{2+} –luminol– H_2O_2 CL system was achieved only via the Co^{3+} initiated Fenton-like reaction (Route 2) after Co^{2+} reacted with H_2O_2 , which was very slow and led to flash CL emission.

CL Tracing of Self-Limited Dephosphorylation

The CL emission period of 25 min with relatively stable signal from the Co SACs-catalyzed luminol– H_2O_2 system was

suitable for real-time tracing biological processes. Therefore, a facile CL imaging method with a prolonged detection window was constructed to probe the self-limited reaction in dephosphorylation by real-time tracing ALP-triggered dephosphorylation (Figure 5a), which is usually ignored in ALP activity measurements with once-off electrochemiluminescence,³⁶ fluorescence,³⁷ and colorimetric³⁸ methods due to their limited time window. After AAP and ALP were separately added into the Co SACs-catalyzed luminol– H_2O_2 system after the CL reaction was initiated for 35 min, the stable imaging signal did not obviously change (Figure 5b). The presence of both AAP and ALP in this system greatly lowered the stable CL signal due to the formation of AA via the dephosphorylation of AAP, which quenched CL emission via eliminating total ROS (the first data in Figure 5c). The quenching was further demonstrated in the presence of 0.25 mM AA (Figure S21), which led to a method for AA detection with a linear regression equation of $I = -677.7 c + 7097.1$ in the concentration range of 50 nM to 10 μM (Figure S22) and a detection limit of 15 nM. Moreover, this method exhibited good repetitiveness (Figure S23). Interestingly, the addition of PO_4^{3-} could inhibit the quenching even at a low concentration of 0.01 mM, illustrating the presence of the PO_4^{3-} -triggered self-limited reaction, which led to increasing CL intensity with the increase of PO_4^{3-} concentration (Figure 5c). Classical colorimetric³⁸ and fluorescent³⁹ measurements with similar results verified the effect of PO_4^{3-} on the dephosphorylation process (Figure S24). Here, the effect of AA on the dephosphorylation of AAP, which was observed by fluorescence detection of the 4-MUP–AAP–ALP system (Figures S25 and 26), could be excluded due to the continuous consumption of generated AA. These results demonstrated the feasibility of the proposed method for probing the PO_4^{3-} -triggered self-limited reaction.

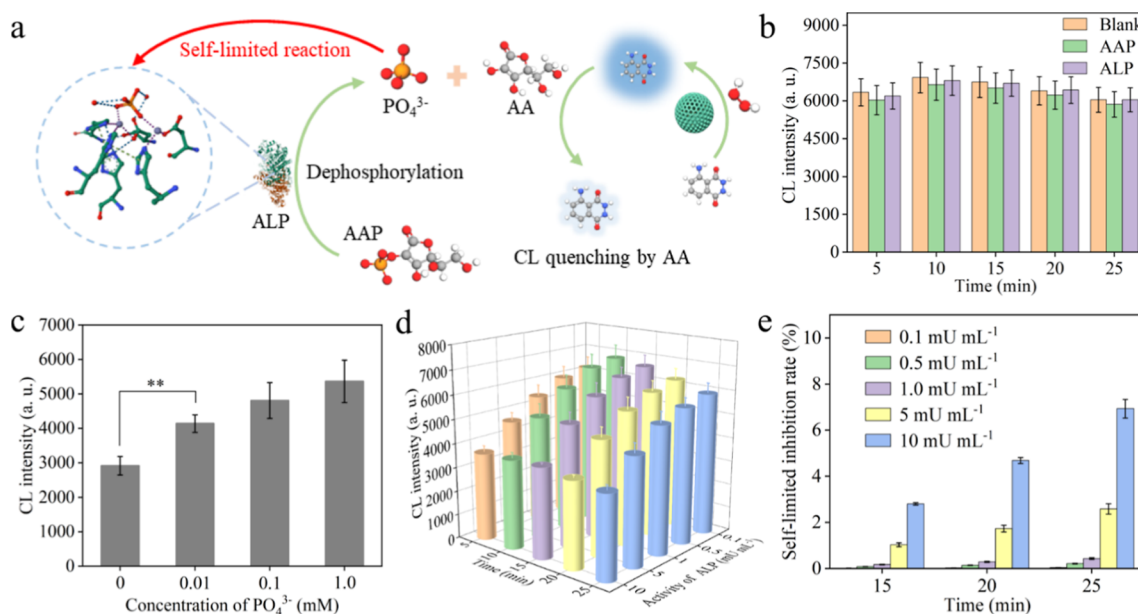


Figure 5. (a) Schematic illustration of the self-limited reaction in ALP-triggered dephosphorylation of AAP and real-time CL tracing with the Co SACs-catalyzed CL reaction. (b) Influences of AAP and ALP on the CL imaging signal of the Co SACs–luminol– H_2O_2 system. (c) CL imaging signals of the AAP–ALP–Co SACs–luminol– H_2O_2 system in the presence of PO_4^{3-} at different concentrations. (d) CL imaging intensity of the Co SACs–luminol– H_2O_2 system after reaction for 35 min and then introducing dephosphorylation of AAP triggered by different activities of ALP for different times. (e) Self-limited inhibition rates of ALP-triggered dephosphorylation of AAP at different times with various ALP activities. Error bars: mean $\pm$ SD ($n = 3$).

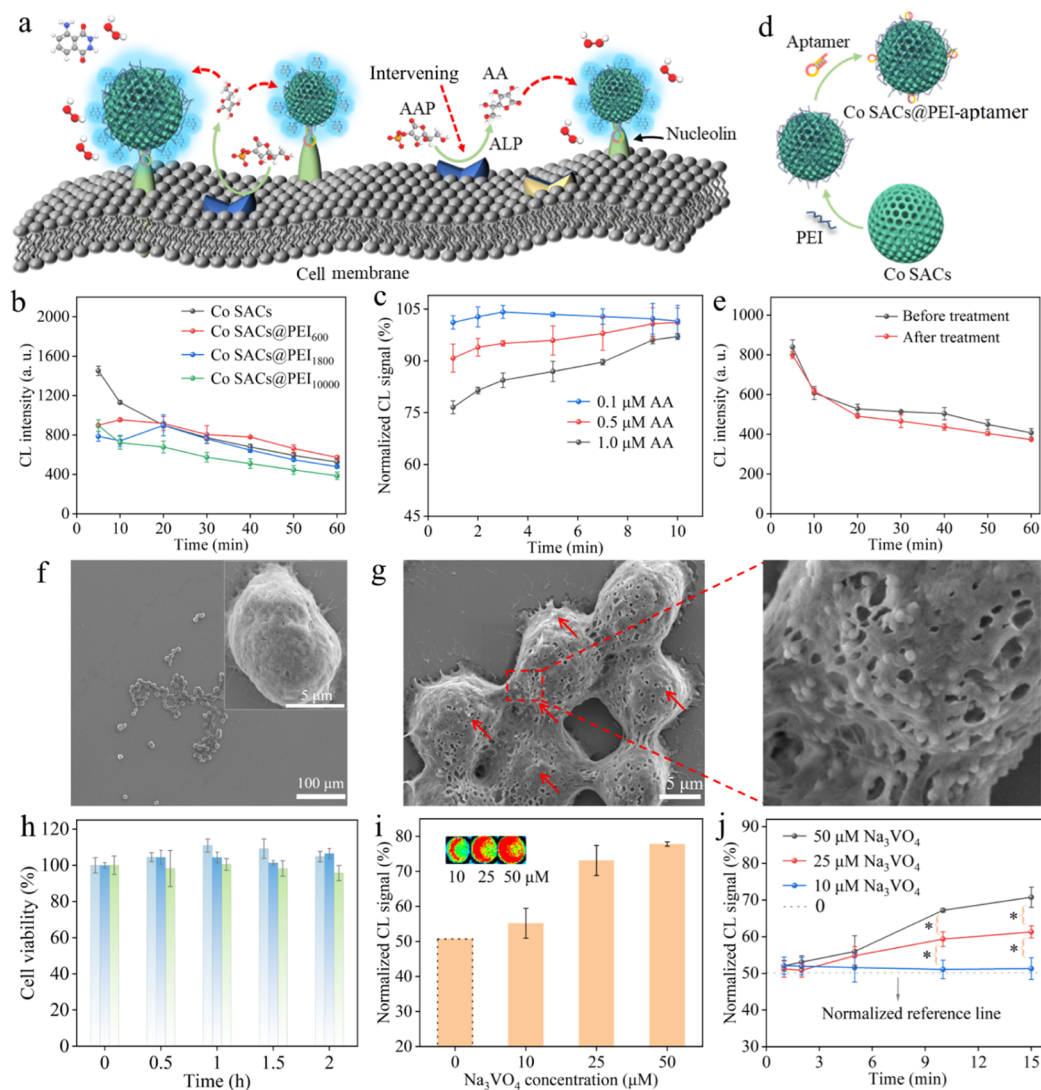


Figure 6. (a) Schematic illustration of real-time CL visualization of ALP-triggered dephosphorylation on the cell membrane. (b) Time-dependent CL imaging intensity of the luminol–H₂O₂ reaction catalyzed by PEI-coated Co SACs at pH 7.4, exposure time: 15 s, signal gain factor = 4×4 , $n = 3$. (c) Normalized CL imaging signals of the Co SACs@PEI–luminol–H₂O₂ system containing 0.1, 0.5, and 1.0 μM AA at pH 7.4 at different reaction times, $n = 3$. (d) Schematic illustration of Co SACs@PEI-aptamer preparation. (e) Time-dependent CL imaging signals of the luminol–H₂O₂ system at pH 7.4, catalyzed with Co SACs@PEI-aptamer before and after being treated with cell culture medium for 1 h. (f,g) SEM images of HeLa cells before (f) and after (g) incubation with Co SACs@PEI-aptamer for 1 h. The red arrows represent part of Co SACs@PEI-aptamer on the cell membrane. (h) Viability of HeLa cells after being incubated with Co SACs@PEI-aptamer (left), Co SACs@PEI-aptamer + luminol + H₂O₂ (middle), and Co SACs@PEI-aptamer + luminol + H₂O₂ + AAP (right) for different times, $n = 5$. (i,j) Normalized CL imaging signals of Na₃VO₄-treated cells after binding Co SACs@PEI-aptamer in the luminol–H₂O₂–AAP system (i) and Co SACs@PEI-aptamer bound cells in the luminol–H₂O₂–AAP system containing 10, 25, and 50 μM Na₃VO₄ (j), $n = 3$. Inset in (i): corresponding CL images.

The effect of ALP activity on the CL imaging signal of the Co SACs–luminol–H₂O₂ system in the presence of ALP-triggered dephosphorylation of AAP resulted from two factors, the generated AA and PO₄^{3−}-triggered self-limited reaction. At the ALP activities up to 10 mU mL^{−1}, the generated AA did not obviously affect the stable CL signal in the tracing period (Figure Sd), illustrating that the Co SACs-catalyzed Fenton reaction was fast enough to produce ROS for consuming the generated AA and maintaining the CL emission. The slight change of the CL signal came from the catalytic kinetic of Co SACs toward the luminol–H₂O₂ reaction, which was consistent with the results shown in Figure 2b. However, with the increasing ALP activity, the CL signal decreased (Figure Sd), which resulted from the quenching of AA generated in the waiting stage of 35 min on CL emission. The

plots of CL intensities detected at different times vs ALP concentration showed good linearity along with similar slopes (Figure S27). To reveal precisely the PO₄^{3−}-triggered self-limited reaction in dephosphorylation of AAP, the slightly changed CL intensities were first corrected with the correction coefficients obtained by dividing them by the highest CL signals at the same ALP concentrations, which produced the plots as shown in Figure S28. Here, the highest CL signals occurred at 10 min. The obtained slopes exhibited a decreasing tendency except that obtained with the signals at 5 min (Table S3), which could be ascribed to the fast-growing self-limited effect. The inhibition rate (R_i) of the self-limited reaction can be calculated with eq 6.

$$R_i = [(I_2 - I_1)/I_1] \times 100\% \quad (6)$$

where I_1 represents the CL intensity calculated with the regression equations at different times and corresponding ALP activity, and I_2 represents the CL intensity calculated with the regression equation obtained at 10 min. Under the certain substrate concentrations, the self-limited reaction showed an increasing inhibition rate with the increase of ALP activity or reaction time (Figure 5e). Therefore, the real-time CL tracing successfully revealed the dynamic inhibition effect of the self-limited reaction on ALP activity in dephosphorylation of AAP, widening the applications of Co SACs and CL bioimaging.

Real-Time Visualization of Dephosphorylation on the Cell Membrane During Drug Treatment

To verify the application of the proposed precise CL tracing protocol under working conditions, HeLa cells with high expression of ALP on the cell membrane⁴⁰ were used to perform ALP-triggered dephosphorylation (Figure 6a). Considering the fact that majority of catalysts show weak CL emission of the luminol–H₂O₂ system under near neutral condition, the CL emission of the Co SACs-catalyzed luminol–H₂O₂ system was first examined at pH 7.4, which showed persistent CL signal (Figure 6b) with ROS-responsive CL mechanism (Figure 6c), similar to that in pH 9.0 CBS. As expected, the persistence was also much better than the Co²⁺-initiated luminol–H₂O₂ system at pH 7.4 (Figure S29). These results indicated that Co SACs overcame the pH limitations to trigger the luminol–H₂O₂ reaction. Here, PEI with abundant amino groups was used as a modifier to improve the labeling efficiency of Co SACs with aptamer (Figure 6d), and the optimal molecular weight was determined as 600 Da due to the steric hindrance (Figure 6b). By conjugating the aptamer of nucleolin overexpressed on the cell membrane⁴¹ to Co SACs, which was demonstrated by the zeta-potential measurements (Figure S30), the catalysts could be conveniently bound to the cell surface. Moreover, the prepared Co SACs@PEI-aptamer exhibited good stability in cell culture medium (Figure 6e), ensuring the feasibility of the constructed protocol.

After HeLa cells were incubated with Co SACs@PEI-aptamer for 1 h, massive Co SACs@PEI-aptamer could be observed on the cell membrane by its specific recognition to overexpressed nucleolin (Figures 6f,g and S31). They showed ignorable cytotoxicity on HeLa cells, as other reagents including luminol, H₂O₂, and AAP (Figure 6h). As an inhibitor of ALP,⁴² Na₃VO₄, which did not interfere with the CL imaging signal (Figure S32), was used as a model to validate the application of the constructed CL tracing protocol in efficacy evaluation of drugs. Prior to Na₃VO₄ treatment, Co SACs@PEI-aptamer bound HeLa cells in the luminol–H₂O₂–AAP system showed approximate 50% of the CL imaging signal in the luminol–H₂O₂ system (Figure 6i), demonstrating the presence of dephosphorylation to form AA. Upon the Na₃VO₄ treatment and then Co SACs@PEI-aptamer binding, the HeLa cells showed increasing CL imaging signal in the luminol–H₂O₂–AAP system with the increasing concentration of Na₃VO₄, indicating the inhibition of Na₃VO₄ to cell surface ALP activity. To real-time evaluation of the inhibition efficacy of Na₃VO₄, different concentrations of Na₃VO₄ were added to the luminol–H₂O₂–AAP system containing Co SACs@PEI-aptamer bound HeLa cells, which showed the recovery of CL emission in the presence of more than 25 μ M Na₃VO₄ after about 5 min (Figure 6j), demonstrating the onset concentration and time for inhibiting ALP activity to be approximately 25 μ M and 5 min, respectively. Therefore, the

constructed real-time CL tracing protocol provided a new avenue for efficacy evaluation of drugs, greatly widening the application potential of Co SACs for tracing biological processes under the physiological environment.

CONCLUSION

Autonomous Co SACs with high activity and good durability in catalyzing the luminol–H₂O₂ CL reaction were synthesized by implanting single-atom Co centers in electron-rich anchoring domains of polydopamine. The presence of delocalized π -electrons around the benzene ring structure and two phenolic hydroxyls achieved the electron transfer from anchoring sites to single-atom Co centers via an internal electron-driven pathway, which accelerated the valence cycle of Co atoms for activity recovery of Co SACs. The boosted durability of Co SACs assured continuous generation of ROS for initiating intensive and persistent CL signal of luminol under both alkaline and neutral conditions. Based on the superior catalytic features of Co SACs, a CL imaging method was constructed for real-time tracing ALP-triggered dephosphorylation of AAP, which revealed the dynamic inhibition of PO₄³⁻-triggered self-limited reaction on ALP activity. The CL tracing protocol also achieved real-time visualization of ALP-triggered dephosphorylation on cell membrane, which led to a new technology for efficacy evaluation of enzymatic inhibitor as a drug, greatly expanding the application potential of Co SACs-prolonged detection window for tracing biological processes under physiological condition and real-time visualizing the molecular information on the cell membrane. To our knowledge, this work presents the first case of SACs with excellent catalytic activity along with good durability in catalyzing a CL reaction, thus provided a potentially generalizable paradigm to design high-performance CL catalysts using an internal electron-driven strategy from ligand to metal centers. Compared with previously reported SACs, the prepared Co SACs exhibited significantly improved catalytic durability by continuously providing electrons from electron-rich anchoring domains to single-atom Co centers for activity recovery, which enabled intensive and persistent CL signal to improve imaging precision under working conditions. More importantly, Co SACs overcame the pH limitations and could also initiate persistent CL imaging signal under the physiological environment, exhibiting great potential in biological tracing for visualizing various molecular information by changing the targets and substrates. This work opens new avenues for designing SACs with high activity and durability via an internal electron-driven strategy and precise bioimaging under working conditions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.6c03486>.

Chemicals and reagents; apparatus; synthesis of relevant nanoparticles; density functional theory calculations; comparative experiments; cell culture; cytotoxicity measurements; and statistical analysis (PDF)

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Notes

The authors declare no competing financial interest.

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