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Encapsulation of ionic nanoparticles in a hydrogel microparticle, i.e. microgel, produces a target-stimulated probe for molecular detection. Selective reactive oxygen species (ROS) triggers the release of cations from the microgel which subsequently turn on the fluorogenic dyes to emit intense fluorescence, permiting rapid detection of ROS or ROS-producing molecules. The ROS-responsive microgel provides the advantages of simple fabrication, bright and stable signals, easy handling, and rapid response, carrying high promises in biomedical applications.

Protection of functional nanomaterials with biocompatible structures like hydrogels is one feasible strategy to improve their biocompatibility and stability under physiological conditions.[1-2] The hydrophilic structures of hydrogels hold large amounts of water in their three-dimensional networks, rendering them high physiochemical similarity to the extracellular matrices.[3] Besides, enclosure of nanomaterials can improve the structural diversity and functionality of hydrogels, making it stimuli responsive.[4] Typically, responses to external stimuli, including metal ions, pH, temperature, light, added nucleic acid fuel, redox potential, etc., can be introduced via modification of the polymer structures of hydrogels.[5-8] Such stimuli-responsive hydrogels have great application potentials in controlled drug release, catalysis, programmed cell growth, and sensing.[5, 7, 9] Alternatively, encapsulation of nanoparticles (NPs) made from noble metal, metal oxide, or transition metal chalcogenides can bring in unique optical or magnetic properties to hydrogels;[10] and embedment of silica or resin NPs can increase their mechanical strength and pressure sustainability.[11]

Among all external stimuli, reactive oxygen species (ROS) are of great interest because of their imperative roles in regulating physiological processes, including signal transduction, gene expression and cell apoptosis.[12] For example, superoxide radical ($O_2^{\bullet-}$) is generated through both enzymatic and non-enzymatic oxidation process in biological systems.[13] Hypochlorite (ClO^-) is useful for destruction of pathogens in human body.[14] H_2O_2 is a stable conversion intermediate among different ROS, has the ability to cross membrane structures for signaling purposes,[15] and is involved in many enzymatic procedures as the substrate or product.[16] ROS sensors typically rely on redox enzymes that are expensive and have short shelf-life; or small chemical probes that need to be designed judiciously to possess the right redox potential and high specificity.[17] The enzyme-mimetic nanomaterials like Au,[18] CoOOH,[19] graphene oxide,[20] etc. have caught people's attention because of their high catalytic efficiency, but they require radical-based indicators with short life-time and thus produce instable signals. ROS-mediated nanoparticle decomposition to release Ag^+ for fluorescence quenching,[21] or to generate spectral shifts of the Ag nanoprisms,[16, 22] can be utilized for ROS detection, but the biocompatibility of such structures is questionable.

Few redox-responsive hydrogels have been reported by far, with more responding to reducing conditions via introduction of thiol groups to the polymer structures,[23-24] than to oxidation conditions, sensing of which is through enzyme encapsulation.[25-26] Herein, we report the ROS-responsive micron-sized hydrogel particles, i.e. microgel, by enclosing the ZnS-containing NPs in the gel network. The enclosed NPs are protected by the microgel from dissolution until encountering ROS, which oxidizes S^{2-} and releases Zn^{2+}. Adding a low content of Cu^{2+} to the enclosed NPs can greatly improve the microgel's responsivity to ROS owing to the formation of hydroxyl radicals (Figure 1a). Coupled with the zinc-responsive dye[27], the microgel can turn the potentially-instable presence of ROS into the stable and bright fluorescent signals, so that they can act as the sensor for ROS as well as for the ROS-producing small molecules of biomedical interest, such as glucose and cholesterol.

We prepared the microgel containing high density of carboxyl groups by emulsion polymerization of N-Isopropylacrylamide and acrylic acid using Bis-acrylamide as the crosslinker. Each gel particle

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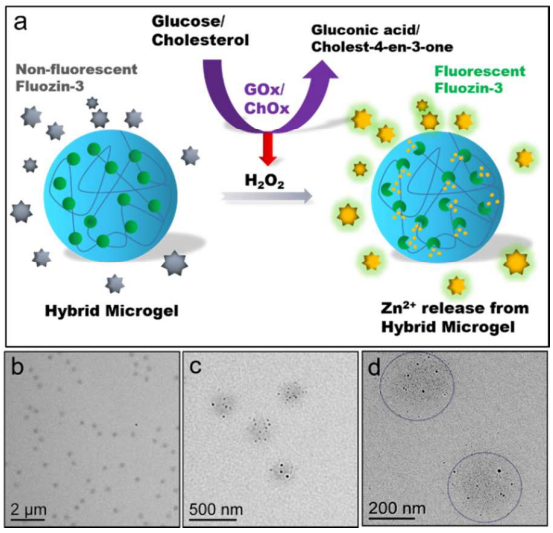

Figure 1. a) Scheme of microgels for detection of glucose and cholesterol. b-d) TEM images of ZnS-enclosed microgels with increasing amplification. The blue circled area in (d) is to show the enclosed nanoparticles inside the microgel.

then served as a microreactor with Zn^{2+} immobilized inside the gel structure via coordination with the carboxyl groups. *In situ* synthesis of small ZnS NPs occurred with the addition of S^{2-}, with the polymer network helping both the nucleation and growth of the NPs, as well as limiting their sizes to less than 10 nm.[10] The size of the microgel before and after *in situ* NP synthesis were measured by the Nanosight™ with the Nanoparticle Tracking Analysis (NTA) software. Before NP encapsulation, the microgel had an average diameter around 431.1+/4.1 nm, and after NP inclusion the average size shifted slightly to 460 nm, both showing comparable size distribution (Figure S1). TEM (Figure 1b, c & d) were used to visualize clusters of small NPs locating within well separated areas (circled by arbitrary boundaries in Figure 1d), which as expected had diameters less than 5 nm. ICP-AES measurement revealed that each hydrogel particle enclosed about 1×10^6 Zn^{2+}, with the molarity of microgel estimated by NTA.

Smaller the NP is, more reactive its ions become; because the majority of the ions are displayed on the NP surface.[27-28] The high reactivity can be utilized to design stimuli-responsive structures, but the stability of the tiny NPs in biological samples is a big concern. Different from the common approaches that protect the NPs with dense surface ligands in the in-solution hydrothermal approaches,[29-30] we protected the small NPs with the hydrogel, the large amount of carboxyl groups in which could stabilize the NPs. We compared the stability of the ZnS-enclosed microgel and the 3-mercaptopropionic acid (MPA)-coated ZnS NPs (11.5 ± 2.7 nm in diameter) when stored in 1×PBS. While the microgel showed negligible release of Zn^{2+} for at least one week, the MPA-coated ZnS NPs dissolved gradually and lost 50% of the Zn content within the same time period (Figure S2). Moreover, the ZnS NPs protected by hydrogel, although smaller in diameter, were more stable in acidic environment: less than 25% of the microgel-enclosed NPs dissolved after 12 hours of storage at pH 5.5, much lower than the > 60% dissolution ratio occurred to the MPA-coated NPs (Figure S3). The high stability can keep the small NP intact even in harsh environments during measurement and quickly respond to target stimulation.

On the other hand, the enclosed NPs are still accessible to small analytes that can rapidly diffuse through the gel network. Metal sulfide can be oxidized by strong oxidants like H_2O_2, which oxidize S^{2-} to S^0 and then to SO_4^{2-} depending on pH (Equation 1 & 2) and the redox potential of the oxidizing reagent:[31-32]

$$S^{2-} + H_2O_2 + 2\,H^+ \rightarrow S + 2\,H_2O \quad (1)$$

$$S^{2-} + 4\,H_2O_2 \rightarrow SO_4^{2-} + 4\,H_2O \quad (2)$$

Sulfide oxidation should release Zn^{2+} to solutions, which can subsequently turn on the Zn-responsive dye, Fluozin-3, converting the oxidation process to fluorescent signals. For quick assessment of whether the as-prepared ZnS-enclosed microgel could be responsive to ROS and turn on the fluorescence of Fluozin-3, we treated the microgel with the representative ROS -- H_2O_2. Indeed, significant fluorescence increase (F/F_0 -1, with F being the fluorescence after reaction with ROS and F_0 being the initial fluorescence background from Fluozin-3) was initiated with 0.8 mM H_2O_2, and reached a plateau with 80 mM H_2O_2. Compared to the MPA-coated ZnS NPs, although the ZnS-enclosed microgel showed a relatively smaller fold of fluorescence increase at the lower [H_2O_2] range (0.8 – 8 mM), which also corresponded to a lower percentage of Zn^{2+} released from the NP. But with a high [H_2O_2] of 80 mM, the microgel released Zn^{2+} 2 times faster than the ZnS NP and reached a plateau fluorescence within 5 minutes (Figure 2). Using Ag^+ to release Zn^{2+} from the microgel and turn on Fluozin-3, we confirmed that this plateau fluorescence was reached because of complete release of Zn^{2+} (Figure S4). This result indicates a high [H_2O_2] gradient is required to deliver sufficient H_2O_2 into the gel to initiate Zn^{2+} release from the NPs; but once the reaction is initiated, its speed is faster for the NPs enclosed in the microgel because of their smaller diameters.

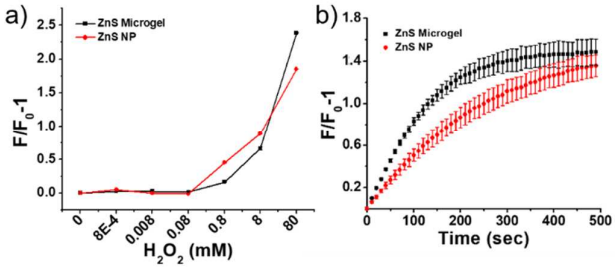

Figure 2 a) Fluorescence response of ZnS NPs or ZnS-enclosed microgel with addition of H_2O_2 (incubated for 15 mins) and (b) their reaction rate with 80 mM H_2O_2. Total [Zn^{2+}] in both microgel and ZnS NP = 1.5 μM, [Fluozin-3] = 3 μM, in 1×PBS buffer at pH 7.4. F_0 and F represent the fluorescence before and after adding H_2O_2, respectively.

To enhance the responsivity to ROS, we chose to supplement Cu^{2+} with Zn^{2+} during cation encapsulation inside microgel, which can convert H_2O_2 to hydroxyl radicals (•OH) via the Fenton-like reaction,[33-34] a stronger oxidant than H_2O_2. Although •OH is highly reactive with organic substrates (rate constants in the range of 10^7 $M^{-1}.s^{-1}$)[35] and cannot travel far away from where it is generated, the microgel structure keeps the Cu^{2+}-based •OH generation site close to the ZnS NPs, thus enhancing ZnS oxidation. Different proportions of Cu^{2+}/Zn^{2+} were added during NP synthesis within the microgel, with the Cu content ranging from 1% to 50% of the Zn content in moles, keeping the total metal amount the same. The effect of H_2O_2-induced Zn^{2+} release was evaluated using the microgel

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containing a total of 1.5 μM M^{2+} (confirmed with ICP-AES). We can see that, the ZnS-enclosed microgel with Cu^{2+} added developed significant fluorescence increase with Fluozin-3 upon incubation with 0.08 mM H_2O_2, but 1% Cu^{2+} showed higher fluorescence increase than the higher percentages (Figure 3a). With the supplement of 1% Cu^{2+}, the microgel released comparable amounts of Zn^{2+} as the MPA-coated ZnS NPs at [H_2O_2] > 0.8 mM but detectible Zn^{2+} release occurred at a [H_2O_2] 10 times lower (Figure S4), overcoming the diffusion problem observed above with the ZnS-enclosed microgel. The Cu-supplement also seemed to reduce the background fluorescence, generating higher fluorescence change.

We confirmed the formation of •OH mediated by Cu^{2+} using chemiluminescence (CL) and electron spin resonance (ESR) spectroscopy.[36] N-(4-Aminobutyl)-N-ethylisoluminol (ABEI) can react with the oxygen-related radicals such as •OH, and produce strong CL (Figure S5a).[37-38] As shown in Figure S5a, the ZnS-enclosed microgel supplied with 1% Cu^{2+} generated significantly larger CL, ~2.4 folds, compared to that without Cu^{2+}. Comparable CL increase was observed among the microgels prepared with 1-10% Cu^{2+}; but a much larger increase, ~10 folds, occurred with the addition of 50% Cu^{2+}. A more specific detection of •OH was carried out by ESR coupled with the radical trapping reagent of 5,5-Dimethyl-1-Pyrroline-N-Oxide (DMPO) (Figure S5b). The characteristic peaks of the typical DMPO-•OH adducts with the peak intensity ratio of 1:2:2:1 were produced with the addition of H_2O_2 to the ZnS-enclosed microgel supplied with 1% Cu^{2+}. This result supports that the supplemented Cu^{2+} can greatly enhance the generation of •OH from H_2O_2 thus speed up Zn^{2+} release from the microgel.

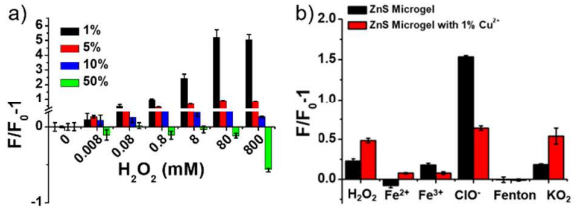

Figure 3. a) H_2O_2 measurement by ZnS-enclosed microgel with supplement of 1, 5, 10, and 50% Cu^{2+} (%Cu^{2+} = Cu^{2+}/Zn^{2+}×100%). b) Responsivity of the ZnS-enclosed microgel without and with the supplement of 1% Cu^{2+} to various ROS. [Fluozin-3] = 3 μM, 1×PBS buffer at pH 7.4, [All reagents] = 10 μM. For Fenton reaction, 10 μM of Fe^{2+} and 10 μM H_2O_2 was used. KO_2 was used to generate $O_2^{\bullet-}$.

However, supplement of Cu^{2+} reduced the overall content of Zn^{2+} enclosed in the microgel. Measurement of the metal composition of each microgel structure with ICP-AES after dissolving the gel with nitric acid to release all the metals (Figure S6) indicated that, with the addition of 1.5 mM pure Zn^{2+}, about 100 μM Zn^{2+} was found in the microgel preparation, which decreased to 50 μM with the addition of 1% Cu^{2+} and to < 20 μM with 5% Cu^{2+}, while keeping total cation concentration at 1.5 mM. When 50% Cu^{2+} added, no Zn^{2+} was found in the gel structure. This is because Cu^{2+} binds more strongly with the carboxylic acids than Zn^{2+}; and the solubility constant of CuS (K_{sp} = 1 x 10^{-36}) is much lower than that of ZnS (K_{sp} = 1 x 10^{-23}), both facilitating CuS formation instead of ZnS during the nucleation and growth processes. The lower Zn^{2+} content with higher %Cu^{2+} greatly decreased the fluorescence signal upon NP dissolution by H_2O_2, making the higher %Cu^{2+} structures less favorable in our sensor design.

We then tested the responsivity of the ZnS-enclosed microgel without or with the supplement of 1% Cu^{2+} to various ROS (Figure 3b). All species were tested at 10 μM using the microgel containing a total [Zn^{2+}] = 1.5 μM. It turned out that without the addition of Cu^{2+}, only the strongest oxidant ClO^- induced the largest fluorescence increase (F/F_0-1 = 1.5) from the ZnS-enclosed microgel, but no significant fluorescence increase was observed with the weaker ROS including H_2O_2. On the other hand, with 1% Cu^{2+}, H_2O_2, ClO^- and KO_2 all showed comparable fluorescence increase (~ 0.5) (Figure 3b). KO_2 produces the unstable $O_2^{\bullet-}$ with a half-life ~0.06 s, it spontaneously scavenges and generates H_2O_2,[39] producing a signal comparable to that of H_2O_2. As discussed above, Cu^{2+} can accelerate production of •OH from H_2O_2 that can rapidly release Zn^{2+} from the microgel. Thus, copper supplement permits responses to H_2O_2, ClO^- and KO_2. However, the iron species of Fe^{2+} or Fe^{3+} alone, and the Fenton reaction system of Fe^{2+}/H_2O_2 did not show any effects. The Fenton reaction should generate •OH, but its high reactivity does not allow it to get close to the NPs before being quenched by the polymer structure, and thus no signal was produced. Therefore, the NP-enclosed microgel we constructed should be responsive to the strong and stable ROS stimuli like ClO^- and H_2O_2, as well as the instable ROS that can spontaneously form H_2O_2.

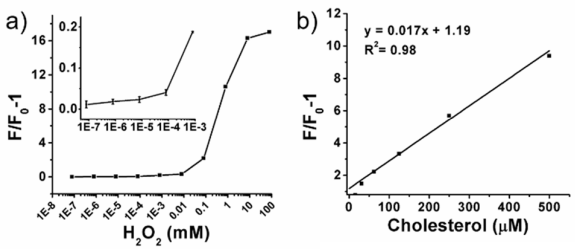

Figure 4. a) Calibration curve of the ZnS-enclosed microgel doped with 1% Cu^{2+} for detection of H_2O_2. The inset enlarges the detection with the lower concentration range. b) Calibration curve for detection of cholesterol. Total [Zn^{2+}] in microgel = 1.5 μM, [Fluozin-3] = 3 μM, 1×PBS buffer at pH 7.4, [EDTA] = 3 μM.

The above studies support that the ROS-responsive microgel we constructed should be valuable for detection of ROS. Since some ROS like H_2O_2 is also involved in many enzymatic processes, the microgel can also be a versatile tool for detection of small molecular biomarkers like glucose and cholesterol. To achieve a better detection performance, we further optimized the experimental condition by reducing the fluozin-3 background using ethylenediaminetetraacetic acid (EDTA) to chelate the interfering background metals (Figure S7a&b). Under the optimized [EDTA] of 3 μM, the ZnS-enclosed microgel with 1% Cu^{2+} supplement detected as low as 80 nM H_2O_2, and achieved a dynamic range of 80 nM to 8 mM (Figure 4a). This limit of detection is about one order of magnitude lower than most fluorescent H_2O_2 sensors reported previously (Table S1)[21, 40-43].

Then, we applied our microgel to detect glucose and cholesterol. The ROS-responsive microgel was added directly to the glucose-containing samples together with GOx at 2 μg/mL and reacted for 30 min (Figure S8). The presence of

microgel did not affect enzyme activity in this one-pot reaction: calculation of the Michaelis–Menten constant K_m using the Lineweaver-Burke plot found a K_m value of 0.54 mM, matching with previous report (Figure S10a). As low as 0.53 µM glucose was detected and a wide dynamic range up to 5 µM was achieved (Figure S9). Similarly, with 5% TX-100 added to improve target solubility (Figure S11), the LOD calculated by the 3σ method for cholesterol was found to be 0.77 µM (Figure 4b), with no impact from the microgel to the enzyme activity of ChOx (Figure S10b).[44] The simple protocol of ROS-responsive microgel to quantify glucose and cholesterol makes it a valuable tool in clinical: when applied to detect these markers in two sets of human sera, each containing 5 patient samples with various levels of glucose or cholesterol, our results agreed well with the true values provided on products information (Table S2).

To summarize, we have designed a unique ROS-responsive microgel system through NP encapsulation and cation release to turn on the fluorescence of the metal-responsive dye. Such a design carries the advantages of high structural robustness, bright and stable fluorescence signal, and easy fabrication and simple implementation. More interestingly, by tuning the NP composition, selective responsivity towards different ROS could be achieved. When applied to detect small molecular markers like glucose and cholesterol, high sensitivity, broad detection range, and accurate and fast measurement in complex biological samples can be achieved. We expect that the NP-enclosed microgels could be adopted in various biomedical applications with minor modifications in their compositions.

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Conflicts of interest

There are no conflicts to declare.

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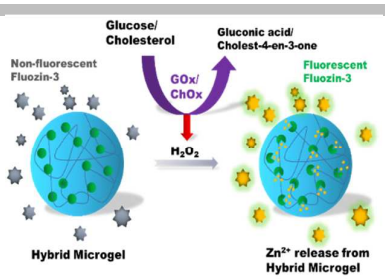
Encapsulation of ionic nanoparticles in microgel produces a reactive oxygen species (ROS)-responsive probe, the encapsulated cations in which could be released by ROS to turn on fluorogenic dyes to realize detection of ROS or ROS-related biomolecules.



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