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Communication

Templated Synthesis of Copper Nanoclusters with a Hybrid Lysozyme-Polymer Material for Enhanced Fluorescence

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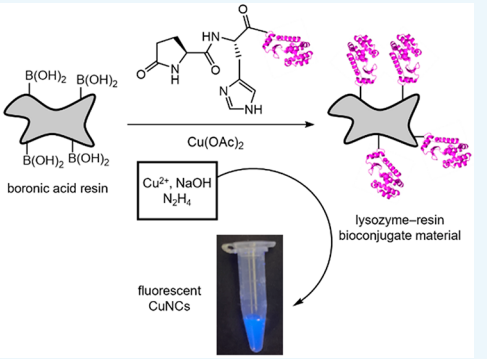
ABSTRACT: Hybrid materials that combine organic polymers and biomacromolecules offer unique opportunities for precisely controlling 3D chemical environments. Although biological or organic templates have been separately used to control the growth of inorganic nanoclusters, hybrid structures represent a relatively unexplored approach to tailoring nanocluster properties. Here, we demonstrate that a molecularly defined lysozyme−polymer resin material acts as a structural scaffold for the synthesis of copper nanoclusters (CuNCs) with well controlled size distributions. The resulting CuNCs have significantly enhanced fluorescence compared with syntheses based on polymeric or biological templates alone. The synergistic approach described here is appealing for the synthesis of biocompatible fluorescent labels with improved photostability.



INTRODUCTION

Metal nanoclusters are atomically precise particles composed of ∼200 atoms or less and have emerged as an important class of material due to their diverse applications as catalysts,[1−3] fluorescent probes,[4−6] sensors,[7−10] and therapeutic agents,[11] among others.[3,12−15] Because metal nanocluster properties are heavily dependent on their morphology, size, and surface chemistry, diverse and well-controlled synthetic methods are required for their production.[3,12,14] Copper nanoclusters (CuNCs) in particular have attracted significant attention compared to other noble metals (e.g. platinum, gold, silver) due to their low cost,[12,16] making them ideal for large-scale applications.[12] Copper is also an essential element in living organisms,[17,18] with limited toxicity making CuNCs appealing for biomedical applications.[19] Many copper nanoclusters fluoresce at visible wavelengths and have attracted interest as imaging[2,13,20] and sensing agents[7,10] However, copper nanomaterials can prove challenging to prepare and handle due to their relatively small reduction potential and corresponding ease of oxidation.[12,15,21]

Typical methodologies for CuNC preparation include top-down approaches, in which bulk materials are broken into nanosized fragments,[12,22,23] as well as bottom-up synthesis, in which ions are chemically or electrochemically reduced to metal zero species which nucleate and grow into small clusters via the appropriate choice of ligand and reaction conditions.[12,24] In either case, clusters are rarely synthesized in high purity and require subsequent size-focusing or purification steps to achieve high-quality samples. An alternative bottom-up approach is to make use of a structurally well-defined template, in which clusters may grow to a prescribed size and shape. Thus, a variety of scaffolds have emerged, including organic molecules,[25,26] polymers,[27−29] and biomolecules,[20,30−32] all of which aim to provide greater control over nanocluster morphology presumably because precise chemical control over these species is more straightforward than tailoring of nucleation and growth kinetics.[12]

Prominent among soft material templates for particle growth are polymers and proteins due to their well-understood and well-controlled 3D structure.[12] Although each can individually provide unique template properties, the fusion of these compounds within a single scaffold material has not been explored. As part of a broader program aimed at the development of efficient methods for the preparation of precise bioconjugates, we recently disclosed the synthesis of a boronic-acid containing poly(ethylene glycol) acrylamide (PEGA) resin, capable of copper-mediated protein immobilization utilizing a minimalist dipeptide tag (pyroglutamate−histidine).[33] The immobilization of tagged proteins within this resin results in a well-defined biomaterial and avoids heterogeneous linkages or a random ensemble of protein orientations.[33] We hypothesized that the homogeneity of the lysozyme−polymer conjugate material would promote batch-to-batch reproducibility by generating a matrix with precise linkages for controlled particle growth, potentially with

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emergent properties unique to the hybrid protein-resin conjugate template. Additionally, the reactive dipeptide tag is easily incorporated and minimally disruptive to protein structure while at the same time readily scalable for bulk material applications. Herein, we describe how hybrid biomaterials derived from site-selective immobilization of pyroglutamate-histidine (pGH) tagged lysozyme onto a PEGA resin matrix can be used to template the formation of CuNCs with enhanced fluorescence emission.

RESULTS AND DISCUSSION

Because prior work has shown lysozyme to be an effective template for CuNC synthesis, here we site-selectively immobilize a pGH-tagged lysozyme onto a boronic acid functionalized resin and investigate the effectiveness of the resulting hybrid biomaterial as a scaffold for CuNC formation, following our previously reported procedure.[33] Briefly, a boronic-acid-functionalized resin, PEGA-B(OH)$_2$ (Figure 1),

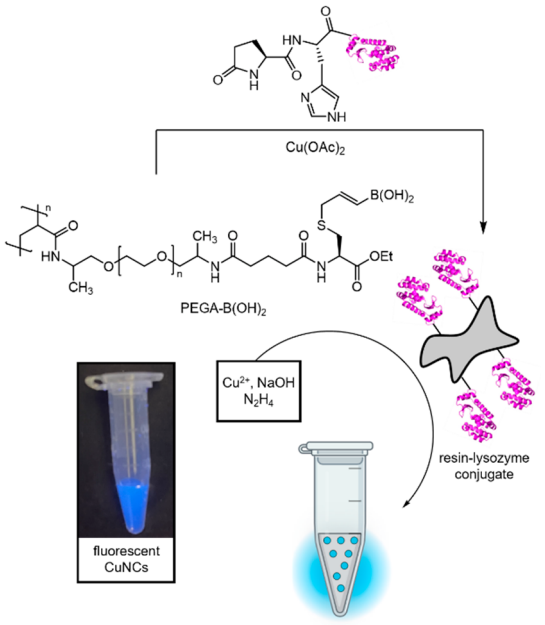

Figure 1. Immobilization of a pGH-tagged lysozyme onto a boronic-acid-functionalized resin followed by templated CuNC synthesis.

was first prepared in two chemical steps from commercially available PEGA resin. Lysozyme incorporating a dipeptide Glp-His tag at the N-terminus was purified from an *E. coli* linked expression system[34] containing a glutamate cyclase enzyme to install the key pyroglutamate (Glp) post-translational modification.[33,35] Next, suspensions of the resin in buffered water were treated with tagged lysozyme protein (10 μM) and Cu(OAc)$_2$ (100 μM) for 18 h. The product hybrid resin was then collected by filtration and washed extensively, providing a resin-lysozyme hybrid for further nanocluster growth studies.

Based on previous protocols,[20,30] CuNCs were synthesized by suspending the washed resin-lysozyme conjugate in 100 μL of Milli-Q water, followed by the addition of 10 μL (25 mM) of aqueous copper(II) sulfate with vigorous stirring at 37 °C. After 10 min, 25 μL (1 M) of NaOH solution was added, during which the reaction mixture turned purple. After 1 h of stirring, 20 μL of aqueous hydrazine (35%) was added dropwise and stirring continued overnight at 37 °C. After allowing the reaction to proceed overnight, the aqueous mixture appeared strongly blue under a 365 nm UV lamp (Figure 2d).

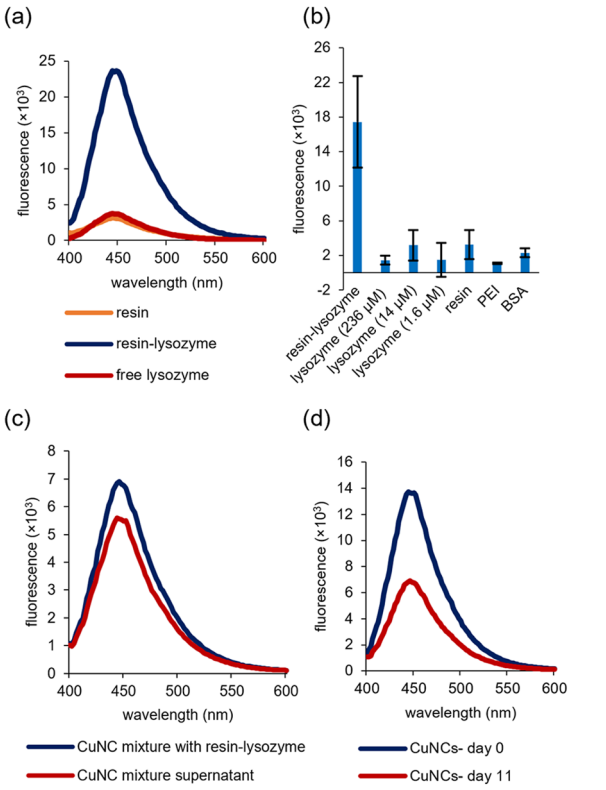

Figure 2. (a) Fluorescence signal and (b) average fluorescence intensities of CuNCs synthesized from "resin only", "resin-lysozyme conjugate", "free lysozyme", "PEI", and "BSA" template structures. (c) Fluorescence of CuNC mixture with "resin-lysozyme conjugate" and only supernatant. (d) Fluorescence of CuNCs synthesized from "resin-lysozyme conjugate" template after 0 and 11 days in solution. Excitation, 360 nm; emission, 444 nm.

The resulting CuNCs exhibited a strong fluorescence signal with a maximum at 444 nm, consistent with previous literature reports of protein-templated CuNCs.[20,30] More interestingly, CuNCs grown with the hybrid protein-resin matrix had a significantly increased fluorescence intensity when compared with particles templated by resin or protein alone (Figure 2a,b). In control experiments with lysozyme alone, we found that variation of the concentration of soluble lysozyme itself over a wide range, from the maximum theoretical protein concentration in the resin-lysozyme system (1.6 μM) up to 14 μM, does not lead to any significant increase in CuNC fluorescence (see Supporting Information). These results indicate that the efficiency of fluorescent nanocluster production is qualitatively and quantitatively different from that achievable with protein or polymer resin alone.

Additional comparisons to other templating structures provide insights into the structural basis of enhanced emission. We produced nanoclusters grown in the presence of polyethylenimine (PEI)[28] and the protein BSA,[13] two other common templates for the growth of fluorescent copper nanoclusters. In both cases, observed emissions were >5-fold lower than that with the resin-lysozyme conjugate material. We previously reported immobilization onto the boronic acid resin (PEGA-B(OH)$_2$) of the proteins GFP and a nanobody-GST fusion (VHH-GFP).[33] However, neither of these immobilized

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conjugates was effective as templates for the production of fluorescent copper nanoclusters, indicating that the nature of the immobilized protein is important for emission of the resulting nanoparticles (see Figure S11).

Conveniently, the protein−polymer template could be readily separated from the produced nanoclusters by centrifugation. Decanting the soluble fluorescent CuNCs away from the protein−polymer precipitate allowed the isolation of soluble CuNCs (≥80% of crude fluorescence intensity, Figure 2c). While CuNCs are presumably formed via templated growth in the hybrid protein-resin matrix, our results indicate that the nanoclusters themselves are not tightly bound to the matrix. Therefore, the preparation here provides a convenient approach to access well-defined nanoclusters that can be readily separated from the synthetic scaffold. The CuNCs made by this method are stable over days in solution, but a significant decline in emission was noted after 11 days (Figure 2d).

To further characterize the size and morphology of the protein-resin synthesized CuNCs, STEM and TEM imaging was conducted of the hybrid material. These data show numerous CuNCs with strong contrast readily distinguishable from the organic matrix in which they are embedded (Figure 3). We also observed CuNCs that were not associated with the organic resin but instead were isolated on the lacey carbon support (Figure 3b), consistent with our solution-phase fluorescence observations (see above). High-resolution TEM imaging also revealed lattice fringes associated with the nanoclusters with a spacing of 0.21 nm, corresponding to the {111} plane of the face-centered cubic (FCC) Cu crystal structure (Figure 3d). Size analysis indicates that the CuNCs are highly uniform, with an average diameter of 1.9 ± 0.3 nm (Figure 3e).

To further confirm the presence of nanocluster-sized (<2 nm) particles resulting from the protein-resin template, CuNCs were tested as catalysts for styrene oxidation, which has been previously used as a benchmark for CuNCs (Table 1).[2] The CuNCs exhibited efficient oxidation at 50 °C, with selectivity for benzaldehyde over other products.[2] While selectivity does erode somewhat at elevated temperatures (entry 2), with significant acetophenone formed alongside benzaldehyde, this likely occurs because of accelerated nanocluster coalescence at elevated temperatures and the loss of catalytically active surface sites.

CONCLUSION

A new material class of protein−polymer resin conjugates has been shown to effectively template the synthesis of high quality CuNCs. The bioconjugate resin is readily separable from the prepared CuNCs, and the hybrid material results in significantly increased fluorescence emission relative to protein or resin templates alone. Moreover, the resulting CuNCs demonstrated excellent catalytic properties through the oxidation of styrene, suggesting that their surfaces remain chemically available. Copper nanoclusters are being explored in diverse sensing and imaging applications, and access to clusters with enhanced emission should provide an improved signal and allow improved detection limits. This work suggests that a high local concentration of lysozyme within the PEGA resin network enhances the formation of fluorescent particles and indicates that more diverse and sophisticated approaches to template for nanocluster growth would enable the preparation of particles with new or optimized properties.

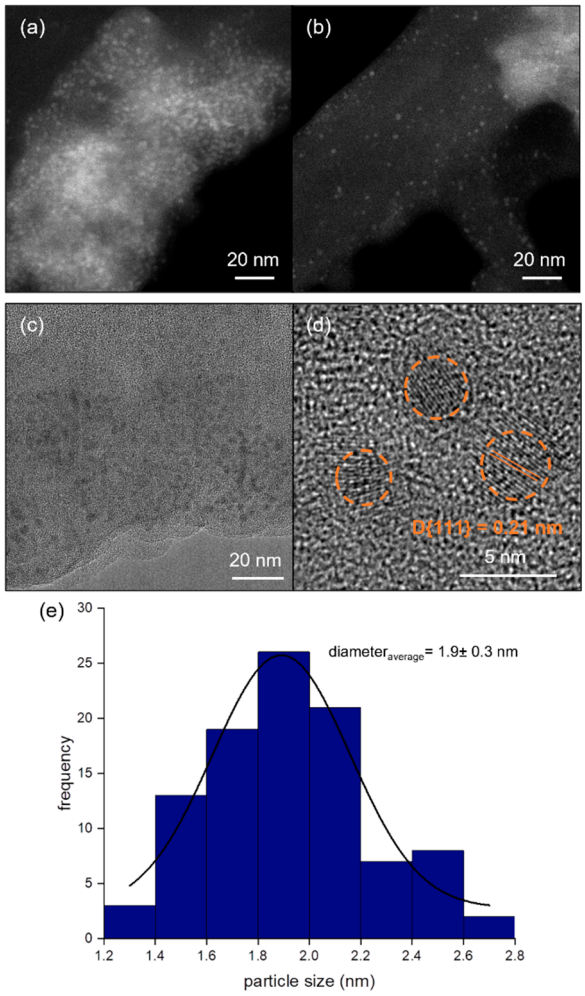
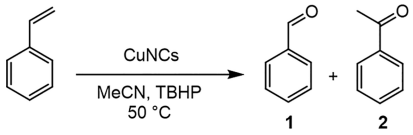


Figure 3. STEM images of CuNCs (a) within resin-lysozyme and (b) separated from the matrix on a lacey carbon imaging substrate. (c,d) TEM images of CuNCs within PEGA-lysozyme. (e) Size distribution of CuNCs from PEGA-lysozyme templated synthesis.

Table 1. Styrene Oxidation Catalyzed by Resin-Lysozyme Templated CuNCs



entry	conditions	% conversion	% yield 1	% yield 2
1	CuNCs, 50 °C	100	100	0
2	CuNCs, 75 °C	100	65	35
3	no CuNCs, 50 °C	0	0	0

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.4c00058.

Synthetic procedures, characterization data, and additional image and spectroscopy data (PDF)

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Notes

The authors declare no competing financial interest.

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