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BIOMIMETIC SYNTHESIS OF CdSe NANOPARTICLES WITH POTENTIAL BIOIMAGING APPLICATIONS

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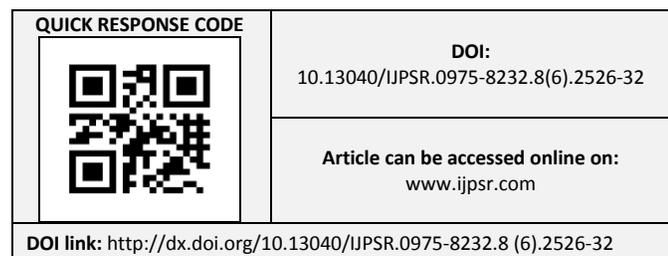
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ABSTRACT: This study presents *in vitro* biomimetic synthesis of CdSe nanoparticles using the enzyme, NADPH dependent-Nitrate reductase purified from fungus, *Fusarium oxysporum*. The synthesis of CdSe nanoparticles was accomplished in the presence of a synthetic peptide having amino acid sequence (γ -Glu-Cys-Glu-Cys)-Gly, which acted as binding molecule. The nanoparticles were synthesized in the size range of 3.9–9.0 nm was analysed using TEM. Further characterization of nanoparticles was done using techniques such as UV, PL, XPS, and FTIR. The as-synthesized CdSe nanoparticles were water dispersible containing free reactive amino groups. These CdSe nanoparticles were conjugated with bi-antennary and tri-antennary glycopeptides opening up the possibilities for their applications in bioimaging. These significant observations will help better understand the mechanism of biosynthesis of fluorescent nanoparticles using fungi. These findings promise an alternative strategy for an eco-friendly, economical, and large-scale synthesis of water dispersible CdSe nanoparticles. These CdSe nanoparticles have promising biomedical-imaging applications without a need for further functionalization.

INTRODUCTION: CdSe quantum dots are some of the most studied nanocrystals due to their use in numerous applications such as solar cells, bio-labels, optoelectronics, and many others¹⁻³. Even though there are numerous routes to synthesize CdSe nanocrystals in sub-10-nm regime, the advantages of biosynthetic processes include eco-friendliness, economy, and ambient characteristics.

Researchers opted for alternative synthetic routes, including biological methods using microbes as a potential alternative to the existing hazardous methods, for a variety of nanoparticles⁴⁻⁶.

Microbes possess complex synthetic machineries that explain the limited exploitation of biosynthetic routes. In general, microorganisms possess biomolecular machineries to overcome the physiological stress created by 'foreign' substances⁷. It is believed that counter-stress measures evolved by microorganisms are responsible for the biosynthesis of nanoparticles. A number of reports have indicated the use of precursors for the synthesis of nanomaterials using microbes that are otherwise toxic to the microbes in question⁸.



A better understanding of the role of different factors involved in the process of detoxification of physiological stress is imperative to exploit the full potential of bio-synthetic routes. In an attempt to understand the biosynthesis of nanoparticles by fungi, the enzymes, sulfite reductase and nitrate reductase and a templating protein were isolated from the extra-cellular broth of fungus *Fusarium oxysporum* and utilized *in vitro* synthesis of gold, silver, and other metal sulfide nanoparticles⁹⁻¹¹. Molecular templates are used to control precisely the fabrication of nanoparticles.

Khan *et al.* have reported the synthesis of gold nanoparticles using trypsin¹². Sarikaya *et al.* have exhaustively reviewed the use of biomolecular templates for the synthesis of nanoparticles¹³. Quantum dots are regarded as one of the best alternative for organic dyes as bio-labels. Stachowski *et al.* have found the synthesis of radio-labeled CdSe/CdS/ZnS quantum dots for *in vivo* applications¹⁴. Biosynthetic methods are economical and scalable; moreover the nanoparticles thus produced can generally be *in situ* functionalized and are water dispersible¹⁵. *In vitro* enzyme based synthesis of nanoparticles paves way for a new strategy for the precise control of particle size while satisfying environmental safety concerns.

Glycopeptides play a vital role in the homeostasis in a biological system that includes many vital process such as fertilization, endocrine system, cell-cell interaction, etc.¹⁶⁻¹⁸. Glycopeptides are widely used as targeting molecules that can be attached to a molecule of interest to be targeted to a particular cellular location. Tsao *et al.* have studied the tissue distribution and imaging potential of⁶⁸ Ga-glycopeptide using Positron Emission Tomography (PET)¹⁹. Richter *et al.* have reviewed the potential of radio-labeled peptides in diagnostic imaging and therapy²⁰.

In this study, we have reported the use of enzyme nitrate reductase purified from fungus, *Fusarium oxysporum* for *in vitro* biomimetic synthesis of water dispersible CdSe quantum dots. We also report the use of a synthetic peptide (γ -Glu-Cys-Glu-Cys)-Gly that acts as a capping agent for *in vitro* synthesis of CdSe quantum dots. The as-

synthesized CdSe quantum dots were conjugated with biomolecules with potential bioimaging applications.

Experimental: All the chemicals and reagents were from standard commercial sources and of the highest purity available. The enzyme nitrate reductase was purified as described by Kumar *et al*¹⁰. The reaction mixture (3 mL) containing CdCl₂ (1 mM), SeCl₄ (1 mM), NaNO₃ (5 mM), 100 μ g of synthetic peptide (SP) having amino acid sequence (γ -Glu-Cys-Glu-Cys)-Gly, 1.0 mM α -NADPH (reduced form of nicotinamide adenine dinucleotide phosphate sodium salt), and 1.66 U of nitrate reductase was incubated under anaerobic conditions at 25 °C.

The reaction mixture was subjected to UV-Vis spectrophotometric measurements performed on Shimadzu dual-beam spectrophotometer (model UV-1601 PC) operated at a resolution of 0.5 nm. Fluorescence was measured by exciting samples at 366 nm, and the emission spectra were recorded from 400 to 700 nm using a spectrofluorimeter, FLS920, Edinburgh Instruments, UK at a scan rate of 300 nm/min. PXRD patterns were recorded in 2 θ range of 20°–80° with step size of 0.02° and time 5 s per step using Philips X'PERT PRO instrument equipped with X'celerator, a fast solid-state detector with Iron-filtered Cu K α radiation (λ =1.5406 Å) as the source. CdSe nanoparticles suspension coated on carbon coated copper TEM grids were subjected to TEM analysis on FEI Tecnai 30 transmission electron microscope operated at an accelerating voltage of 300 kV.

Dynamic light scattering measurements were performed on Brookhaven 90 Plus/Bi-MAS Instrument (Brookhaven Instruments, New York), equipped with a 15 mW solid state laser at a wavelength of 657 nm, and scattering signals were collected at 90° at an average of 6 runs per scan. Chemical analysis of a drop-coated film of CdSe nanoparticles suspension on a conductive substrate was carried out on ESCALAB MK II set-up X-ray photoelectron spectrometer (V. G. Scientific, UK) with Al K α as the exciting source ($h\nu$ = 1486.6 eV) operating at an accelerating voltage of 10 kV and 20 mA at a pressure of about 10⁻⁸ Pa.

The core level binding energies (BEs) were aligned with respect to C 1s binding energy (BE) of 285 eV. The pI of the synthetic peptides was calculated from peptide property calculator available online (<http://www.innovagen.com/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp>). The free amino groups were estimated as described elsewhere²¹⁻²².

A modified version of the method used to estimate carboxyl group was used for the conjugation of CdS/CdSe nanoparticles with bi/tri-antennary glycopeptides. The reaction mixture (3 mL) containing 100 µg of CdS/CdSe nanoparticles, 50 µg of bi/triantennary glycopeptide in 50 mM MES/HEPES buffer (75:25 v/v) pH 6.0 and 50 mM EDC was incubated at 30 °C for 45 min. CdS/CdSe - bi/ tri - antennary glycopeptides conjugates were purified by passing the mixture through Sephadex G-25 matrix pre-equilibrated with 20 mM phosphate buffer at pH 7.2 containing 150 mM NaCl.

RESULTS AND DISCUSSION: The reaction mixture containing CdCl₂, SeCl₄, NaNO₃, the synthetic peptide (SP) (γ-Glu-Cys-Glu-Cys)-Gly, NADPH, and nitrate reductase, when incubated at 25 °C resulted in the formation of CdSe quantum dots (**Fig. 1**). The synthesis was monitored by following the appearance of absorption band centered at 366 nm that indicated the formation of CdSe nanocrystals. No absorption band at 366 nm was found in the absence of nitrate reductase or NADPH, and when the denatured enzyme was used (data not shown). This observation confirms involvement of enzyme in the reduction of Cd²⁺ and Se⁴⁺ to stable CdSe nanoparticles utilizing nitrate as substrate and NADPH as cofactor. We used the fungus, *Fusarium oxysporum*, for the extracellular biosynthesis of CdSe quantum dots; the same was used in earlier study to purify the nitrate reductase²³.

The as-synthesized CdSe nanocrystals were subjected to preliminary investigation using dynamic light scattering to determine the size distribution of CdSe nanocrystals. The nanocrystals were in the range of 3.9 to 9.0nm. The absence of SP did not result in the synthesis of CdSe quantum dots as indicated by the absence of absorbance at

366 nm. A possible mechanism of the biosynthesis of CdSe nanoparticles using enzymes in the presence of synthetic capping peptides is proposed. The SP which has a pI of 3.1 behaves as an anionic polyelectrolyte in solution at pH 7.2. The precursors, cadmium chloride (CdCl₂) and selenium tetrachloride (SeCl₄), completely dissociate in aqueous solution generating Cd²⁺ ions and Se⁴⁺ ions, respectively.

In neutral or slightly alkaline solution, the peptide having pI 3.1 carries a net negative charge of -2.1 owing to the functional groups of constituent amino acids, thereby attracting Cd²⁺ and Se⁴⁺ ions due to electrostatic interactions. This might result in the formation of nuclei for nanoparticles. The reduction of the electrostatically bound cadmium and selenium cations results in the formation of stable CdSe nanocrystals stabilized by the SP. It is worth to note that CdSe nanoparticles of larger size were synthesized upon incubation of the same precursors in the culture broth of the fungus, *Fusarium oxysporum*²³. Similarly, Zhou et al. demonstrated the potential of His-tag proteins in the crystallization of ZnS nanocrystals²⁴.

The absorption spectra of the as-prepared CdSe in aqueous solution showed a peak corresponding to the first excitonic transition. The relative blue shift in the UV-Visible spectrum corresponding to 366 nm indicates the formation of CdSe quantum dots with a band gap of 2.73eV, which is relatively large as compared to that of bulk CdSe (698 nm, 1.78eV) (**Fig. 1**). This is in close agreement with the quantum size effect associated with direct gap semi-conductors. Luminescence characteristics of the nanocrystals were studied by exciting the nanocrystals in aqueous solution at 366 nm.

The emission band centered at 454 nm (**Fig.1**) could be attributed to the band gap or near band gap emission resulting from the recombination of electron-hole pairs in the CdSe nanocrystals with a band gap of 2.73 eV. This corresponded well with the photoluminescence achieved for CdSe quantum dots. The half width of the emission peak of ≈ 90 nm indicated that the size distribution of particles was not even.

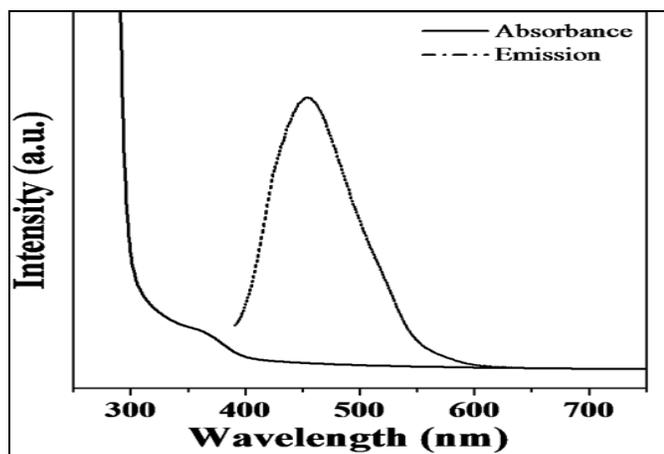


FIG. 1: ABSORBANCE AND PHOTOLUMINESCENCE SPECTRA OF AS-SYNTHESIZED CdSe NANOPARTICLES

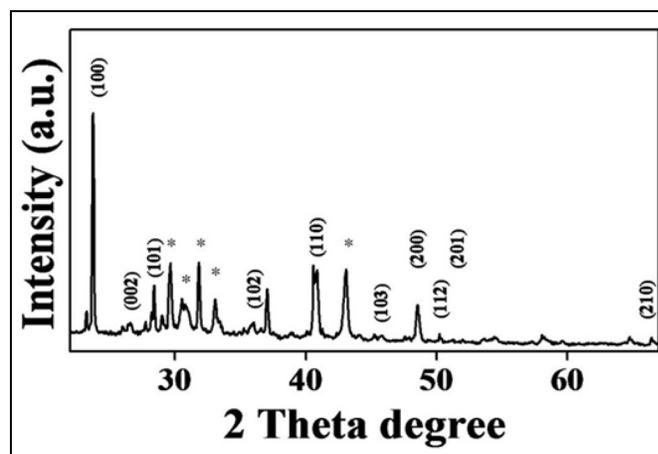


FIG. 2: XRD SPECTRA OF AS-SYNTHESIZED CdSe (ASTERISKS INDICATE CdO PEAKS)

X-ray diffraction analysis of the sample showed well-defined Bragg's reflections indicating that the particles were crystalline in nature and possessed wurtzite structure (Fig. 2). The diffraction measurements also indicated that the CdSe nanocrystals were in hexagonal phase having cell parameters of $a = b = 4.30$ and $c = 7.02$ and $\alpha = \beta = 90^\circ$ and $\gamma = 120^\circ$. The peaks corresponding to cadmium oxide were also observed (indicated by (*) asterisk) which might be due to the oxidation of Cd atoms present on the surface of CdSe nanoparticles.

X-Ray photoelectron spectroscopy analysis of the as-synthesized CdSe nanoparticles showed core level binding energies for Cd and Se that agree with the values reported for CdSe. Cd3d spectrum could be resolved into two peaks (Cd3d_{5/2} and Cd3d_{3/2}) with a spin orbit splitting factor of ≈ 7.0 eV (Fig. 3.A) corresponding to binding energies of 405.5 and 412.5 eV, respectively. The Se3d peak observed at binding energy of 54.8 eV agreed with the core level binding energy reported for Se (Fig. 3.B).

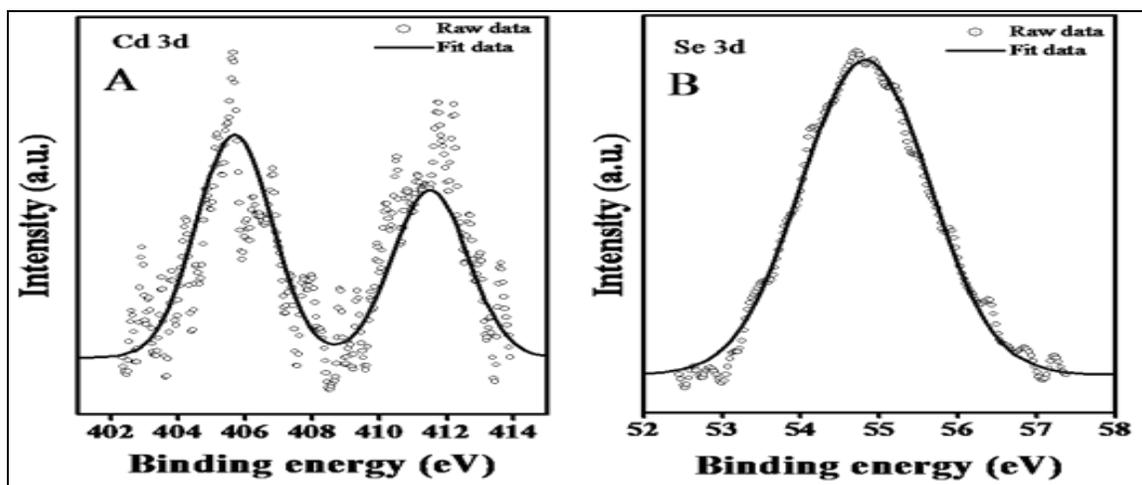


FIG. 3: NARROW SCAN X-RAY PHOTOELECTRON SPECTRA OF AS-SYNTHESIZED CdSe NANOPARTICLES; CORE LEVEL SPECTRA OF A. Cd, and B. Se, IN AS-SYNTHESIZED CdSe QUANTUM DOTS

TEM observations showed that the CdSe nanocrystals were discrete, and essentially spherical in shape (Fig. 4. A). The particle size histogram was constructed by counting ~ 100 individual particles from different TEM images. The formation of spherical nanoparticles might be

due to its energetically favourable nature. The nanoparticles were found to be in the range of 3.9 to 9.0 nm with an average size of ~ 5.5 nm (Fig. 4.B). HR-TEM images showed that the nanoparticles possess good crystallinity, with discrete lattice fringes (Fig. 4.C & D).

The corresponding reduced FFT images of individual nanocrystals could be indexed to CdSe nanocrystals. The lattice fringes observed on the CdSe nanocrystals could be indexed to $\langle 112 \rangle$ and $\langle 101 \rangle$ phases of CdSe with corresponding d-spaces of 1.83 and 3.31 Å, respectively.

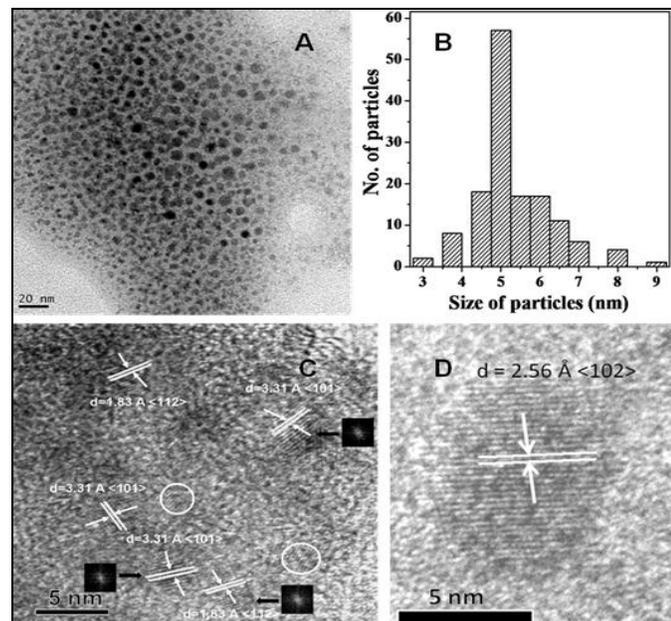


FIG. 4: TRANSMISSION ELECTRON MICROGRAPHS OF AS-SYNTHESIZED CdSe NANOPARTICLES, A. TEM IMAGES TAKEN AT DIFFERENT MAGNIFICATIONS, B. HISTOGRAM OF SIZE DISTRIBUTION OF PARTICLES CALCULATED FROM TEM IMAGES (THE SOLID LINE REPRESENTS THE GAUSSIAN FIT TO THE HISTOGRAM), C. HR-TEM IMAGE (REDUCED FFT IMAGES ARE PLACED BESIDE INDIVIDUAL NANOCRYSTAL. INDIVIDUAL CdSe NANOCRYSTALS ENCIRCLED), D. HR-TEM IMAGE OF AN INDIVIDUAL CdSe NANOPARTICLE.

The estimation of free amino and carboxyl group present on the as-synthesized CdSe nanoparticles showed presence of a reactive amino group while no free carboxyl groups were detected. The amino group might have been contributed by the SP bound to CdSe nanoparticles. The conjugation of as-synthesized CdS and CdSe nanoparticles was carried out by using a modified protocol used for the estimation of carboxyl group.

The mentioned synthesis of nanoparticle-glycopeptide conjugates was carried out at pH 6.0 by using the free amino group of the capping peptide associated with the nanoparticles, for cross-linking with reactive carboxyl group present on bi/tri-antennary glycopeptides. The reaction might

have utilized water-soluble 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) to catalyze reactions between nanoparticle-capping peptide amino group and the acid group of bi-/tri-antennary glycopeptides. In this reaction, the concentration of bi-/tri-antennary glycopeptides was kept twice as that of the nanoparticles in order to facilitate the formation of nanoparticle-glycopeptide.

When passed through the size exclusion matrix Sephadex G-25, the elution profile of nanoparticle-bi/tri-antennary glycopeptides conjugates also showed a shift in the retention time as compared to the retention times of the individual CdSe nanoparticles and bi/triantennary glycopeptides indicating an increase in the size of the CdSe nanoparticles due to the attachment of glycopeptides on the surface of CdSe nanoparticles. The formation of conjugates of CdSe-bi-antennary glycopeptides and CdSe-tri-antennary glycopeptides were further corroborated by the emission profile of CdSe-glycopeptide conjugates, which showed a blue-shift in the emission profile (CdSe-bi-antennary glycopeptide – 443 nm and CdSe-tri-antennary glycopeptides – 437 nm) as compared to the naked CdSe nanoparticles (454 nm) (Fig. 5).

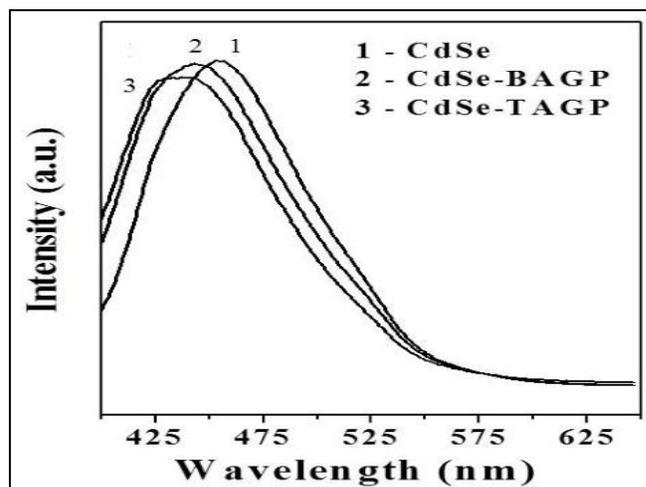


FIG. 5: COMPARATIVE PHOTOLUMINESCENCE SPECTRA OF CONJUGATES OF CdSe-BI-ANTENNARY GLYCOPEPTIDES (CdSe-BAGP), AND CdSe-TRI-ANTENNARY GLYCOPEPTIDES (CdSe-TAGP) WITH THAT OF BARE-CdSe NANOPARTICLES

The formation of CdSe-glycopeptides conjugates indicates the potential of the surface reactive amino groups to be utilized in the conjugation of these

CdSe nanoparticles with other biomolecules such as proteins, polysaccharides, etc. This obviates the need for further functionalization of the as-synthesized CdSe nanoparticles for potential biomedical applications.

In this report, we have attempted to shed some light on the biomolecular factors that might be responsible for the biosynthesis of CdSe nanoparticles by the fungus, *Fusarium oxysporum*. We have demonstrated that the enzymes produced by fungus, *Fusarium oxysporum* could be used for the production of water-dispersible CdSe nanoparticles. In addition, we have proved that synthetic peptide, (γ -Glu-Cys-Glu-Cys)-Gly could be used as a capping peptide for the synthesis of CdSe nanoparticles that could be used for biomedical applications. This method is simple, efficient, straight-forward, economical, and eco-friendly for the production of water-dispersible CdSe quantum dots.

CONCLUSION: Although numerous methods exist for the synthesis of fluorescent nanoparticles, synthetic methodologies that address environmental concerns associated with the large-scale synthesis of nanoparticles are still at large. Enzyme mediated biosynthesis of nanoparticles has the potential to emerge as the alternative process without compromising efficiency. The identification of the biomolecules responsible for biosynthesis by fungus would open up the efficient use of biosynthesis in the synthesis of a wide variety of nanoparticles. Although significant advances have been made in the size and shape control of different nanoparticles, the use of biomolecules for the synthesis of quantum dots with a narrow size distribution may offer significant advantages over other methods viz., possibility of exhaustive combination of biomolecules, dispersibility in aqueous media, functionalization of nanoparticles, reduced toxicity, etc. The establishment of a mathematical model to understand the above protocol would help in the better understanding and subsequent exploitation of the synthesis and the role of different factors influencing the growth, size, and shape of nanocrystals.

CONFLICT OF INTEREST: The authors have no financial conflicts of interest.

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