Facile Peptides Functionalization of Lanthanide-Based Nanocrystals through Phosphorylation Tethering for Efficient in Vivo NIR-to-NIR Bioimaging

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Supporting Information

ABSTRACT: Peptide modification of nanoparticles is a challenging task for bioapplications. Here, we show that noncovalent surface engineering based on ligand exchange of peptides for lanthanide based upconversion and downconversion near-infrared (NIR) luminescent nanoparticles can be efficiently realized by modifying the hydroxyl functional group of a side grafted serine of peptides into a phosphate group (phosphorylation). By using the phosphorylated peptide with the arginine-glycine-aspartic acid (RGD) targeting motifs as typical examples, the modification allows improving the selectivity, sensitivity, and signal-to-noise ratio for the cancer targeting and bioimaging and reducing the toxicity derived from nonspecific interactions of nanoparticles with cells. The in vivo NIR bioimaging signal could even be detected at low injection amounts down to 20 μg per animal.

Surface modification plays a predominant role in determining the behavior and function of nanoparticles in biological systems such as bioimaging and targeted therapy.1,2 A variety of both covalent and noncovalent modification means, using poly(ethylene glycol) (PEG),3−5 silica,6,7 chitosan,8 proteins,9−11 peptides,12,13 and so on, have been developed to improve the surface properties of nanoparticles. Of these methods, noncovalent surface engineering with high-affinity binding peptides is particularly noteworthy, as it exhibits unparalleled versatility, biocompatibility, simplicity, and scalability. Surface modification with absorbed proteins and specific binding peptides has been shown to alter the nanomaterial cell interaction and nanomaterial toxicity.14,15 However, the mechanism for the noncovalent surface engineering of peptides is still not clear. Peptides show an intrinsic limitation of the stability to bind metal ions if only natural amino acids are used, which bear only simple oxygen ligands with a moderate affinity for metal ions binding. Therefore, the design of high affinity metal-binding peptides is a challenging task, although some reports demonstrated that the amino acid sequence, rather than the composition, plays a key role in determining the peptide binding to inorganic materials.16,17

Modifying the hydroxyl functional group of a serine, threonine, or tyrosine residue into a phosphate group can change its propensity to bind metal ions, especially if the residue is positioned among other potential metal-binding residues.18 The importance of metal-phosphopeptide interactions is highlighted by their role in diverse biological functions, including nutrition and biomineralization, as well as potentially pathogenic roles in protein cross-linking and aggregation. On the other hand, with the high affinity of phosphopeptides toward metal-ions, immobilized metal ion affinity chromatography (IMAC) has been the most frequently used method for the enrichment of phosphopeptides using different metal ions on various base materials and commercial kits are available from different suppliers.19−21

We thus hypothesized that noncovalent surface engineering of peptides for lanthanide metal based nanoparticles can be efficiently realized by modifying the hydroxyl functional group of a side grafted serine of peptides into a phosphate group (phosphorylation) (Figure 1). Lanthanides belong to a group of

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metals that occur in the trivalent oxidation state. They are known to be hard “acceptors” with a strong preference for oxygen-containing anions such as phosphates to which they form very tight ionic bonds. In the phosphorylated amino acid molecules, phosphate groups have stronger acidity than carboxylic acid group, with a higher charge after dissociation in solution, thus have stronger combination ability with high positive charged rare earth ions. What’s more, the predominant binding mode of the phosphate anion and monosubstituted phosphates to the lanthanide surface was of a “tridentate” nature, stronger than that of “bidentate” carboxylic acids.

In the present work, by using synthetic Arg-Gly-Asp-Ser (RGDS) peptides combined with lanthanide based near-infrared (NIR) upconversion nanoparticles (UCNPs) as a model system (Figure 1), we studied the metal-binding properties in detail and showed that the RGDS(p) obtained by dramatic enhancement of its metal ion affinity without effect on their original target recognition performance. For the first time, we used first-principles calculations to confirm a stronger absorption force of phosphate oxygen to the Gd atom than the absorption force of carboxyl oxygen to the Gd atom, which is consistent with the experimental observations. This novel initial observation of phosphorylation dependent metal binding by a peptide was also confirmed by the efficient in vivo cancer target performance of the peptide coated NIR-to-NIR luminescent UCNPs and downconversion nanoparticles (DCNPs), respectively. The modification allows higher near-infrared (NIR) bioimaging efficiency owing to the higher binding amount of phosphopeptide. The NIR-to-NIR bioimaging signal could even be detected at a low injection amount down to 20 μg per animal.

### EXPERIMENTAL SECTION

**Synthesis of the Phosphopeptides.** After a mixture of 2-CI-trityl-Cl resin (500.0 mg, 0.75 mmol/g), Fmoc-Ser-(HPO₃Bzl)-OH (0.2 mmol), and DIEA (300.0 μL) in DMF (5 mL) was shaken 1.5 h on a vortex mixer at room temperature, the resin was filtered off and washed several times with MeOH, DCM, and DMF. The Ser(HPO₃Bzl)-linked resin was then used for the construction of full length peptides. The protocols employed were deprotection of Fmoc with 20% piperidine in DMF (15 min) and peptide coupling using 5 equiv of amino acid, 4.5 equiv of HCTU, and 10 equiv of DIEA. All coupling reactions were set to perform 1 h at room temperature. In the synthesis of RGDS(p), amino acids Fmoc-Asp(But)-OH, Fmoc-Gly-OH, and Fmoc-Arg(pbf)-OH were sequentially installed to construct peptides on the resin. The peptide-loaded resin was treated with a mixture of TIPS/TFA (5.95, 10 mL) for 2 h at room temperature. The resin was filtered off and washed with TFA. The crude product was dissolved in water and purified by HPLC (conditions, Supelco Discovery C18, 250 mm × 10 mm, suitable ratio of acetonitrile–0.1% TFA in water–0.1% TFA, 4 mL/min) to give the final product. In the synthesis of RADS(p), amino acids Fmoc-Ser(HPO₃Bzl)-OH, Fmoc-Asp(But)-OH, Fmoc-Ala-OH, and Fmoc-Arg(pbf)-OH were sequentially installed.

**Characterization.** TEM of nanoparticles was performed on a JEOL 2011 transmission electron microscope with an accelerating voltage of 200 kV. The upconversion spectra were characterized on a Hitachi fluorescence spectrometer F4500 instrument equipped with a 0–2 W adjustable continuous-wavelength laser (980 nm, Beijing Hi-Tech Optoelectronic Co., China) as the excitation source. SWIR spectra was measured on an Ocean Optics UV–vis-NIR CCD (QE65000) equipped with an 800 nm CW laser (20 W/cm²). Dynamic light scattering (DLS) and zeta potential experiments were carried out on a Malvern Zetasizer 3600 (Malvern Instruments). Confocal luminescence images were made using an Olympus FV1000 confocal laser scanning microscope with a continuous-wave (CW) NIR laser at 980 nm as the excitation source.

**Synthesis of UCNPs/DCNPs.** The synthesis of the NaGdF₄:Yb, Er or NaGdF₄:Yb, Tm (UCNP)/NaGdF₄:Nd (DCNP) cores with a size of ~15 nm in this work was similar to the synthesis previously reported by Prasad et al. In a typical method for the synthesis of NaGdF₄:20% Yb, 2% Er@NaGdF₄ UCNPs, 0.78 mmol of anhydrous GdCl₃, 0.20 mmol of YbCl₃, and 0.02 mmol of ErCl₃ were added to a 100 mL core solution, 0.1% TFA in water (0.1% TFA, 4 mL/min) to 15 nm in this work was similar to the synthesis previously reported by Prasad et al.③ In a typical method for the synthesis of NaGdF₄:20% Yb, 2% Er@NaGdF₄ UCNPs, 0.78 mmol of anhydrous GdCl₃, 0.20 mmol of YbCl₃, and 0.02 mmol of ErCl₃ were added to a 100 mL flask containing 10 mL of oleic acid and 15 mL of 1-octadecene. The mixture was heated at 150 °C for 30 min before cooling down to 50 °C to remove the water content from the solution. Shortly thereafter, 10 mL of methanol solution containing NH₄F (2.75 mmol) and NaOH (2.5 mmol) was added and the resultant solution was stirred for 30 min to remove the methanol. After methanol was evaporated, the solution was heated to 300 °C under argon for 1 h and then cooled down to room temperature. The resulting 15 nm NaGdF₄:Yb,Er nanoparticles were precipitated by addition of ethanol, collected by centrifugation at 6000 rpm for 5 min, washed with ethanol several times, and dispersed in 10 mL of cyclohexane. To obtain the 18 nm NaGdF₄:Yb, Er@NaGdF₄ UCNPs, 2.5 mL of the purified 15 nm NaGdF₄:Yb,Er initial core solution was mixed with 4.0 mL of OA and 6.0 mL of ODE. The flask was pumped at 70 °C for 30 min to remove cyclohexane and residual air. Subsequently, the system was switched to Ar flow and the reaction mixture was further heated to 280 °C at a rate of ~20 °C/min. Then Gd-OA (0.05 M) and Na-TFA-OA (0.20 M) host shell precursors were alternately introduced by dropwise addition at 280 °C and the time interval between each injection was 15 min. The amounts of the shell precursors for each addition were calculated and summarized in Table S1. Finally, the obtained NaGdF₄:Yb,Er@NaGdF₄ UCNPs with a diameter of 18 nm were centrifuged and washed as above and dispersed in cyclohexane.

**Synthesis of Phosphopeptide RGDS(p) Modified Nanoparticles.** The oleic acid capped UCNPs/DCNPs (containing 2 μmol Ln³⁺) in 0.2 mL of chloroform was slowly added into a water solution (0.5 mL) containing 1 mg of...
RGDS(p), and the solution is vigorously stirred overnight. Afterward, the NPs could be clearly transferred into the upper water layer from the chloroform layer due to the phosphopeptide attachment. The water solution was transferred to a microtube. After vigorously sonication, excess RGDS(p) was recovered from RGDS(p)-UCNPs/RGDS(p)-DCNPs by centrifugation and washing. The dispersion was filtered through a 0.22 μm membrane filter to remove large aggregates. Then the product of RGDS(p)-UCNPs/RGDS(p)-DCNPs were collected by dialysis with MWCO 3000 Da dialysis bag to remove unbounded peptides.

**In Vitro Bioimaging for Cancer Cells with the Phosphopeptide Modified UCNPs.** U87MG cells (∼10⁶/dish) were seeded in confocal dishes in 1 mL of Eagle’s minimum essential medium (MEM) medium supplemented with 10% FBS and 1% antibiotics and incubated in a CO₂ incubator for 24 h at 37 °C. Then, 100 μg/mL of RGDS(p)-UCNPs in HEPES was added and incubated in a CO₂ incubator for 30 min at 37 °C. Then the treated U87MG cells were washed with HEPES (3 × 1 mL). The cell samples were subsequently imaged by confocal laser scanning microscopy with wide-field or scan excitations with 980 nm continuous-wavelength (CW) laser.

**Specific NIR-to-NIR in Vivo Bioimaging of Cancer with the Phosphopeptide Modified UCNPs.** Nude mice borne U87MG tumor on the left hind-leg for targeted imaging were administered 100 μL of solution of RGDS(p)-UCNPs or RADS(p)-UCNPs at a concentration of 2 mg/mL (approximately ∼200 μg per animal) through tail vein injection. After 3 h of blood circulation, the mice were imaged using the modified upconversion luminescence in vivo imaging system under 980 nm laser excitation (0.2 W/cm²).

**Highly Sensitive NIR-to-NIR in Vivo Bioimaging with the Phosphopeptide Modified DCNPs.** Nude mice borne U87MG tumor on the left fore-leg for targeted imaging were administered 100 μL of solution of RGDS(p)-DCNPs or RGD-DCNPs at concentrations of 0.8, 0.4, and 0.2 mg/mL (approximately ∼80 μg, 40 μg, 20 μg per animal) through tail vein injection, respectively, to investigate the bioimaging feasibility by InGaAs camera. After 3 h of blood circulation, SWIR bioimages were taken for each animal upon 800 nm irradiation (0.2 W/cm²) with appropriately equipped filters.

**Animal Experiments and in Vivo Imaging Setup.** Male nude mice (weighing 30–40 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All animal experimental procedures were in agreement with institutional animal use and care regulations. Upconversion in vivo UCL imaging was performed with a modified LB983 NightOWL II (Berthold Technologies GmbH & Co. KG, Germany) using an external 0–2 W adjustable 980 nm CW laser as the excitation source. Downconversion in vivo imaging was performed with a modified NIRvana CCD camera (Princeton Instrument) that had an external 0–2 W adjustable 800 nm CW laser as the excitation source.

### RESULTS AND DISCUSSION

**One-Step Phosphopeptide Surface Modification for the Lanthanide Nanoparticles.** Phosphopeptides with specific sequences was easily synthesized by phosphorylation of the hydroxyl to the phosphate group of a side chain serine-containing amino acid (Figure 1). High-quality NIR-to-visible UCNPs comprised of hexagonal NaGdF₄:18%Yb³⁺/2%Er³⁺@NaGdF₄ were synthesized using the successive layer-by-layer (SLBL) strategy that has been developed by our group (Figure 2a).39,40 We used designed RGDS(p) phosphopeptides to exchange the oleic acid ligands on UCNPs surfaces through coordinating interactions (Figure 1). The as-synthesized UCNPs were originally dispersed in the organic solvent chloroform and kept stable with the capping agent of hydrophobic oleic acids. After 2 h incubation with the RGDS(p) phosphopeptides in the aqueous solution, the RGDS(p) demonstrated a greater chelating capability and completely pulled out the UCNPs from the bottom oil phase (chloroform) into the upper layer of aqueous phase. The oleic acids capped hydrophobic UCNPs were successfully transformed into RGDS(p) capped hydrophilic UCNPs (RGDS(p)-UCNPs). Representative TEM (Figure 2a) and optical photographic images (Figure 2b, inset) of the resulting RGDS(p)-UCNPs show that these nanoparticles are well-
dispersed in water and their shape is unchanged from that of the OA-capped hydrophobic UCNPs (Figure 2a); thus, these RGDS(p)-UCNPs were hydrophilic and remained monodispersed (Figure 2ac). The corresponding upconversion spectrum of RGDS(p)-UCNPs in water is similar to that of the OA-capped UCNPs in chloroform (Figure 2b); the integrated green/red emission ratio decreased due to the surface quenching of water molecules. Attachment of the phosphopeptides onto the UCNPs was further confirmed by the appearance of characteristic amide (1670, 1540, 1420 cm⁻¹) and phosphate (1200–800 cm⁻¹) vibrations in the FT-IR spectrum (Figure S1). To further confirm the noncovalent phosphopeptide modification of the UCNPs, fluorescent rhodamine B (Rhb)-labeled phosphopeptides (Rhb-RGDS(p)) were used to prepare Rhb-RGDS(p)-UCNPs. RET from UCNPs to RhB under excitation at 980 nm yielded a characteristic RhB emission band (at approximately 580 nm) (Figure 2d), which was accompanied by a decrease in the green upconversion emission bands at approximately 555 nm due to the UCNPs. The productivity of the RGDS(p)-modified UCNPs is 88.1% in comparison with that of RGDS modified UCNPs (1.4%), indicating the efficient ligand exchange performance of the RGDS(p) for the UCNPs. Compared to the original RDGS peptide, the phase-transfer efficiency of UCNPs by RGDS(p) is elevated more than 60-fold (Figure S2). Moreover, RGDS(p)-UCNPs were well-dispersed in physiological buffers, including fetal bovine serum (FBS), lysozyme solutions. In high-concentration salt solutions such as 3 M NaCl (Figure S3), the excellent stability of RGDS(p)-UCNPs was maintained for at least 4 weeks.

**First-Principle Calculation Simulations of the Peptide Modification on Nanoparticles.** To shed more light on the interaction of phosphate group (phosphorylated peptides) and carboxyl group (nonphosphorylated peptides) with the NaGdF₄ nanocrystal surface, we performed first-principle calculations based on density functional theory (DFT) for ground-state geometry and absorption energy of the carboxyl-modified and NaGdF₄ nanocrystal ([001] plane), (Figure 3 and Table S1). The DFT calculations include the generalized gradient approximation (GGA), using a plane wave basis (kinetic energy cutoff 500 eV) and projector augmented-wave pseudo potential. The absorption energy is calculated with the following formula.

$$\Delta E = E_{\text{tot}} - (E_{\text{slab}} + E_{\text{mole}})$$

where $E_{\text{tot}}$ stands for the total energy of slab with absorbed molecule, $E_{\text{slab}}$ for the energy of the slab and $E_{\text{mole}}$ for the energy of a single molecule. In the carboxyl-NaGdF₄ model, the absorption energy is 2.18 eV while that is 5.41 eV in the phosphate-NaGdF₄ model, representing the fact that phosphate-NaGdF₄ absorption is much more energetically stable than carboxyl-NaGdF₄. After the occurrence of the adsorption, the distance between O and Gd (d_{O-Gd}) is 3.59 Å in the phosphate-NaGdF₄ model and 4.12 Å in the carboxyl-NaGdF₄ model, respectively (Figure 3), indicating a stronger absorption force of phosphate oxygen to Gd atom than the absorption force of carboxyl oxygen to Gd atom. Our calculations are consistent with our experimental observations showing that the absorption of phosphate group on NaGdF₄ nanocrystal surface is much stronger than carboxyl group.

**Cytotoxicity and Integrin Specificity.** To investigate the cytotoxicity of RGDS(p)-UCNPs, an CCK-8 assay with the U87MG cells was used to determine the effect of CCK-8 on cell proliferation after 24 h. No significant differences in the proliferation of the cells were observed in the absence or presence of 0.5–10.0 mg/mL RGDS(p)-UCNPs (Figure S4). The cellular viabilities were estimated to be greater than 90% after 24 h. These data show that RGDS(p)-UCNPs (<1 mg/mL) can be considered to have low cytotoxicity. Integrin receptor specificity of RGDS(p)-UCNP was further demonstrated by a competition assay. The U87MG cells were preincubated with a 10-fold excess of unlabeled RGDS(p) peptide at 37 °C for 30 min and then incubated with RGDS(p)-UCNP at 37 °C for 30 min. Only weak UCL signal was observed in the competition experiment (Figure S5). To demonstrate the $\alpha_\text{v}\beta_\text{3}$ integrin specificity of the probe in vitro, the human breast cancer cell line MCF-7 (expressing low levels of integrin $\alpha_\text{v}\beta_\text{3}$) were chosen for the control experiments. The living cells were incubated with RGDS(p)-UCNPs or UCNPs. As shown in Figure S6, very weak UCL emission could be detected.

**Specific NIR-to-NIR in Vivo Bioimaging for Cancer.** To further confirm the highly specific cell-adhesion activity of phosphopeptides coated nanoparticles, RGDS(p) and RAD(p) phosphopeptides were modified on the 18 nm-UCNPs, respectively, for U87MG living cell targeting. The RAD peptide lack of specific cell-adhesion activity was used as a negative control. Efficient targeting of RGDS(p)-UCNPs revealed obviously green and red UCL signals on U87MG cells, in which the green signal was 16-times higher than that of the nontargeted RAD(p)-UCNPs control (Figure 4a). Qualification analysis of the UCL signal ($\lambda_{\text{em}} = 550 \pm 50$ nm) of RGDS(p)-UCNPs across the line reveals strong UCL intensity (counts >1500) and nearly no background fluorescence (Figure S7).

We also studied the specific in vivo cancer targeting performance of RGDS(p) modified NaGdF₄:20%Yb⁺/0.2% Tm³⁺@NaGdF₄ UCNPs, which had been demonstrated with deeper tissue penetration depth previously with efficient 800 nm upconversion emission under 980 nm laser excitation (Figure S8). For in vivo bioimaging, nude mice borne U87MG tumor on the left hind-leg were administrated RGDS(p)-UCNPs or RAD(p)-UCNPs HEPES solution (approximately 200 µg per animal) through tail vein injection.
After 3 h of blood circulation, the mice were imaged using the modified upconversion luminescence in vivo imaging system. The luminescent signal of the RGDS(p)-UCNPs targeted groups was demonstrated to be 4 times higher than those of the RADS(p)-UCNPs targeted groups due to the specific targeting of RGD amino acid sequence toward the integrin \( \alpha_v \beta_3 \) produced of the U87MG tumor site (Figure 4b). Quantitative biodistribution studies for Gd element showed that RGDS(p)-UCNPs exhibited an enhanced specific accumulation at tumor sites by 4 h and about 7 times higher enrichment than RADS(p)-UCNPs (Figure 4c), further demonstrating that the enhanced accumulation of UCNPs depended on the highly specific targeting of phosphopeptide coating.

**Highly Sensitive NIR-to-NIR in Vivo Bioimaging for Cancer.** To further evaluate the cancer targeting sensitivity of the phosphopeptide-modified nanoparticles, the lanthanide \( \text{NaGdF}_4:5\%\text{Nd}^{3+}@\text{NaGdF}_4 \) DCNPs NIR-to-NIR in vivo imaging system was used (quantum yield >20%); this system can be excited by near-infrared (800 nm) radiation and emits short-wavelength infrared (SWIR, 1060 nm) radiation (Figure 5c). Excitation at 800 nm lies in the first “tissue-transparent window”, which features low water absorption, low heat generation, and low tissue damage and is considered an ideal excitation wavelength. Furthermore, regarding the SWIR emission at 1060 nm, optical simulations predicted a second SWIR (1000−2300 nm) “tissue-transparent window”, which features lower tissue autofluorescence and up to a 1000-fold reduction in scattering losses, thus enabling unprecedented improvements in detection depth and resolution.

As a proof-of-concept experiment, nude mice bearing U87MG tumors on the left fore-leg for targeted imaging were administered RGDS(p)-DCNPs, RADS(p)-DCNPs, or DCNP (ligand-free) at concentrations of 0.8, 0.4, and 0.2 mg/mL (approximately 80, 40, and 20 \( \mu \)g per animal) by tail vein injection to investigate the feasibility of bioimaging using an InGaAs camera. After 3 h of blood circulation, bright 1060 nm SWIR signals were detected using RGDS(p)-DCNPs (Figure 5a,b). Moreover, when the injected amount was as low as 20 \( \mu \)g per animal, the 1060 nm SWIR signals remained detectable. In comparison, the SWIR signals obtained when using the RADS(p)-DCNPs were weak, and signals were detected only when the RADS(p)-DCNPs were used at higher amounts (>40 \( \mu \)g per animal) (Figure 5b). Even if RAD amino acid sequence has no specific interactions with the U87MG tumor, they were
also slightly enriched at the tumor site due to the enhanced permeability and retention (EPR) of the tumor vasculature. When used as contrast agents, the ligand-free DCNPs (0.8 mg/mL) showed very weak SWIR signals, because the cation surfaces of DCNPs have a strong impact on their circulation in blood.

Thus, we demonstrated that, due to the high binding capacity of phosphopeptides, the specific phosphopeptide-modified nanoparticles provided greater bioimaging sensitivity than bare nanoparticles or nonspecific peptides conjugated nanoparticles. Quantitative biodistribution studies showed that RGDS(p)-DCNPs exhibited enhanced specific accumulation at tumor sites at 4 h and were enriched at much higher levels than RADS(p)-DCNPs (Figure 5d), further demonstrating that the accumulation was mediated by integrin αβ3 binding. It is worth noting that nonspecific nanoparticles uptake and retention took place primarily in the liver and the spleen, with little accumulation in the kidney or the lung. This pattern of organ uptake and distribution was similar to that of nanoparticle probes in the previous reports. However, these liver and spleen accumulation will metabolize in a period of time and decrease unexpected adverse effects.

**CONCLUSION**

In conclusion, this work demonstrated a simple and stable method for one-step bioconjugation of peptide ligands to as-prepared hydrophobic upconversion/downconversion nanoparticles that is generally applicable to lanthanide-based nanoparticles. By phosphorylating specific serine side-chains, the affinity of these peptides to nanoparticles dramatically enhanced without affecting their target recognition. Our one-step bioconjugation method for functionalizing the molecule-specific active peptides to upconversion nanoparticles will greatly advance in vitro and in vivo imaging efficiency of upconversion/downconversion nanoparticles in analytical and biomedical areas, which might be ideally suited for cancer target diagnosis and cancer therapy applications.

**ASSOCIATED CONTENT**

* Supporting Information
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Materials and methods and additional figures and table (PDF)

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**Notes**
The authors declare no competing financial interest.

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