

Light-driven catalytic regulation of enzymes at the interface with plasmonic nanomaterials

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# 1 Light-driven catalytic regulation of enzymes at 2 the interface with plasmonic nanomaterials

3  
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16

## 17 **ABSTRACT**

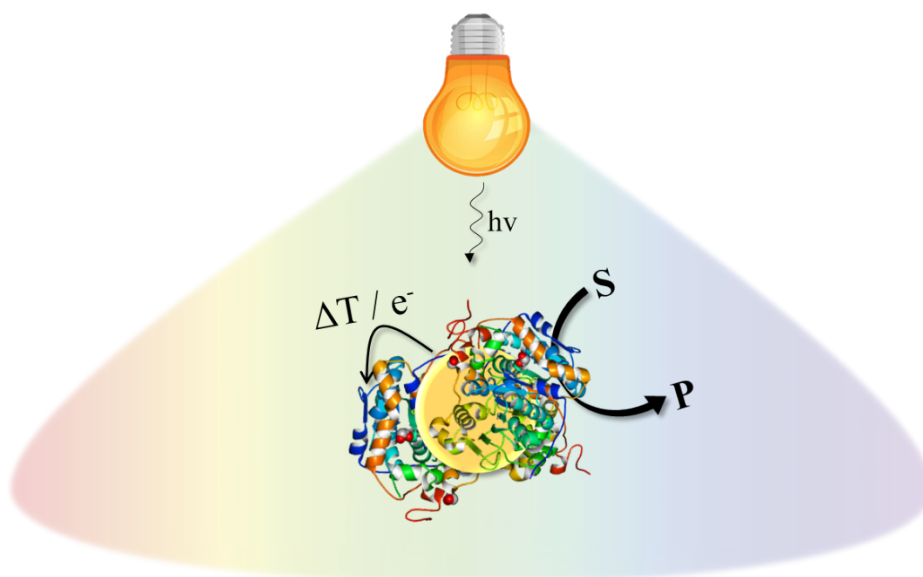
18 Regulation of enzymes is highly relevant toward orchestrating cell-free and stepwise  
19 biotransformations, thereby maximizing their overall performance. Plasmonic  
20 nanomaterials offer a great opportunity to tune the functionality of enzymes, through their  
21 remarkable optical properties. Localized surface plasmon resonances (LSPR) can be used  
22 to modify chemical transformations at the nanomaterial's surface, upon light irradiation.  
23 Incident light can promote energetic processes, which may be related to an increase of  
24 local temperature (photothermal effects), but also to effects triggered by generated hot-  
25 spots or hot-electrons (photoelectronic effects). As a consequence, light irradiation of the  
26 protein-nanomaterial interface affects enzyme functionality. To harness these effects to  
27 finely and remotely regulate enzyme activity, the physicochemical features of the

28 nanomaterial, properties of the incident light and parameters governing molecular  
29 interactions must be optimized. In this perspective article, we discuss relevant examples  
30 that illustrate the use of plasmonic nanoparticles to control enzyme function through  
31 LSPR excitation. Finally, we also highlight the importance of expanding the use of  
32 plasmonic nanomaterials to the immobilization of multi-enzyme systems for light-driven  
33 regulation of cell-free biosynthetic pathways. Although this concept is living its infancy,  
34 we encourage the scientific community to advance in the development of novel light-  
35 controlled biocatalytic plasmonic nanoconjugates and explore their application in  
36 biosensing, applied biocatalysis, and biomedicine.

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### 39 **ToC Graphic**



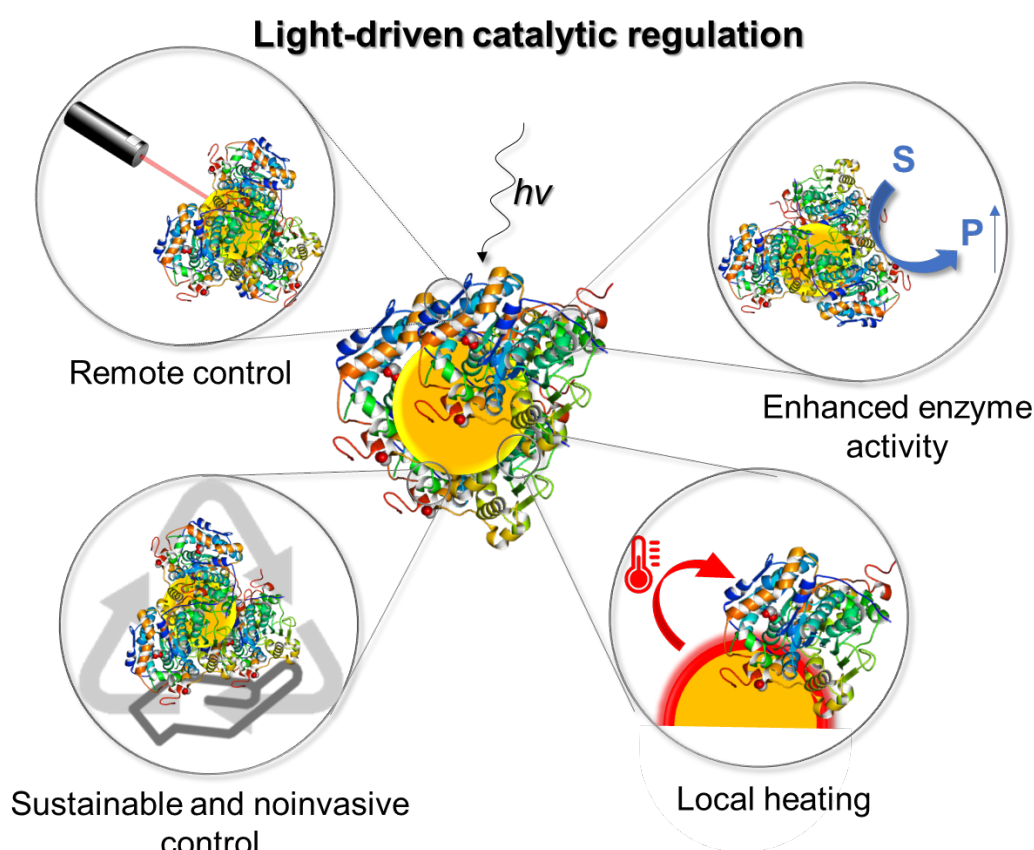
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42 Remote control of extracellular enzyme functionality through external stimuli  
43 provides access to more complex and concurrent cell-free biosynthetic routes. Enzyme  
44 activity regulation additionally offers the possibility of orchestrating several enzymes to  
45 simultaneously work in tuned cascade reactions, which maximizes the efficiency of  
46 biosynthetic pathways. Inside the cell, enzymes are tightly regulated, both at genomic and  
47 proteomic levels,<sup>1</sup> however such a regulation is lost when they are isolated. Therefore,  
48 breakthroughs in this topic are expected to advance toward enzymatic designs with a finer  
49 control for fully artificial applications. Light is one of the most efficient stimuli to  
50 remotely control enzymatic activity. The bio-conjugation of enzymes to light-responsive  
51 molecules and polymers has been the most common approximation hereto.<sup>2-4</sup> Interfacing  
52 enzymes onto plasmonic nanomaterials has been recently introduced as a promising  
53 alternative tool to design bioconjugates with tailored properties for remote control of  
54 biocatalysis. As any immobilization protocol, the conjugation of enzymes onto  
55 nanoparticles must be designed to enhance both the activity and the stability of the  
56 resulting bioconjugates. Hence, nanoparticle size and shape, as well as the conjugation  
57 chemistry, are fundamental aspects to control the orientation, density and binding strength  
58 that ultimately influence the functionality of the immobilized enzymes.<sup>5, 6</sup> Not only the  
59 immobilization of enzymes on plasmonic nanomaterials may enhance their functional  
60 properties,<sup>7, 8</sup> but optimization of the interactions between enzymes and plasmonic  
61 nanoparticles additionally offers opportunities to develop nanoengineered materials for  
62 light-controlled biocatalysis.<sup>9, 10</sup> For example, combination of the remarkable biological  
63 functions of enzymes and the unique optical properties of plasmonic nanomaterials can  
64 contribute to applications including cancer therapy,<sup>11-13</sup> biosensing,<sup>14-16</sup> applied  
65 biocatalysis and biotransformations,<sup>17, 18</sup> and intra- or extra-cellular nanosurgery.<sup>19-21</sup>

66 The suitability of plasmonic nanomaterials to tune enzyme activity arises from  
67 their tunable localized surface plasmon resonances (LSPR), in the UV-Vis and near-  
68 infrared wavelength ranges.<sup>22-24</sup> Under incident light, conduction electrons in metal  
69 nanoparticles display coherent collective oscillations, thereby generating intense  
70 absorption and scattering at specific wavelengths, resulting in LSPR signals.<sup>25, 26</sup> It is  
71 well-known that the LSPR response is largely dictated by the dimensions and morphology  
72 of the plasmonic nanoparticles, but it is also highly sensitive to the interaction between  
73 biomolecules and the nanoparticles' surface.<sup>27, 28</sup> Therefore, LSPR excitation in resonance  
74 with light irradiation can be used as a potential tool to regulate protein functionality at the  
75 interface with the nanomaterial. For example, enzymes immobilized on the surface of

76 nanoparticles can be readily triggered by light irradiation, to enhance their catalytic  
 77 activity.<sup>9, 10, 17, 29</sup> Harnessing LSPR, biocatalysis can be remotely regulated upon light  
 78 irradiation to fine tuning artificial reaction biocascades.<sup>9, 10, 17, 29-31</sup> Hence, the use of light  
 79 is a precise, sustainable, noninvasive and remote method to foster innovative solutions in  
 80 the biocatalysis field through merging enzymes and plasmonic nanoparticles (Figure 1).<sup>32</sup>  
 81 <sup>33</sup> This perspective provides an overview of this emerging research field, in an attempt to  
 82 present and discuss the most exciting studies where light controls the enzyme activity at  
 83 the interface with plasmonic nanomaterials, and ultimately offering our own outlook.  
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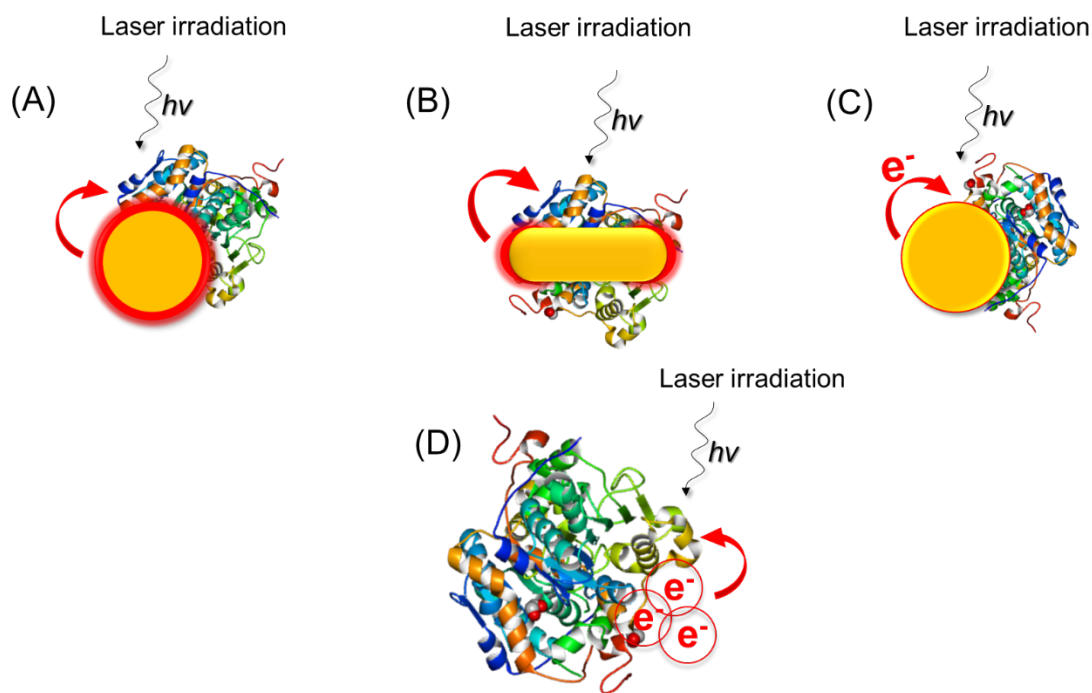
85  
 86 **Figure 1.** Schematic representation of some foreseen prospects for light-driven enzymatic  
 87 activity regulation onto plasmonic nanoparticles. For clarity purposes, enzymes and  
 88 nanoparticles are presented in similar sizes, but this is just a graphic representation, not  
 89 necessarily the real scale.

90  
 91

92 LIGHT AS THE MAJOR PLAYER IN THE FIELD: MECHANISMS BEHIND LIGHT-  
 93 DRIVEN BIOCATALYSIS INTERFACING PLASMONIC NANOPARTICLES

94 Light irradiation can introduce enhanced energetic processes through different  
95 mechanisms into plasmonic nanoparticles, depending on the nanoparticle features, such  
96 as their size and morphology. For example, anisotropic nanoparticles can concentrate the  
97 generated energy at sharp corners or tips, with a significant enhancement of several orders  
98 of magnitude.<sup>34</sup> Plasmonic effects are thus expected to influence the functional properties  
99 of enzymes (activity and stability) in their neighborhood. Unfortunately, we are still  
100 lacking fundamental knowledge and understanding to properly explain the mechanisms  
101 underlying this phenomenon. It is well-established that chemical reactions triggered by  
102 plasmonic effects in nanoparticles are mainly driven by one or more of the following  
103 physical effects: (i) temperature increase promoted by incident light, also termed  
104 thermoplasmonics (Figure 2A); (ii) enhancement of the electromagnetic near-field at the  
105 nanoparticles surface, creating “hotspots” of concentrated energy per unit volume (Figure  
106 2B); and (iii) light-induced hot electrons, where light promotes the transfer of an electron  
107 from the plasmonic nanoparticle to an adjacent molecule on their surface (Figure 2C).<sup>34</sup>  
108 On the other hand, in photo-biocatalysis, enzyme activity is enhanced by directly shining  
109 light onto the enzymes, usually oxidoreductases, which harbor a photoactive cofactor.<sup>35</sup>  
110 <sup>36</sup> The mechanisms driving such photobiocatalytic reactions are mainly related to the  
111 photoexcitation of such photoactive cofactors, which shuttle electrons between the  
112 substrates and the products (Figure 2D).<sup>37-40</sup> Hence, on the basis of this fundamental  
113 knowledge, some attempts have been made to address the study of the mechanisms  
114 governing light-driven biocatalysis of enzymes interfacing with plasmonic nanoparticles,  
115 which we briefly review in this section.

116



117

118 **Figure 2.** Schematic representation of potential mechanisms of enzymes interfacing with  
 119 plasmonic nanomaterials, upon laser irradiation. (A-C) Concepts from nanoplasmonics  
 120 through temperature increase (A), “hot-spots” generated from the enhancement of the  
 121 optical near-field (B), transfer of hot electrons to the enzyme structure (C). (D) Concepts  
 122 from photo-biocatalysis with photoinduction of electron transfer to the enzyme. For  
 123 clarity purposes, enzymes and nanoparticles are presented in similar sizes, but this is just  
 124 a graphic representation, not necessarily the real scale.

125

126 The photo-induced increase of local temperature on plasmonic nanoparticles is  
 127 arguably the most intensively explored effect to explain the mechanisms involved in  
 128 enhanced enzymatic activity.<sup>9, 10, 12, 13, 17, 29, 30, 41</sup> Gold, the most common plasmonic  
 129 nanomaterial, features inherent properties such as low specific heat capacity associated  
 130 to high thermal conductivity, which render it an ideal candidate for thermally enhanced  
 131 processes.<sup>8</sup> In principle, the activity of enzymes immobilized on the surface of plasmonic  
 132 nanoparticles should rise along with the local temperature increase induced by light,<sup>12</sup>  
 133 according to Arrhenius equation. However, a local high temperature can also promote a  
 134 partial or even complete deactivation of the enzymes, due to conformational changes.  
 135 This dichotomy makes it extremely important to precisely tune the heating ability of the  
 136 involved plasmonic nanoparticles,<sup>42</sup> so that the enzyme surroundings can reach an optimal  
 137 temperature ensuring maximum activity while avoiding deactivation.

138 On the other hand, hardly any studies are available that explore electron transfer  
 139 between excited plasmonic nanoparticles and enzyme structures. One of the few, if not

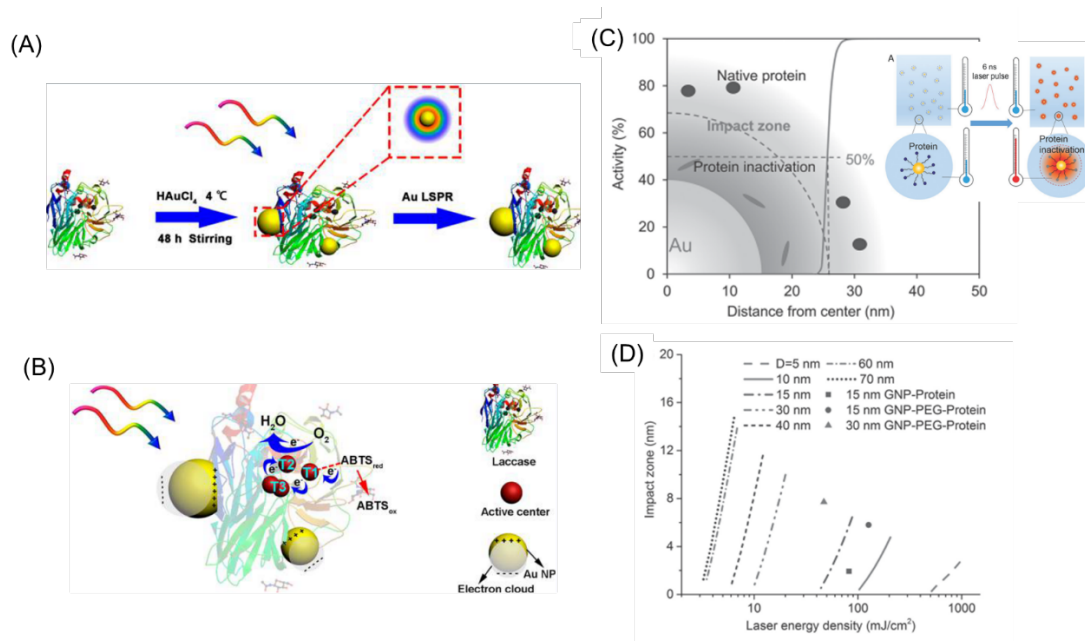
140 the only example that addresses these light-driven electron transfer mechanisms uses a  
141 laccase immobilized onto gold nanoparticles (AuNPs) under green light.<sup>30</sup> Laccase is a  
142 multicopper oxidase that transfers electrons from the substrate to the molecular oxygen  
143 towards two copper clusters found at the enzyme's active site. More specifically, laccases  
144 work through a copper atom (T1 site) that takes electrons from a reducing substrate, to  
145 subsequently transfer those electrons to a trinuclear copper center (T2/T3 sites) that  
146 ultimately shuttles the electrons to the molecular oxygen as the final electron acceptor to  
147 produce water. It has been observed that green light irradiation increases the local  
148 temperature at the surface of AuNP biohybrids, promoting conformational changes in the  
149 immobilized laccase that likely alter electron transfer within its active site. Therefore, the  
150 local temperature increase at AuNPs surfaces seems to unpair the electron transfer  
151 between T1 and T2/T3 sites, which consequently diminishes the laccase activity (Figure  
152 3A and B).

153 Mechanistic studies concerning electron transfer upon LSPR excitation are  
154 usually carried out using redox enzymes, because their mechanisms intrinsically involve  
155 the transfer of electrons. In an illustrative example, Wang *et al* identified hot charge  
156 carriers generated on AuNPs under visible light excitation (450, 532, 652, and 808 nm)  
157 in a plasmon-accelerated electrochemical reaction (PAER).<sup>43</sup> The experiments confirmed  
158 the presence of hot charge carriers, responsible for the enhanced electrocatalytic oxidation  
159 of glucose, as a model system. Although enzymes were not explored in this study, it  
160 highlights opportunities to exploit PAER with enzymes to regulate biocatalysis and  
161 elucidate the mechanisms involving electron transfer. For example, in the oxidation of  
162 D-glucose catalyzed by glucose oxidase (GOx), the mechanism involves molecular  
163 oxygen as an electron acceptor and a flavin cofactor as an electron shuttle.<sup>44</sup> The same  
164 concept could be expanded to other flavin oxidases that oxidize a diversity of substrates,  
165 such as alcohols, amino acids, and cholesterol, also using oxygen as the final electron  
166 acceptor.

167 In this context, it is clear that different events can concurrently occur upon light-  
168 driven biocatalysis through LSPR excitation, but some of them (i.e. temperature increase)  
169 are easier to identify than others (electronic effects). An interplay of different mechanisms  
170 can also result in one mechanism triggering another. Despite the few mechanistic studies  
171 involving plasmonic regulation of enzyme properties, we consider that the interactions  
172 between enzymes and nanomaterials are essential to harness the plasmonic effects upon  
173 light irradiation, as it happens in electrobiocatalysis, where electrons go back and forth

174 between the enzyme active sites and the electrodes.<sup>8</sup> In these cases, the distance, the  
175 number of attachments, the orientation and the density of the immobilized enzyme have  
176 been proven to play a central role in the interplay between biocatalysts and materials, for  
177 a variety of applications ranging from biosensing to biotransformations.<sup>45</sup> As an example,  
178 Kang *et al.* observed that the photothermal inactivation of  $\alpha$ -chymotrypsin immobilized  
179 on AuNPs (irradiated at 532 nm) was strongly dependent on its distance to the AuNP  
180 surface.<sup>13</sup> Because of the rapid local temperature gradients generated in the region termed  
181 impact zone, enzyme deactivation occurred immediately under nanosecond laser pulses  
182 (Figure 3C). The authors additionally observed that the size of AuNPs (5-70 nm) was also  
183 a determining factor behind the temperature gradient responsible for protein inactivation:  
184 the larger particles induce larger impact zones at low laser power (Figure 3D). In this  
185 scenario, small AuNPs (5 nm) did not cause enzyme inactivation, while larger AuNPs  
186 (>50 nm) readily inactivated the enzymes upon laser irradiation. The AuNPs surface-to-  
187 volume ratio and the laser energy, related to the efficiency of heat dissipation to the  
188 surroundings, were argued to explain enzyme inactivation under irradiation conditions.  
189 Since the local temperature in the enzyme surroundings strongly depends on the distance  
190 between enzyme and nanoparticle surface, the heating and dissipation capacity of the  
191 nanoparticles, together with the intrinsic enzyme thermal stability, should determine the  
192 optimal immobilization distance in light-controlled biocatalysis. We suggest that further  
193 studies are needed to address the impact of protein orientation and the separation between  
194 enzymes and nanomaterials on photothermal effects. We foresee that protein engineering  
195 and chemical biology will facilitate these studies, *e.g.* by creating new enzyme variants  
196 with engineered surfaces, so as to precisely control their interaction with plasmonic  
197 nanomaterials. We therefore stress that, using plasmonic nanoparticles to manipulate  
198 enzyme activity is still highly challenging and demands a clear understanding of the  
199 mechanisms responsible for plasmon-enhanced biocatalysis. We envision plenty of  
200 opportunities to elucidate and dissect the mechanisms underlying LSPR effects on  
201 enzyme functionality. For instance, light-driven plasmonic effects may control the  
202 intermolecular interactions or bond breaking to timely and remotely activate or deactivate  
203 enzymes of interest.

204



205

206 **Figure 3.** Light effect on enzymes interfacing with plasmonic nanomaterials.  
 207 Temperature effect (A) and electron transfer effect (B) in laccase-AuNPs bioconjugates,  
 208 under green light irradiation. Reproduced with permission from ref. 30. Copyright 2015  
 209 American Chemical Society. (C) Impact zone on the nanoparticle surface for  $\alpha$ -  
 210 chymotrypsin inactivation and (D) effect of AuNP size and laser energy on impact zone.  
 211 Adapted with permission from ref. 13. Copyright 2017 John Wiley and Sons. For clarity  
 212 purposes, enzymes and nanoparticles are presented in similar sizes, but this is not  
 213 necessarily the real scale.

214

215

216 PLASMONIC NANOMATERIALS AS AN OPPORTUNITY TO INTRODUCE  
 217 REMOTE CONTROL IN ENZYME ACTIVITY

218 The response of plasmonic materials under irradiation conditions, and thus their  
 219 effect on enzyme functionality, can be regulated by tuning parameters of the incident light  
 220 such as wavelength, power, and illumination time. Some examples illustrate how light  
 221 decreases the activity of enzymes immobilized on plasmonic nanoparticles. Guo *et al.*  
 222 observed<sup>30</sup> a decrease in the activity of laccase-AuNP bionanohybrids by 8.1% under  
 223 visible light ( $\lambda > 420$  nm) illumination for 3 minutes, with recovery upon turning the light  
 224 off. This effect was wavelength-dependent, since green light ( $\lambda = 500 - 560$  nm) induced  
 225 the lowest enzyme activity, likely due to resonance with the LSPR of spherical AuNPs ( $\lambda$   
 226  $\sim 520$  nm). Interestingly, the intensity of green light - ranging 0 - 60  $\text{mW} \cdot \text{cm}^{-2}$  - followed  
 227 a negative correlation with the activity of the immobilized laccase. Similar results were  
 228 obtained for horseradish peroxidase (HRP) immobilized onto spherical AuNPs upon laser

229 irradiation at  $\lambda = 532 \text{ nm}$ ,<sup>41</sup> suggesting that enzyme inactivation is related to released heat  
230 under resonance conditions. Another interesting example of enzyme inactivation by heat  
231 generation was provided by Thompson *et al.*<sup>12</sup> By using gold nanorods (AuNRs) ( $\lambda$  at  
232  $\sim 785 \text{ nm}$ ) in resonance with incident near-infrared (NIR) laser irradiation ( $\lambda = 785 \text{ nm}$ ),  
233 the authors showed selective control of a cascade reaction induced by glucose oxidase  
234 (GOx) immobilized on AuNRs and free horseradish peroxidase (HRP), through selective  
235 inactivation of GOx (Figure 4A). As the bi-enzyme system is controlled through the  
236 activity of one of the enzymes – GOx – this is a one-time (shot) control because once  
237 GOx is inactivated upon light irradiation, the bi-enzyme cascade cannot be subsequently  
238 recovered.

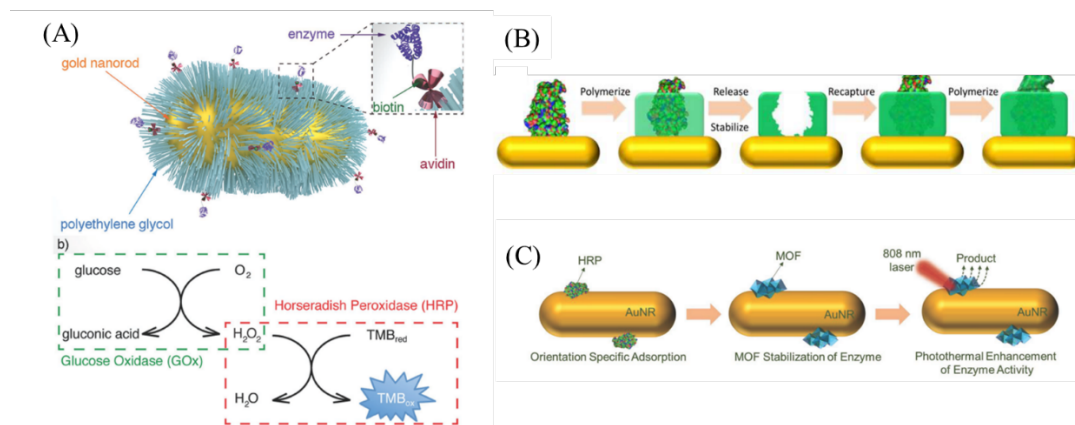
239 Light-driven biocatalysis on plasmonic nanoparticles becomes more attractive  
240 when using thermophilic enzymes, since they are highly active and stable at high  
241 temperature ( $>70 \text{ }^\circ\text{C}$ ). The local heating triggered by the LSPR effect may be thus  
242 transferred to thermophilic enzymes immobilized on plasmonic NPs, thereby enhancing  
243 their catalytic activity. This concept has been demonstrated for several classes of  
244 temperature-resistant enzymes. Li *et al.* compared the enzymatic performance of three  
245 enzymes with different thermal stability, conjugated to AuNRs and irradiated with a NIR  
246 laser ( $808 \text{ nm}$ ).<sup>17</sup> The results were compared for a mesophilic enzyme pig pancreatic  
247 lipase (PPL), a thermophilic protease from *Sulfolobus tokodaii* strain T7 (ST0779), and a  
248 hyperthermophilic protease from *Aeropyrum pernix* strain K1 (APE1547), with optimal  
249 temperatures of 37, 70 and 90  $^\circ\text{C}$ , respectively. Under laser irradiation, the activity of the  
250 enzymes immobilized onto AuNRs increased by 75.3%, 118.4% and 218.4%,  
251 respectively, compared to their free enzyme counterparts. It was interesting to confirm  
252 that more thermophilic enzymes underwent a higher activity enhancement by the  
253 photothermal effect as a consequence of LSPR illumination. To further concentrate the  
254 heating generated by the plasmonic effect in the enzyme surroundings, Blankschien *et al.*  
255 trapped AuNRs conjugated with a thermophilic glucokinase from *Aeropyrum pernix* into  
256 alginate macrobeads.<sup>29</sup> Under 800 nm laser irradiation, the enzymatic activity was found  
257 to increase by 60%, compared to alginate-trapped enzyme, whereas a negligible effect  
258 was observed by illuminating the same biohybrid in dispersion (outside the beads). This  
259 study shows how a surrounding matrix can preclude fast heat dissipation, thereby  
260 preserving the generated heat to enhance enzymatic activity. However, entrapment of the  
261 biohybrids in macroporous hydrogels brings along a 10-fold decrease in enzymatic

262 activity, likely due to mass transport restrictions for the substrate to reach the enzyme  
263 active sites. To overcome mass transport limitations, other solutions have been proposed  
264 to confine the heat generated by photothermal effects at the nanoscale. Tadepalli *et al.*  
265 explored two strategies to encapsulate bionanohybrids through either *in situ*  
266 polymerization or by means of metal-organic frameworks (MOF) (Figure 4B and C).<sup>9, 10</sup>  
267 The enzymatic activity of HRP immobilized on AuNRs and protected by either a polymer  
268 layer or MOF architecture, was stimulated by approximately 110% and 80% under laser  
269 irradiation (808 nm, 400 mW·cm<sup>-2</sup>), respectively, compared to the dark reaction. These  
270 strategies showed that local heating may be preserved at the enzymes surroundings,  
271 thereby stimulating their activity at the NP interface. Hence, nanoencapsulation seems to  
272 be a suitable strategy to promote photothermal effects in enzymes of different nature and  
273 origin (besides thermophilic) without jeopardizing their stability under the resulting high  
274 local temperatures induced by LSPR illumination.

275 From the studies reported during the past decade, we conclude that the activity of  
276 enzymes immobilized on plasmonic nanomaterials upon light irradiation depends on  
277 various parameters related to enzyme nature and type, nanomaterial properties  
278 (morphology, size, LSPR signal) and laser features (power, wavelength, illumination  
279 time). It is important to mention that nanoparticle concentration in the dispersion,  
280 regarding the density of the immobilized enzyme, is decisive to tune the heat output  
281 transfer in these systems, and therefore controlling bionanohybrid activity under  
282 irradiation.<sup>46, 47</sup> Finally, we identify scenarios where the enzyme activity either increases  
283 or decreases upon light irradiation. However, the vast majority of studies are focused on  
284 quantifying the effect of light once the enzyme is immobilized without accounting for the  
285 enzyme activity lost upon conjugation. To evaluate the effect of light on the enhancement  
286 of enzyme functionality, we encourage reporting the specific activity of the enzyme before  
287 and after immobilization, under irradiation and non-irradiation conditions.

288

289



290

291 **Figure 4.** Examples of enzyme inactivation or activation onto plasmonic nanoparticles  
 292 upon laser irradiation. (A) Strategy to selective targeting of GOx or HRP immobilized  
 293 onto PEG-AuNRs, upon NIR laser irradiation. Reproduced with permission from ref. 12.  
 294 Copyright 2017 John Wiley and Sons. (B,C) Schematic representation of strategies to  
 295 confine the heat generated at AuNRs to enhance HRP enzymatic activity by *in situ*  
 296 polymerization (B) and by using MOFs (C). Reproduced with permission from ref. 9.  
 297 Copyright 2017 American Chemical Society. Reproduced with permission from ref. 10.  
 298 Copyright 2018 John Wiley and Sons.

299

### 300 CELL-FREE PLASMONIC REGULATION OF BIOSYNTHETIC PATHWAYS

301 Metabolic networks within living cells have evolved for billions of years to  
 302 maximize fitness and ensure optimal cell survival and reproducibility within specific  
 303 environments. Hence, the cell metabolism relies on a multitude of chemical reactions  
 304 catalyzed by highly chemo-, regio- and stereo-selective enzymes. These biological  
 305 catalysts are perfectly orchestrated to work simultaneously within the cell milieu, under  
 306 similar reaction conditions. Although some enzymatic reactions are compartmentalized,  
 307 the vast majority of them occur inside the cytosol. For this reason, the regulation of  
 308 enzyme activity is extremely important to efficiently route biosynthetic pathways. Within  
 309 the cells, such metabolic pathways are mainly regulated at the genomic level by  
 310 enhancing or suppressing the expression of the genes encoding the enzymes involved in  
 311 the target pathway, and at the protein level through allosterism and post-translational  
 312 modifications. In the former case, gene regulation pathways respond to both metabolite  
 313 accumulation and environmental changes, by either increasing or decreasing the  
 314 intracellular enzyme concentrations. In contrast, the latter case promotes conformational  
 315 changes in enzymes, thereby modulating their activity through partial proteolysis or  
 316 chemical modification.

317 Human beings exploit metabolic networks for their survival but also for  
318 technological purposes. Since the beginning of biotechnology in the 7<sup>th</sup> century B.C,  
319 ancient societies made use of enzymes for food processing (wine, beer, bread...).  
320 Nowadays, enzymes are routed to catalyze non-natural biosynthetic pathways demanded  
321 by modern biotechnological applications in chemical manufacturing, biosensing and  
322 biomedicine.<sup>31</sup> These applications often require the enzyme to work far from  
323 physiological conditions. Therefore, driving the enzymes out of their natural environment  
324 makes us face technological challenges related to their functionality toward non-natural  
325 substrates and their responsiveness to external stimuli. Protein engineering has  
326 successfully adapted the functionality of a myriad natural enzymes to artificial synthetic  
327 reactions,<sup>48, 49</sup> yet the regulation of such artificial cascades must be engineered.

328 In recent years, many approaches have been developed to artificially regulate the  
329 catalytic activity of isolated enzymes. With the revolution of cell-free synthetic biology,  
330 more complex biosynthetic pathways are assembled to work under non-physiological  
331 conditions. Defining synthetic biology as the discipline that integrates the engineering  
332 principles behind biological processes, materials chemistry and engineering can  
333 contribute to expand this concept beyond DNA modifications. Through the incorporation  
334 of biological machinery (i.e multi-enzyme systems) at the interfaces between stimuli-  
335 responsive materials (i.e. plasmonic nanoparticles), biological processes can gain novel  
336 functionalities. This revolution is still seeking radical solutions to control the activity of  
337 enzymes, so that their performance can be tuned in the context of cell-free biosynthetic  
338 pathway.

339 Enzyme cascades based on NADH-dependent alcohol dehydrogenases have been  
340 controlled using light as an external stimulus, through different approaches. The  
341 combination of enzymes and materials with photochromic functionality is usually  
342 exploited for this aim. The most common approach comprises mixing soluble enzymes  
343 and photoactive materials in one-pot, which couples a photocatalytic step with a  
344 biocatalytic one.<sup>50</sup> Whereas the enzymes consume the redox cofactor to perform the  
345 reaction of interest, the light-irradiated material replenishes the pool of the cofactor  
346 demanded by the enzyme. A similar strategy has been recently adopted for the  
347 photocatalytic *in situ* production of H<sub>2</sub>O<sub>2</sub> employed by peroxygenases.<sup>51</sup>  
348 Events where light remotely controls the activity of more than one enzyme attached to  
349 the surface of light-responsive or plasmonic nanomaterials are however scarce. The  
350 assembly of two NADH-dehydrogenases in DNA origami scaffolds is one of the few

351 examples where light can directly control the activity of two enzymes.<sup>52</sup> This system  
352 relies on DNA strands functionalized with azo groups that control the relative position of  
353 each enzyme, bridging them together and activating the cascade under visible light, but  
354 separating and inhibiting them under UV irradiation. More complex systems where more  
355 than two enzymes are simultaneously controlled by light have not been reported so far.  
356 We can definitely see here an opportunity for the use of plasmonic effects (photothermal  
357 or electron transfer) interfacing with multi-enzyme systems. In fact, one can envision in  
358 the long term a cohort of bionanoplasmonic hybrids whose catalytic activities are  
359 activated under different irradiation conditions (lasers with different wavelengths). In this  
360 scenario, different enzymes could be remotely activated through either simultaneous (two  
361 lasers at a time) or sequential (one laser after another) light stimuli to orchestrate a given  
362 enzymatic cascade reaction on demand. An enzyme cascade composed of thermophilic  
363 enzymes and thermolabile cofactors transforming temperature-sensitive substrates would  
364 be an excellent proof of this concept. While the enzymes interfacing the nanoplasmonic  
365 materials operate at their optimal temperature under irradiation conditions due to local  
366 heating, the bulk remains at room temperature avoiding the deterioration of both  
367 substrates and cofactors.

368

## 369 CHALLENGES AND PERSPECTIVES

370 Light-regulation at the interface between enzymes and nanomaterials is an  
371 outstanding concept to control enzymatic activity through remote stimuli. Regulation of  
372 enzymes is particularly relevant for sequential enzymatic reactions, since pairing the  
373 activities of all involved enzymes is paramount to optimally drive the chemical flux  
374 toward the target product. When enzyme cascades simultaneously work in one pot, their  
375 activities must be orchestrated to avoid accumulation of inhibiting intermediates and toxic  
376 by-products. Within living cells, such biosynthetic orchestration is met through  
377 sophisticated genomic and proteomic regulation pathways that control the expression  
378 level and post-translational modifications of the enzyme working in the cell milieu. When  
379 using enzymes for *ex vivo* applications, enzyme regulation becomes an arduous task that  
380 requires artificial actuators to remotely exert control over the enzymatic activities. These  
381 challenges open up the opportunity of using plasmonic nanomaterials, which harness the  
382 incident light to tune enzyme functionalities at the protein-nanomaterial interface.

383 Heat generated at the surface of plasmonic nanoparticles is attractive to  
384 manipulate the catalytic properties of enzymes, but effective strategies to protect enzyme

385 activity are still challenging. Even though progress has been made for thermophilic  
386 enzymes, little has been developed for mesophilic enzymes in terms of enzymatic activity  
387 enhancement. Strategies for remote-control of mesophilic enzymes are highly relevant  
388 because these enzymes represent the majority of applications in biocatalysis and  
389 biotransformations, overall when compared to thermophilic ones. Hence, plasmonic  
390 nanoparticles can introduce numerous advantages for remote-control of biocatalysis using  
391 mesophilic enzymes if we can find the optimal trade-off between activity and stability  
392 under light-induced local heating. Besides increasing the local temperature, LPSR effects  
393 can also generate hot-electrons at the surface of plasmonic NPS, which may induce  
394 electronic effects on enzymatic mechanisms. Unfortunately such light-driven plasmonic  
395 effects have been rarely exploited to control enzyme activity. Here, we identify a  
396 scientific niche to remotely regulate the activity of redox enzymes with catalytic  
397 mechanisms relying on electron transfer. New discoveries in this direction are yet to  
398 come.

399 Controlling more than one enzyme simultaneously is an unmet challenge using  
400 plasmonic nanomaterials. Even using phenomena other than plasmonics, few examples  
401 have been reported where light itself can control a multi-enzyme process. Therefore, the  
402 creativity window stays open toward the design of novel biohybrids, where the interface  
403 between materials and enzymes comes to play in the presence of an external stimulus  
404 such as light. Hence, enzymatic material interfaces are needed to allow efficient light  
405 control over enzyme functionality. Protein engineering is a promising route toward  
406 gaining control over orientation, number of attachments and distance between the enzyme  
407 and the plasmonic nanomaterial.

408 The revolution of synthetic biology has arrived to cell-free systems, where many  
409 isolated enzymes work simultaneously in the same pot and under the same conditions. It  
410 is thus the right time to introduce regulators that exert control over cell-free enzymatic  
411 systems to up- or down-regulate enzymes without altering the reaction medium. To that  
412 aim, such remote regulators must respond to external stimuli as plasmonic nanomaterials  
413 do to light. Hence, we envision enzymes immobilized on plasmonic nanomaterials as new  
414 tools to endow cell-free synthetic biology with spatio-temporal control. Additionally, the  
415 immobilization of enzymes on light-responsive materials, such as plasmonic  
416 nanoparticles, is likely to pave the way to more futuristic applications like the chemical  
417 communication between compartmentalized cell-free systems, or even living cells. More

418 rational interfaces between enzymes and nanomaterials must therefore be created and new  
419 theoretical knowledge must be acquired to better understand the mechanisms governing  
420 the enzyme functionality at the interface with plasmonic nanomaterials.

421

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