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1 **Capillary electrophoresis with dual detection UV/C⁴D for monitoring myrosinase-mediated hydrolysis**
2 **of thiol glucosinolate designed for gold nanoparticle conjugation**

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18 **List of abbreviations:** AuNPs, gold nanoparticles; C⁴D, contactless capacitively coupled conductivity
19 detector; CPA, corrected peak area; GLs, glucosinolates; GL-SH, thiol glucosinolate; ITCs,
20 isothiocyanates; K_m, Michaelis–Menten constant; NPs, nanoparticles; TDLFP, transverse diffusion of
21 laminar flow profiles; V_{max}, maximum velocity.

22

23 **Keywords:** capillary electrophoresis, conjugation, contactless conductivity detector, enzyme assay, gold
24 nanoparticles

25 **Abstract**

26 Capillary electrophoresis (CE) with dual UV and conductivity detection was used for the first time to
27 monitor the functionalization of gold nanoparticles (AuNPs), a process catalyzed by an enzyme, myrosinase
28 (Myr). A thiol glucosinolate (GL-SH) designed by our group was used as substrate.

29 Hydrolysis of free and immobilized GL-SH was characterized using off-line and on-line CE-based
30 enzymatic assays. The developed approaches were validated using sinigrin, a well-referenced substrate of
31 Myr. Michaelis-Menten constant of the synthesized GL-SH was comparable to sinigrin, showing that they
32 both have similar affinity towards Myr. It was demonstrated that transverse diffusion of laminar flow
33 profiles was well adapted for in-capillary Mixing of nanoparticles (AuNPs) with proteins (Myr) provided
34 that the incubation time is inferior to 20 min. Only low reaction volume (nL to few μ L) and short analysis
35 time (< 5 min) were required. The electrophoretic conditions were optimized in order to evaluate and to
36 confirm the AuNPs stability before and after functionalization by CE/UV based on surface plasmon
37 resonance band red-shifting. The hydrolysis of the functionalized AuNPs was subsequently evaluated using
38 the developed CE-C⁴D/UV approach. Repeatabilities of enzymatic assays, of electrophoretic analyses and of
39 batch-to-batch functionalized AuNPs were excellent.

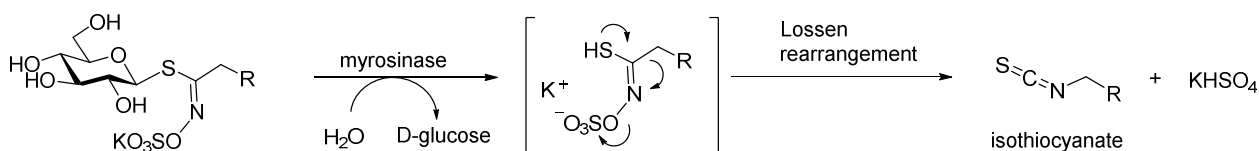
40 **1. Introduction**

41 Capillary electrophoresis (CE) is a cost-effective and a powerful tool for enzyme, substrate and inhibitor
42 screening and characterization [1-5]. There are two main approaches for studying enzymes by CE. In off-line
43 or pre-capillary assays [6], the enzymatic reaction takes place in a vial outside the capillary and a few
44 nanoliters of the reaction mixture are injected into the capillary. The product formed is then separated from
45 excess reagents, detected and quantified. In on-line assays [7-9], the enzymatic reaction takes place directly
46 in the CE capillary that acts as an enzymatic nanoreactor. The small volume requirement enables analysis of
47 reactants in very limited supply [7-11]. Kinetic and affinity constants (Michaelis-Menten or inhibition
48 determinations; V_{\max} and K_m , IC_{50}) ranging from nM to mM can be determined [1-3,7-11]. Enzymes can also
49 be used tethered to a monolithic support or to particles in order to improve their stability and reusability
50 [12,13]. The compatibility of CE with various detectors *e.g.* UV-visible absorption, laser induced
51 fluorescence (LIF), contactless capacitively coupled conductivity detector (C^4D) [14,15] and mass
52 spectrometry (MS) makes it suitable for various enzymatic systems. Structural information is obtained by
53 CE-MS, for example after in-line digestion of peptides and proteins [16-21]. CE- C^4D allows the
54 quantification of small inorganic ions even at ppt levels [22,23].

55 On the other hand, CE is useful for investigating the quality and the stability (aggregation) of nanoparticles
56 (NP) functionalized with a variety of molecules [24-27]. NP (1-100 nm) exhibit unique chemical and
57 physical properties depending on their chemical composition, shape, size, surface, and local environment
58 [28-32]. Gold nanoparticles (AuNPs) are one of the most studied nano-objects. Physicochemical
59 characterization of AuNPs is mainly performed using light scattering [33], electron or atomic force
60 microscopies, atomic spectroscopy, and electroanalytical techniques [34,35]. Separation methods are also
61 used such as size exclusion chromatography, flow field fractionation as well as gel and capillary
62 electrophoresis [24-27,36]. The surface of AuNPs has been continuously modified through binding of
63 functional groups or molecules to provide stability, selectivity and functionality for various applications

64 [37,38]. AuNPs have been reported in separation sciences [26,39], electronics, optical and electronic
 65 devices, catalytic applications, and in bio-nanotechnology (diagnosis, imaging and drug delivery) because of
 66 their excellent biocompatibility and low toxicity [28-31,35-38,40]. For example, the *in-vitro* anticancer
 67 effect of broccoli phytochemicals, mainly glucosinolates (GLs), isothiocyanates (ITCs) and flavonoids, was
 68 enhanced when conjugated to AuNPs as a drug delivery system [41]. Moreover, extracts of watercress and
 69 of *Brassica oleracea L.* [42], containing high quantities of GLs, were used to biosynthesize AuNPs with
 70 biocide properties.

71 In this work, CE was employed as a technique of choice to monitor an original conjugation strategy for
 72 AuNPs that we introduce. For this, a glucosinolate bearing a terminal thiol moiety (GL-SH) was designed
 73 and synthesized (Figure 1). The hydrolysis of this GL-SH by the myrosinase (Myr), a thioglucoside
 74 glucohydrolase, will allow capping the AuNP surface with an ITC precursor (Schema 1) [43]. This latter can
 75 be used as a bio-conjugation tool allowing efficient threading of molecules of interest onto AuNP. Indeed,
 76 the myrosinase-glucosinolate reaction, a unique biochemical transformation in the nature [44], produces
 77 highly reactive ITCs that can further react with nucleophiles for *in vitro* chemical ligation [45,46]. To our
 78 knowledge no glucosinolate capped AuNP has neither been developed, nor studied using CE.



80 Schema 1: Myrosinase-mediated hydrolysis of a glucosinolate into isothiocyanate and sulfate

81 Before conducting enzymatic reaction, CE analyses were optimized to simultaneously detect GL-SH by UV
 82 absorption and sulfate by C⁴D. This allows a simultaneous follow-up of both the decrease of the substrate
 83 GL-SH and the increase of the sulfate as the by-product of the catalyzed reaction. To monitor the hydrolysis
 84 by Myr of free and immobilized GL-SH on AuNPs, CE with dual detection UV/C⁴D was used. Our previous
 85 off-line CE-C⁴D assay [47] was optimized and adapted for this study. For economy features, an on-line Myr
 86 assay based on transverse diffusion of laminar flow profiles (TDLFP) [48,49] was developed. The linearity

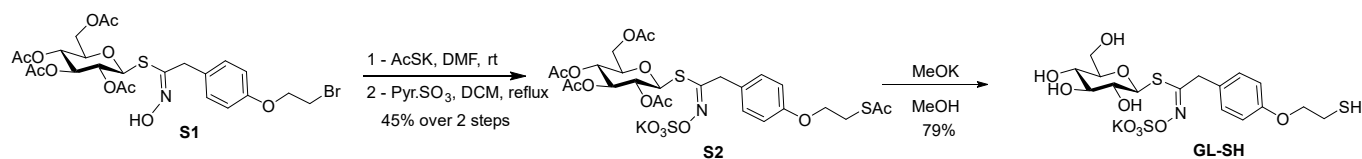
87 of the Myr action on the synthesized GL-SH was assessed. For validation purposes, we analyzed the sinigrin,
 88 a well-referenced substrate of Myr, using both the off-line and the novel on-line approaches. Citrate-capped
 89 AuNPs were chosen since they are the most frequently used particles, as they often represent a first step of
 90 synthesis before being grafted [25]. Their stability was also monitored using CE based on the approach
 91 proposed by Haes et al. [24] since a significant red-shifting of the surface plasmon resonance band is
 92 induced by aggregation [24,38]. Repeatability of enzymatic assays, of electrophoretic analyses and of batch-
 93 to-batch functionalized AuNPs were evaluated in order to confirm the reliability of the developed CE
 94 methods.

95

96 2. Materials and methods

97 2.1 Chemicals

98 AuNP (20 nm o.d., stabilized suspension at 1.09 nM in citrate buffer), citric acid (C₆H₈O₇, purity ≥ 99.5%),
 99 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS, C₆H₁₁NH(CH₂)₃SO₃H, purity ≥ 98 %), glacial acetic
 100 acid (AcOH, CH₃CO₂H, purity ≥ 99.99 %), L-histidine (C₆H₉N₃O₂, purity ≥ 99.5 %), orthophosphoric acid
 101 (H₃PO₄, 85 % w/w in water), potassium phosphate dibasic trihydrate (K₂HPO₄, 3H₂O, purity ≥ 99.0 %),
 102 potassium phosphate monobasic (KH₂PO₄, purity ≥ 99.0 %), sodium dodecyl sulfate (SDS, C₁₂H₂₅NaO₄S,
 103 purity ≥ 98.5 %), sodium hydroxide (NaOH, purity ≥ 98 %), sodium sulfate (Na₂SO₄, purity ≥ 99.0 %),
 104 sodium tetraborate (Na₂B₄O₇, purity ≥ 99 %), (-)sinigrin hydrate (C₁₀H₁₆NO₉S₂·K·xH₂O, purity 99.0 %) were
 105 and thioglucosidase from *Sinapis alba* (myrosinase (Myr), EC 3.2.1.147, 25 U, ≥100 units.g⁻¹) were
 106 purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Bidistilled 18 MΩ cm water was from
 107 Carlo Erba (Val de Reuil, France). All chemicals were used as received. All product bottles were placed in
 108 tightly closed plastic bags to preserve them from aerosol contaminations [50] and then stored at 4°C.



Schema 2: Thiol glucosinolate (GL-SH) synthesis

Thiol glucosinolate (GL-SH) was synthesized using methodologies developed within our group from known bromoglucosinolate **S1** (schema 2, [46]). Nucleophilic substitution with potassium thioacetate, followed by sulfatation using pyridinium sulfur trioxide led to the corresponding acetylated thioglucosinolate **S2** which was then deacetylated using modified Zemplen conditions to deliver the desired **GL-SH** in good yield (79 %).

2.2 Equipment and operating conditions

Experiments were carried out on an HP³D capillary electrophoresis system (Agilent, Waldbronn, Germany). Agilent software 3D-CE Chemstation (rev B.04.02) was used to pilot the CE. This instrument was equipped with an on-capillary TraceDec C⁴D detector (Innovative Sensor Technologies GmbH, Strasshof, Austria) and a UV-Vis diode-array detector. The following parameters were fixed for the C⁴D: frequency medium; voltage 0 dB; gain 100 %; offset 130; filter: frequency 1/3 and cut-off 0.05. The C⁴D detection signal was acquired with the Tracemon software (Istech, version 0.07a).

Unless otherwise stated, CE analyses were conducted in an uncoated silica capillary of 31 cm total length and 50 μm i.d. (Polymicro Technologies, Phoenix, AZ, USA). The effective detection length was 8.5 cm to the UV detection and 19 cm to the C⁴D (short-end injection). New capillaries were conditioned by performing the following rinses: 1 M LiOH (15 min), water (5 min) and background electrolyte or BGE (10 min). Between runs, the capillary was rinsed with the BGE for 3 min. Rinse cycles were conducted at 950 mbar. At the end of each working-day, the capillary was rinsed for 10 min with water before storing it overnight.

Polypropylene flasks, containers, CE vials and polyethylene olefin snap caps were used since they have little extractable material unlike glass. To avoid inadvertent contamination, nitrile – powder free – gloves were worn during solution preparation. To ensure good-quality cleaning, all volumetric equipment, vials and snap caps were rinsed with water (18 M Ω cm), and then soaked in water for an overnight period.

2.3 Solutions

All solutions were prepared with ultra-pure bidistilled water, filtered through a 0.45 μm polyvinylidenedifluoride (PVDF) Millex-HV Syringe Filter (Millipore, Molsheim, France) before use and stored at 4° C when not in use. Phosphate buffer ($\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$) was used as an incubation buffer (IB) at an ionic strength (I) of 33 mM. Its pH was adjusted to 7.0 by addition of 1 M NaOH. For electrophoretic separation, each tested BGE was degassed by ultra-sonication before use. The BGE solution in the separation vials was changed every three runs. BGE parameters were given by Phoebus software (Analis, Namur, Belgium). The pH was measured with a MeterLab PHM201 pH-Meter (Radiometer Analytical, Villeurbanne, France).

Stock solutions of each glucosinolate substrate (100 mM), Na_2SO_4 (100 mM) and Myr ($0.5 \text{ U}\cdot\text{mL}^{-1}$) were prepared by dissolving the appropriate quantity of each compound – accurately weighed – in the IB. Working solutions were prepared by appropriate dilution of the corresponding stock solution in the IB. Substrates must be used at large excess compared to enzyme (about 100 times). For this, Myr was used at $0.05 \text{ U}\cdot\text{mL}^{-1}$ for all enzymatic assays.

The response function of the C^4D detector was examined by plotting the corrected-peak areas (CPA) *versus* SO_4^{2-} concentrations. The calibration curve was performed over the concentration range 0.015–15 mM sulfate. Three independent determinations were performed at each concentration. Repeatability was expressed as relative standard deviation (RSD) on the migration times and on the CPA.

For the functionalization of nanoparticles, 40 μL of aqueous solution of GL-SH (10 mM) was added to 210 μL of AuNP citrates and the solution kept at ambient temperature for 18 h. Then, the solution was centrifuged at 10000 rpm during 30 min at 10 °C in order to settle the functionalized gold nanoparticles (NP-GL) and discard the supernatant containing unbounded GL-SH. The residue of NP-GL was suspended in 250 μL of water and centrifuged again. The whole process was repeated twice. The cleaned NP-GL were suspended in 210 μL -volume of IB. Figure 1 shows the mechanism of the AuNP functionalization.

2.4 Evaluation of gold nanoparticles stability by CE-UV

To evaluate gold nanoparticle stability, a silica capillary of 40 cm total length (31.5 cm effective length and 50 μm i.d) was used. 10 μL of NP citrates or NP-GL were placed in a micro-vial of the CE instrument auto-sampler at room temperature. The sample was then injected into the capillary at the anode (normal-end injection) by applying a positive pressure of 50 mbar for 30 s (injected volume 57 nL). The BGE was 10 mM CAPS-70 mM SDS buffer (pH 10) and the electrophoretic separation was performed at 25 $^{\circ}\text{C}$ under + 20 kV (current of 61 μA).

The stability of AuNPs was then estimated by calculating the ratio (R) of the absorbance at 520 nm over the absorbance at 600 nm (equation 1) [24]:

$$R = \frac{\text{absorbance (520)}}{\text{absorbance (600)}} \quad (1)$$

2.5 Enzymatic study

- Pre-capillary assays: This pre-capillary assay is based on the one we developed earlier in our laboratory [47]. Unless otherwise stated, enzymatic reaction was conducted at 37 ± 1 $^{\circ}\text{C}$. The reaction mixture (11 μL) contained 1 μL of the Myr (0.05 $\text{U}\cdot\text{mL}^{-1}$) and 10 μL of the substrate solution dissolved in the incubation phosphate buffer (pH 7.0). It was poured in a micro-vial of the CE instrument auto-sampler.

The linearity of the Myr activity toward the thiol GL, free or immobilized on AuNPs, was evaluated by monitoring simultaneously the increase over the time of the product SO_4^{2-} by the C^4D and the decrease of the substrate GL-SH by UV detection at 230 nm (due to the presence of the aromatic ring chromophore). For this, the enzymatic reaction was conducted with GL-SH at 1.6 mM and the incubation time was varied using a time interval of 10 min.

To estimate K_m and V_{max} values, the incubation time was 10 min so that the substrate conversion does not exceed 10 %. The stock solution of each tested glucosinolate was diluted to obtain a range of concentrations, from 0.01 to 3 mM for GL-SH and 0.01 to 2.5 mM for sinigrin.

181 Before CE analyses, the enzymatic reaction was stopped by heating the mixture with an oil bath at 130 °C±5
182 °C for 5 min. The reaction mixture was then injected into the capillary at the cathode (short-end injection) by
183 applying a negative pressure of -50 mbar for 10 s (24.6 nL). Unless otherwise stated, the electrophoretic
184 separation was performed at 25 °C under +20 kV in the His-AcOH buffer (current of 30 µA). Blank assays
185 were conducted according to the same procedure without adding the Myr or the substrate.

186 The solutions used to assess the calibration curve for SO₄²⁻ underwent the same treatments in order to avoid
187 any error due to evaporation in the oil bath. The reaction rate V_i (mM.min⁻¹) was calculated as the ratio of
188 the concentration of SO₄²⁻ formed *per* time interval.

189 - ***On-line assays:*** The TDLFP approach was used for in-capillary mixing of Myr with the GL. After filling
190 the capillary with the BGE, the reagents were introduced as follows: IB (-25 mbar for 10 s; ~12.3 nL), Myr
191 (-50 mbar for 5 s; ~12.3 nL), GL (-50 mbar for 5 s; ~12.3 nL) and IB (-50 mbar for 10 s; ~24.6 nL). The
192 concentrations of the reactants in the solutions used for on-line assays were twice the concentration used for
193 pre-capillary assays; 0.1 U.mL⁻¹ for Myr, 0.02 to 6 mM for GL-SH and 0.02 to 5 mM for sinigrin. After in-
194 capillary mixing, the concentration of each reactant is expected to be divided by two since two plugs of
195 reactants were introduced in the capillary.

196 For on-line assays of the hydrolysis of NP-GL, the TDLFP sequence was adapted as follows: IB (-25 mbar
197 for 10 s; ~12.3 nL), NP-GL (-50 mbar for 5 s; ~12.3 nL), Myr (-50 mbar for 5 s; ~12.3 nL) and IB (-50 mbar
198 for 10 s; ~24.6 nL). As for the off-line assays, the incubation time was optimized and a voltage of +20 kV
199 was applied for separation in the His-AcOH buffer.

200 - ***Evaluation of V_{max} and K_m constants:*** For each enzymatic assay, each point was repeated (n=3). The
201 nonlinear curve-fitting program PRISM® 5.04 (GraphPad, San Diego, California, USA) was used to evaluate
202 V_{max} and K_m according to equation (2):

203
$$V_i = \frac{V_{\max} \times [S]}{K_m + [S]} \quad (2)$$

204 Where V_i is the reaction rate ($\text{mM}\cdot\text{min}^{-1}$), K_m is the Michaelis–Menten constant (mM), V_{max} is the maximum
205 reaction velocity ($\text{mM}\cdot\text{s}^{-1}$) and $[S]$ is the concentration (mM) of substrate (GLs).

206

207

208 **3. Results**

209 **3.1. Analysis of GL-SH and sulfate by CE-C⁴D/UV**

210 Before conducting any enzymatic reaction, the CE analyses were optimized in order to detect simultaneously
211 GL-SH by UV at 230 nm and sulfate small anion by C⁴D. This would allow to monitor simultaneously the
212 decrease of the GL-SH if hydrolyzed by Myr and the increase of sulfate as the product of the catalyzed
213 reaction.

214 Firstly, the $\text{H}_3\text{PO}_4\text{-KH}_2\text{PO}_4$ buffer (pH 3, ionic strength of 40 mM) was used as BGE. In these conditions,
215 GL-SH was detected at 1.3 min with good repeatability of migration time ($\text{RSD}_{\text{tm}} = 0.45\%$; $n=3$). The
216 linearity was successfully assessed in the 0.02–2 mM (10-1000 ppm) concentration range with a
217 determination coefficient (r^2) of 0.999 by plotting CPA *versus* GL-SH concentrations. In CE, any fluctuation
218 in migration time will cause an error in peak area determination unless this latter is normalized for migration
219 time. However, sulfate peak was not detected with the C⁴D. This may be explained by the high effective
220 mobility (μ_{eff}) of the co-ion phosphate used in the BGE ($\mu_{\text{eff}} \sim -25 \times 10^{-5} \text{ cm}\cdot\text{V}^{-1}\cdot\text{cm}^{-1}$) as well as the overall
221 high conductivity of this BGE due to the presence of K^+ cations. Indeed, it is known that the difference
222 between the μ_{eff} of the BGE co-ion and the μ_{eff} of the analyte sulfate ($\mu_{\text{eff}} \sim -60 \times 10^{-5} \text{ cm}\cdot\text{V}^{-1}\cdot\text{cm}^{-1}$) needs to
223 be as high as possible in order to obtain high C⁴D sensitivity [14,15,50]. Keeping the pH and the ionic
224 strength constant, histidine/citric acid was used as BGE since the μ_{eff} of citrate is lower than phosphate's ($-$
225 $11 \times 10^{-5} \text{ cm}\cdot\text{V}^{-1}\cdot\text{cm}^{-1}$). However, similar results were obtained. Subsequently, a BGE histidine/acetic acid
226 with a higher pH of 4.2 (ionic strength of 40 mM) was used. The buffer capacity of this BGE is better than
227 the one for histidine/citric acid; 58 mM *versus* 17 mM, respectively. The μ_{eff} of acetate is equal to -14×10^{-5}

228 cm.V⁻¹.cm⁻¹ which is close to the μ_{eff} of citrate. At pH 4.2, the electroosmotic flow (EOF) is more intense
229 than at pH 3.0, which decreases the apparent mobility of the anions analyzed in reverse mode *i.e.* migrating
230 in the opposite direction as EOF. In these conditions, GL-SH was successfully detected by UV at 2.4 min as
231 well as sulfate by C⁴D at 0.9 min (Figure 2.a). The linearity of GL-SH peak area *versus* concentration was
232 confirmed in the 0.08–2 mM range with an excellent r² of 0.9999. Good repeatability was obtained for CPA
233 and t_m (RSD < 1.5 %; n=6). The calibration curve was also assessed over the wide concentration range
234 0.015–15 mM for sulfate. The r² value obtained was of 0.9994 confirming the linearity of the C⁴D response
235 for sulfate detection. Excellent repeatability for sulfate was also obtained confirming the choice of the BGE
236 for the CE-C⁴D/UV analyses.

237 **3.2 Myrosinase-mediated hydrolysis of GL-SH characterized by CE-C⁴D/UV**

238 **3.2.a Linearity of GL-SH hydrolysis catalyzed by Myrosinase**

239 First, the linearity of the Myr action on the synthesized GL-SH was assessed using the pre-capillary
240 enzymatic assay presented in section 2.5 and the CE-C⁴D/UV method above developed. As shown in Figure
241 2.a., a sulfate peak was detected after 10 min of incubation, at 37 °C. Moreover, Figure 2.b shows that the
242 peak area of the GL-SH quantitatively decreases while the sulfate increases over time. The enzymatic
243 reaction is linear for an incubation time of 40 min approximately. A plateau is reached after almost 60 min
244 of reaction time, which corresponds to the total consumption of the GL-SH present in the incubation
245 mixture. The results of this straightforward assay already confirm that the tested GL-SH is a substrate of the
246 myrosinase.

247 Subsequently, V_{max} and K_m parameters were determined to evaluate the affinity of this unnatural GL towards
248 the myrosinase and to compare it to sinigrin, a natural well-referenced substrate.

249 **3.2.b. Michaelis-Menten study of GL-SH hydrolysis using off-line and on-line CE-based assays**

250 The Michaelis-Menten curves of the GL-SH as well as of sinigrin were assessed using an IB at pH 7.0 which
251 corresponds to the optimum pH value for the myrosinase to be active [51]. Sinigrin was used for validation

252 of the developed assay and for comparison with the GL-SH in terms of affinity towards Myr. The Figure 3
253 shows the Michaelis-Menten curves obtained using the off-line assay for the sinigrin and the GL-SH. V_{\max}
254 and K_m values are reported in the Table 1. Results show that the unnatural GL-SH has affinity in the same
255 range as the natural substrate sinigrin even though its structure is bulkier.

256 In order to decrease the consumption of substrate and enzyme from few microliters to few nanoliters, an on-
257 line CE assay was developed for the first time to study myrosinase kinetics. This on-line assay is based on
258 TDLFP for reactant mixing and triggering of the enzymatic reaction inside the capillary. The order of
259 reactant plugs was based on the diffusion coefficient (D_m) of each reagent [48]. For this, the reactant
260 injection started with the Myr (0.1 U.mL^{-1}) since it has the lowest D_m followed by the GL substrate (sinigrin
261 or GL-SH) having the highest D_m . This is important to better control the incubation time of the enzymatic
262 reaction triggered by transverse diffusion inside the capillary [48,49]. A plug of IB was injected first to
263 prevent inactivating the enzyme by the BGE. This plug must have a rectangular shape to avoid its transversal
264 diffusion and therefore, its injection occurred at low pressure for longer injection time than for reactant
265 injection; -25 mbar for 10 s. A long parabolic plug of IB was also injected (-50 mbar for 10 s) at the end of
266 the injection sequence to ensure a good mixing of the enzyme with its substrate. Taking into account the
267 dilution occurring in the capillary after reactant mixing, the final enzyme concentration in the capillary is
268 expected to be around 0.05 U.mL^{-1} , thus comparable to the one used in pre-capillary assays. However, it is
269 well established that the dilution effect of the final IB plug is difficult to forecast [48,52].

270 In these conditions, a quantifiable peak of the product SO_4^{2-} was obtained after 10 min of incubation inside
271 the capillary of sinigrin or of GL-SH with the Myr with $\text{RSD}_{\text{CPA sulfate}}$ inferior to 0.47 % ($n = 3$).
272 Subsequently, this on-line CE assay was used to determine the V_{\max} and K_m constants. Results presented in
273 Figure 3 and Table 1 are consistent with those obtained using the off-line assay showing that the unnatural
274 GL-SH has affinity in the same range as the natural substrate sinigrin. Moreover, it can be noticed that for
275 both glucosinolates, the V_{\max} values obtained from the on-line approach are slightly lower than the one

276 obtained off-line, whereas the on-line K_m values are slightly higher. This can be explained by the dilution
277 phenomena inherent to the TDLFP method [48,52].

278 To conclude, V_{max} and K_m values obtained by both off-line and on-line approaches confirm that the
279 synthesized GL-SH is a good substrate of Myr with a kinetic profile comparable to the sinigrin one. These
280 results encouraged us to use the GL-SH to functionalize the surface of AuNPs following the schema shown
281 in Figure 1, with the aim of setting up NPs that may easily serve as practical platforms for bioconjugaison.

283 **3.3 Monitoring of the hydrolysis of functionalized AuNPs**

284 **3.3.a Stability of commercial AuNPs**

285 The high surface energy of noble metal nanoparticles can induce aggregation in solution. For this, when
286 working with AuNPs, it is essential to identify whether they are forming reversible (flocculated) or
287 irreversible (agglomerated) clusters in solution. Haes et al. [24] introduced a CE method to evaluate the
288 stability of the AuNPs based on the fact that if the majority of the NPs are in their isolated form in solution,
289 they will absorb strongly at 520 nm and weakly at 600 nm (red-shifting) [24,38]. Consequently, they
290 established that NP stability can be estimated by taking the ratio (R) of the absorbance at both wavelengths
291 (equation 1).

292 In this study, the CE-UV analysis of the commercial citrate stabilized AuNPs was optimized. Indeed, it is
293 known that despite the high degree of stability in water or buffer solution, gold citrate nanoparticles are
294 unstable inside a capillary. The electrostatically attached citrate molecules can be lost by the influence of the
295 electric field. Moreover, several parameters may induce uncontrolled aggregation such as temperature
296 variations, stirring or modification of the surface chemistry as for example by adding in this study the GL-
297 SH. For this, it was important to control the stability of the AuNPs during experimentations.

298 First, a background electrolyte borate (pH 9.2, I = 25 mM) was used for the analysis of these anionic NPs.

299 The peak of NP citrates was distorted and had a low intensity. Even at higher ionic strength of 50 mM, peak

300 shape was still not acceptable. This may be due to the rather low stability of citrate-capped AuNPs inside the
301 capillary [53]. Subsequently, a 10 mM CAPS containing 70 mM SDS (pH 10, I = 50 mM) was used as BGE.
302 The presence of the surfactant SDS is expected to improve AuNP stability and to reduce uncontrolled
303 aggregation [38]. With this BGE, a symmetrical peak was detected at 2.6 min (CPA = 62.2) by absorption at
304 520 nm (Figure 4.a). This peak is characteristic of isolated AuNPs. A less intense peak (CPA = 16.0) was
305 obtained when the electropherograms were recorded at 600 nm indicating the presence of small quantities of
306 aggregated AuNPs (Figure 4.a). Good repeatabilities (n=6) were obtained on the migration time (RSD = 0.4
307 %) and on the CPA (RSD = 1.7 %).

308 The ratio R could thus be calculated (equation 1). It was found to be 3.9 ± 0.1 (n = 3) indicating that the
309 commercial citrate-capped AuNPs were well flocculated in solution. Indeed, R is close to 3.5-4 for
310 flocculated nanoparticles and decreases for aggregated NPs approaching eventually 0 [24]. Since the
311 stability of citrate AuNPs was confirmed, their functionalization by the GL-SH could be conducted.

312 **3.3.b. Functionalization of AuNPs**

313 Functionalization of the citrate AuNPs with GL-SH is based on a ligand exchange reaction between the
314 external thiols of the GL-SH and the existing labile capping ligands on AuNPs *e.g.* citrates. This equilibrium
315 process allows the synthesis of mixed monolayer-protected AuNPs. Different temperatures (4 °C, 19 °C and
316 25 °C) were tested (other conditions in section 2.3). The functionalized NPs, presented in this manuscript as
317 NP-GL, were then analyzed by CE-UV using the conditions optimized in the previous section 3.3.a.

318 When the functionalization was carried out at low temperature 4 °C, the electropherograms were similar to
319 those plotted for the analysis of commercial citrate AuNPs with a peak detected at 2.6 min. A peak at 2.9
320 min (Figure 4.b) was obtained when working at higher temperature. Moreover, excellent repeatabilities (n =
321 6) on migration time (RSD = 0.3 %) and on CPA (RSD = 1.2 %) were obtained when conducting the
322 functionalization at 25 °C. The shift in the migration time *i.e.* in the electrophoretic mobility of these anions

323 - since the electroosmotic flow is constant - indicates a modification of the surface of the AuNPs. This is
324 certainly due to the citrate on the AuNPs being displaced by the GL-SH.

325 Figure 4.b also shows that the GL-SH free in solution can be detected at 230 nm with a longer migration
326 time equal to 3.6 min. GL-SH migrates as an anion, like the citrate AuNPs and the NP-GL.

327 It is essential to note that, no free GL-SH was detected when analyzing the NP-GL, which confirms the
328 efficiency of the centrifugation/washing steps of the NPs. Moreover, the ratio R was also calculated for the
329 functionalized AuNPs. The value obtained was 3.8 ± 0.1 (n=6) indicating that the NP-GL were still
330 flocculated in solution. The hydrolysis of these glucosinolate functionalized nanoparticles was therefore
331 studied using the CE-C⁴D/UV approach developed in section 3.1.

332 **3.3.c. Myrosinase-mediated hydrolysis of NP-GL characterized by CE-C⁴D/UV**

333 First, the on-line assay was conducted due to the interesting economy of this approach. The NP-GL were
334 injected before Myr since they have a lower D_m (other conditions in section 2.5). The sulfate was
335 successfully detected at 0.9 min with a CPA increasing with the incubation time (5, 10 and 20 min). On the
336 other hand, no free GL-SH was detected simultaneously using UV detection. This is a very interesting result
337 showing that: (i) the sulfate detected is produced by the action of the Myr exclusively on the GL-SH
338 immobilized on the AuNPs; and (ii) the Myr is still able to catalyze the hydrolysis of the GL-SH even when
339 immobilized on the AuNPs. Moreover, this also demonstrates that TDLFP can be used for mixing nano-
340 material with the protein Myr of much smaller size. However, this mixing process is limited by the
341 sedimentation of the NPs inside the capillary. Indeed, when the incubation time is relatively high, higher
342 than 20 min, the reaction was limited resulting in a relatively small increase of the sulfate peak.

343 Consequently, the pre-capillary approach was then evaluated. As for on-line assay, the sulfate was
344 successfully detected by the C⁴D at 0.9 min with good repeatability (RSD = 0.39 %; n = 3) and no free GL-
345 SH was detected by UV. The advantage of this approach over the on-line assay is that the incubation time
346 could be increased to several hours. The CPA of the product SO_4^{2-} detected by the C⁴D for the hydrolysis of

347 two batches of NP-GL prepared freshly or after one week of conservation at 4°C were plotted as a function
348 of the incubation time (Figure 5). The interval of time was equal to 10 min and the functionalization of the
349 NPs was conducted at 25 °C. This time-dependent following of the enzyme reaction (continuous assay) is
350 important for evaluating the myrosinase activity towards its substrate, the NP-GL in this case. CE results
351 show that NP-GL are stable at 4°C for at least one week and that the batch-to-batch reproducibility is
352 acceptable. Indeed, it can be seen that all the three curves in Figure 5 present the same trend, corresponding
353 to the progress curve of a typical enzyme reaction. The sulfate quantity increases in a linear manner for an
354 incubation time less than 40 min ($r^2 > 0.985$) which compares well with the linearity obtained for the
355 hydrolysis of the free GL-SH (Figure 2.b). However, the plateau is reached for GL-SH immobilized on the
356 AuNP after almost 150 min of incubation (Figure 5) whereas it was reached after 60 min for the free GL-SH
357 (Figure 2.b). The slower kinetics observed can be explained by diffusion limitations due to reactant
358 immobilization [54,55]. On the other hand, it is worthy to note that when conducting NP-GL
359 functionalization at 19 °C, the quantities of SO_4^{2-} released were lower than those obtained with NP-GL at
360 25°C. The grafting of AuNPs at 25 °C is thus more efficient.

361

362 **4. Conclusion**

363 In this work, CE with dual detection $\text{C}^4\text{D}/\text{UV}$ was introduced as an all-in-one tool for monitoring an original
364 process of functionalization of gold nanoparticles based on the myrosinase-glucosinolate reaction. First, a
365 CE method was developed to simultaneously detect by UV any decrease of the GL-SH substrate when
366 hydrolyzed by Myr, and by C^4D any increase of sulfate as the product of the catalyzed reaction. The linearity
367 of GL-SH and sulfate peak area *versus* concentration was confirmed with an excellent r^2 higher than 0.999.
368 Good repeatabilities were also obtained confirming that the electrophoretic conditions chosen were adapted
369 for the dual detection system, C^4D and UV. Offline and online CE-assays both confirmed that the
370 synthesized GL-SH is a good substrate of Myr with a kinetic profile comparable to the sinigrin one. In

371 addition, CE was established as a tool to follow-up and to confirm the stability of citrate AuNPs before and
372 after the functionalization of their surface by GL-SH. The hydrolysis of glucosinolate functionalized
373 nanoparticles was subsequently studied using the developed CE-C⁴D/UV approach. It was demonstrated that
374 Myr was able to hydrolyze the functionalized nanoparticles. The rapidity and the economy of this technique
375 allowed to follow this entire process using low reaction volumes and with only few minutes of analysis time.
376 Moreover, it was possible to confirm the compatibility of the myrosinase-glucosinolate system with the on-
377 line CE based assays with an important advantage in term of reagent consumption.

378 This work opens the way to further promising investigations concerning the use of CE to follow the
379 functionalization of AuNPs using the enzymatically produced isothiocyanate function as a reactive moiety to
380 anchor biologically relevant biomolecules.

382 **Declaration of interests**

383 The authors declare that they have no known competing financial interests or personal relationships that
384 could have appeared to influence the work reported in this paper.

385 The authors declare the following financial interests/personal relationships, which may be considered as
386 potential competing interests.

387
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391 **Figure list**

392 **Figure 1.** Mechanism of gold-nanoparticles functionalization by GL-SH (1st step) and their hydrolysis to
393 obtain isothiocyanate valuable for bio-conjugation (2nd step).

394 **Figure 2.** Myrosinase-mediated hydrolysis of GL-SH monitored by CE-C⁴D/UV. **a)** Example of
395 electropherograms; **b)** Linearity study of the enzymatic reaction.

396 Experimental conditions: Reaction mixture (11 μ L total volume): incubation buffer KH₂PO₄/K₂HPO₄ (pH
397 7.0, ionic strength 33 mM); myrosinase (0.05 U.mL⁻¹); GL-SH (1.6 mM). Incubation at 37 °C for 10 min.
398 Capillary *electrophoresis*: silica capillary: 31 cm x 8.5 cm x 50 μ m i.d. (short-end injection); BGE:
399 histidine/acetic acid buffer (pH 4.4, ionic strength 40 mM). Electrophoretic separation: +20 kV at 25 °C
400 (current 31 μ A). Short-end injection: -50 mbar \times 10 sec (24.6 nL). Rinse between analyses: 3 min BGE. C⁴D
401 detector: frequency medium, voltage 0 dB, gain 100 %, offset 130, filter: frequency 1/3 and cut-off 0.05. UV
402 detection of GL-SH was conducted at 230 nm.

403 **Figure 3. a)** Michaelis-Menten plots for myrosinase-mediated hydrolysis of **a)** sinigrin and **b)** GL-SH
404 obtained by off-line and on-line assays.

405 For experimental conditions: see figure 2 and section 2.5.

406 **Figure 4.** Electropherograms obtained **a)** for commercial NP citrates at 520 nm and 600 nm, and **b)** for NP
407 citrates (520 nm), NP-GL (520 nm) and free GL-SH (230 nm).

408 Experimental conditions: silica capillary: 40 cm x 31.5 cm x 50 μ m i.d.. BGE: 10 mM CAPS-70 mM SDS
409 buffer (pH 10). Electrophoretic separation: +20 kV at 25 °C (current 61 μ A). Normal injection: 50 mbar \times 30
410 sec (57 nL). Rinse between analyses: 3 min BGE.

411 **Figure 5.** Myrosinase-mediated hydrolysis of NP-GL characterized by CE-C⁴D/UV using off-line CE-based
412 enzymatic assays.

413 Reproducibility between batches was evaluated as well as the stability of the functionalized gold NP.

414 Experimental conditions: Fig. 2.

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550

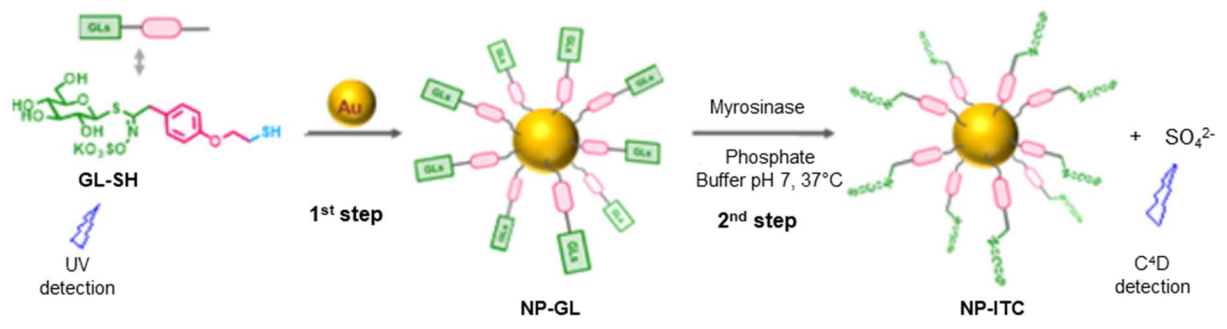


Figure 1

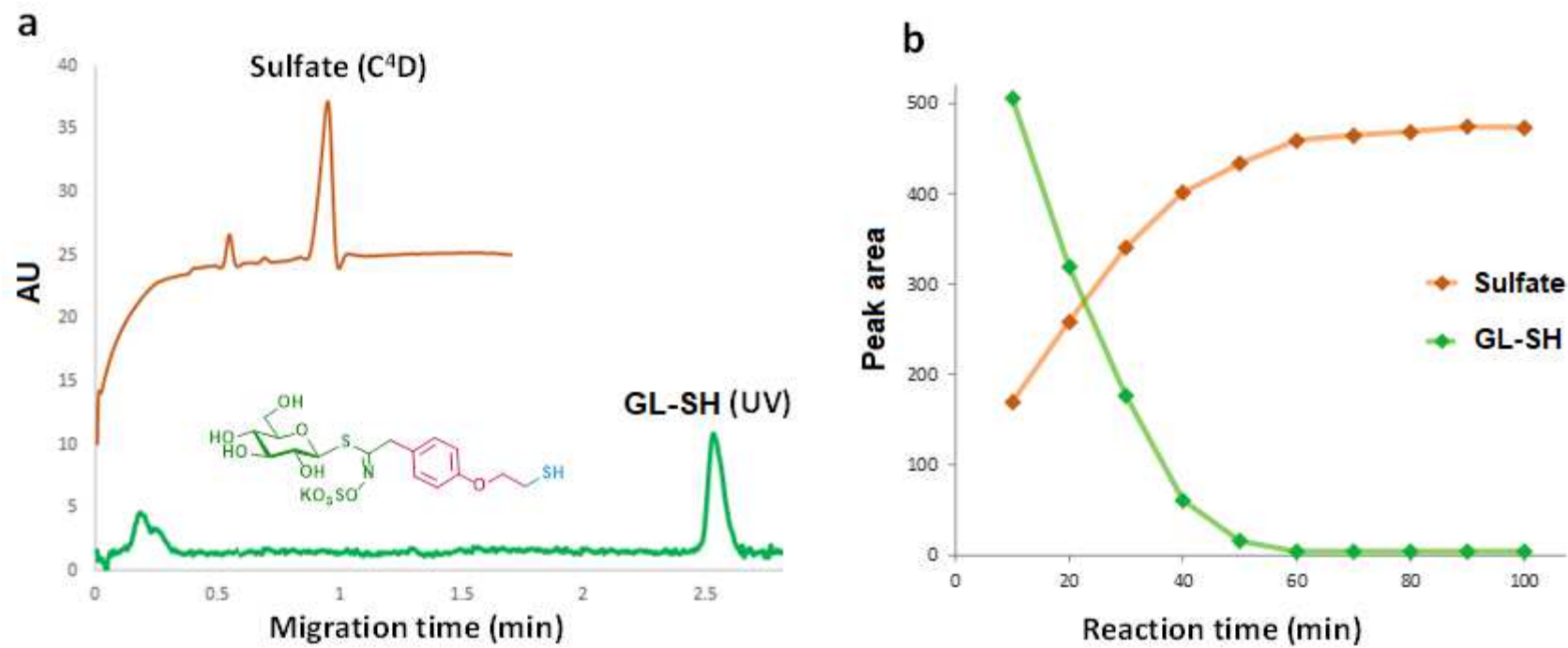


Figure 2

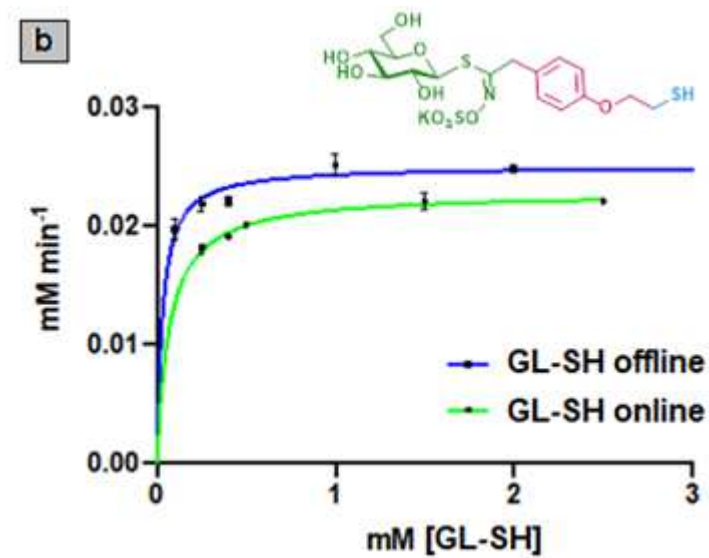
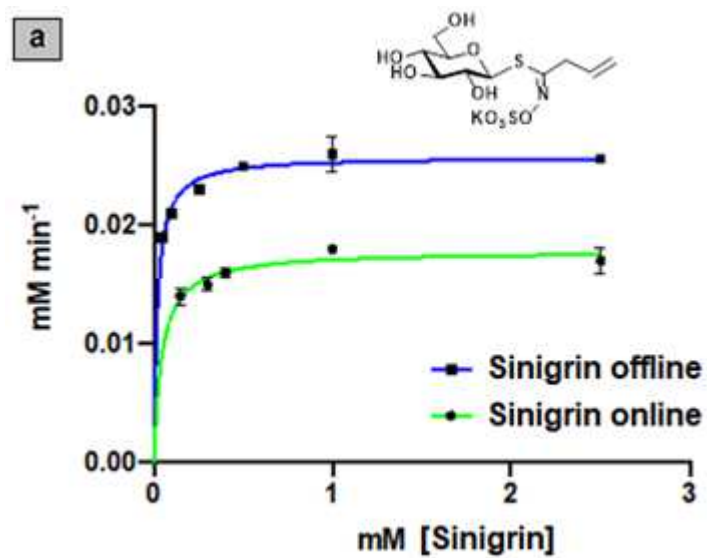


Figure 3

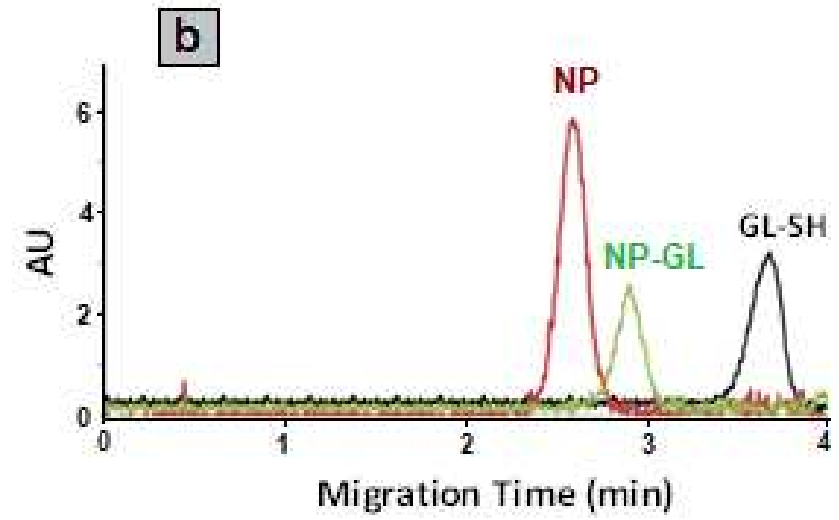
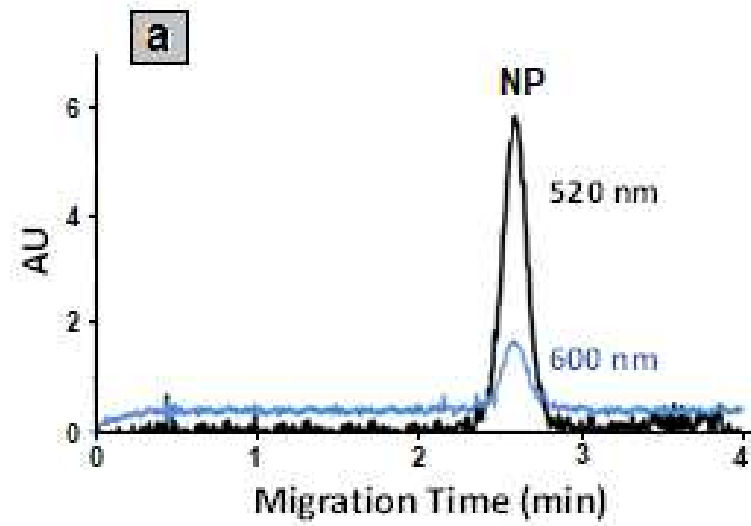


Figure 4

Hydrolysis of glucosinolates immobilized on gold nanoparticles (NP-GL)

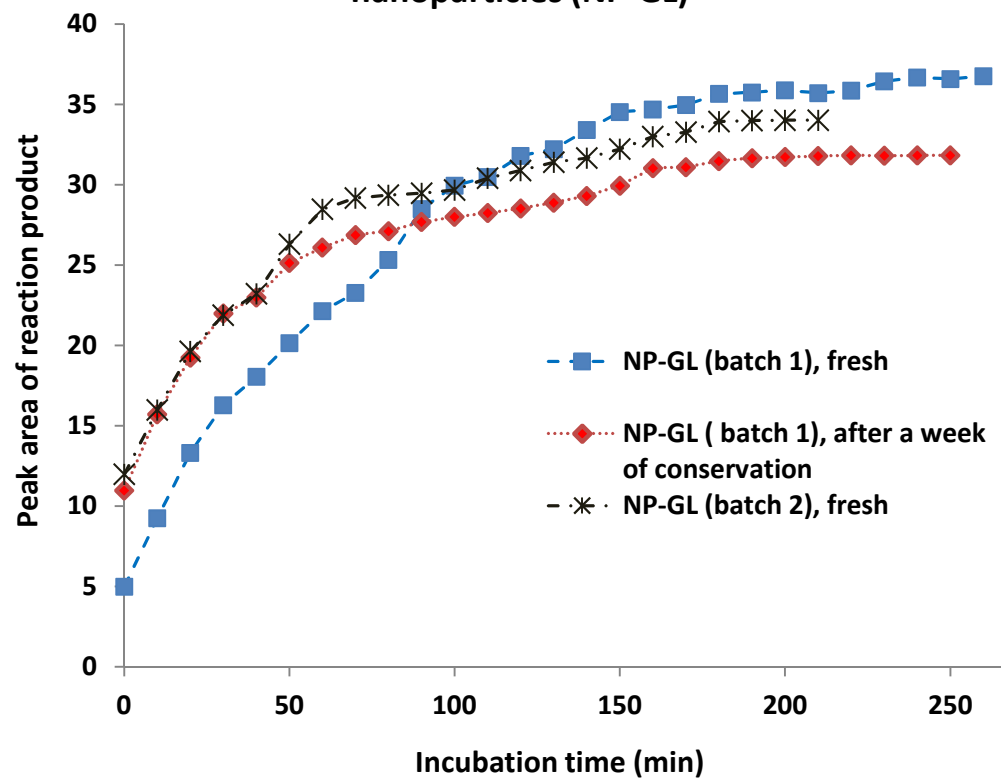


Figure 5

Table 1 : K_m and V_{max} for sinigrin and GL-SH using off-line and on-line CE-C⁴D/UV assay

	Off-line		On-line	
	V_{max} ($\mu\text{M min}^{-1}$)	K_m (μM)	V_{max} ($\mu\text{M min}^{-1}$)	K_m (μM)
Sinigrin	25.8 ± 2.1	20.0 ± 2.6	17.8 ± 0.9	44.1 ± 3.9
GL-SH	24.9 ± 1.3	30.6 ± 3.6	22.7 ± 1.7	69.7 ± 4.1

Hydrolysis of glucosinolates immobilized on gold nanoparticles (NP-GL)

