Supplementary Information

A Simple, Fast and Highly Sensitive Colorimetric Detection of Zein in Aqueous Ethanol via Zein-Pyridine-Gold Interactions

Alejandro Latorre-Sanchez and Jose A. Pomposo*

*E-mail: <u>Josetxo.pomposo@ehu.eus</u>

1. Materials and methods.

Methyl methacrylate (99 %, \leq 30 ppm MEHQ as inhibitor), 4-vinylpyridine (95 %, 100 ppm hydroquinone as inhibitor), 1,1'-azobis(cyclohexanecarbonitrile) (98 %), 1,4-diiodobutane (\geq 99 %, containing copper as stabilizer), sodium tetrachloroaurate(III) dihydrate (99 %), hydrazine monohydrate (64-65 %, reagent grade, 98 %), zein, deuterated chloroform (99.8 atom % D, containing 0.03 % (v/v) TMS), diethyl ether (\geq 99.8 %, containing BHT as inhibitor) and hexane (\geq 99 %) were purchased from Sigma-Aldrich. Purified water was obtained from a Thermo Scientific apparatus (Barnstead TII Pure Water System). Tetrahydrofuran (\geq 99.9 %, stabilized with 250 ppm of BHT) and ethanol (\geq 99.9 %) were purchased from Scharlab. Methyl methacrylate and 4-vinylpyridine were purified by passing through neutral alumina.

1.1. Synthesis and characterization of the precursor of pyridine-functionalized single-chain nanoparticles (ScNPs).

In a typical procedure, methyl methacrylate (11 ml, 102.8 mmol), 4-vinylpyridine (1.26 ml, 11.7 mmol) and 1,1'-azobis(cyclohexanecarbonitrile) (1.4 ml of prepared initiator solution 0.022 M in benzene) were mixed. The reaction mixture was degassed by passing argon for 15 min. The copolymerization reaction was carried out at 90 °C for 3 h. The resulting precursor was isolated by precipitation in diethyl ether and further drying under dynamic vacuum (Yield (%) = 20; M_w (SEC/MALLS, dn/dc = 0.083) = 998 KDa, M_w/M_n = 1.2; composition (¹H NMR) = 15 mol % of 4-vinylpyridine).







Methyl methacrylate

4-Vinylpyridine

Precursor

The ¹H NMR spectrum in $CDCl_3$ of the precursor is shown below, with the corresponding proton assignments (1):



Figure 1: ¹H NMR spectrum of the precursor. The proportion of each monomer is calculated considering that signal "a" corresponds to 2 protons of the pyridine group and signal "c" corresponds to 3 protons of the methyl methacrylate group.

1.2. Synthesis and characterization of pyridine-functionalized ScNPs.

In a typical reaction, the precursor (100 mg, 0.15 mmol of 4-vinylpyridine) was dissolved in THF (200 ml) at room temperature. Then, 1,4-diiodobutane (10 μ l, 0.075 mmol) was added and the mixture was maintained at 70 °C under reflux and stirring. The progressive folding/collapse process was followed through simultaneous SEC/MALLS measurements as illustrated below. A progressive shift to longer retention time and, hence, to reduced hydrodynamic size was observed with reaction time. After 5 days, the reaction was stopped because at longer reaction time the incipient formation of aggregates was observed. Finally, the ScNPs were isolated by precipitation in hexane and further drying under dynamic vacuum (yield (%) = 85).



Figure 2: SEC traces for the precursor and ScNPs after 1, 2 and 5 days of reaction.

A comparison of the ¹H NMR spectra in $CDCl_3$, FTIR spectra and dynamic light scattering (DLS) results between the precursor and the nanoparticles is shown below:





Figure 3: (a) ¹H NMR spectrum of the precursor (red) and ScNPs (blue). (b) Enlargement of ¹H NMR spectrum (a).

In the ¹H NMR spectrum of the ScNPs, signals from protons coming from quaternized pyridine groups are visible upon enlargement of the spectrum (1). The amount of quaternized pyridine groups was estimated to be *ca*. 10 %. The integrated spectrum of the ScNPs is shown below.



Figure 4: Integrated ¹H NMR spectrum of the ScNPs. Signal "a" corresponds to 1 proton of pyridine group and signal "a" corresponds to 1 proton of quaternized pyridine group.



Figure 5: FTIR spectra of the precursor (green) and ScNPs (blue).

By comparing the FTIR spectra of the precursor and ScNPs, changes in the bands corresponding to C-H, C-N and C-C stretching and CCH bending were observed upon quaternization (2).



A reduction in hydrodynamic size was clearly observed from DLS measurements. An average hydrodynamic radius of 14 nm was determined for the precursor, whereas the ScNPs showed an average hydrodynamic radius of 8 nm.

1.3. Procedure for the simple, fast and highly sensitive colorimetric detection of zein in aqueous ethanol.

In a first step, independent solutions of ScNPs, NaAuCl₄ and zein were prepared. Hence, ScNPs (5 mg, 7.5 x 10^{-3} mmol of 4-vinylpyridine units) were dissolved in 10 ml of an ethanol/water (75% /25%) mixture at room temperature. NaAuCl₄ (2.9 mg, 7.3 x 10^{-3} mmol) was dissolved in 1 ml of ethanol/water (75% /25%) mixture at room temperature and after that, a dilution of this solution was prepared to a final concentration of 0.29 mg/ml. Finally, zein (5 mg) was dissolved in 10 ml of ethanol/water (75% /25%) mixture at room temperature.

In a second step, different final solutions were prepared at room temperature by taken first 2 ml of the ScNPs solution, adding 1 ml of the diluted gold solution drop by drop, and subsequently zein (zein was added in solution only when the amount was not enough to be added by weight), according to the data reported in the following table. When all components were mixed, 1 drop of hydrazine (25 μ l) was finally added.

Added volume of ScNPs solution (ml)	Added volume of diluted gold solution (ml)	Added volume of zein solution (ml)	Zein amount (mg)	Added volume of solvent (ml)	Final volume (ml)	Zein concentration in final solution (µg/ml)
2	1	0	18	3	6	3 000
2	1	0	6	3	6	1 000
2	1	0	3	3	6	500
2	1	0	1.5	3	6	250
2	1	1.2	0.6	1.8	6	100
2	1	0.6	0.3	2.4	6	50
2	1	0.3	0.15	2.7	6	25
2	1	0.15	0.075	2.85	6	12.5
2	1	0	0	3	6	0

Table 1: Composition of different solutions used for the colorimetric detection of zein.

1.4. Determination of zein in a real sample.

In a 10 mg of real freeze-dried sample, a first extraction with water (1 ml) was carried out to remove salts that could interfere in the experiment. A second extraction with the ethanol/water (75% /25%) mixture (3 ml) was carried out to extract zein. After that, the sample was filtered. The problem solution was prepared by adding 2 ml of ScNPs solution and 1 ml of diluted gold solution to 3 ml of filtered corn sample. After that, 1 drop of hydrazine was added. The color of the resultant solution is shown below (Fig. 7b) in comparison with a solution without zein (Fig. 7a).



Figure 7: (a) AuNPs solution without zein (as control), (b) AuNPs solution in a real sample (containing zein).

The UV-vis technique showed a surface plasmon resonance peak located at 529 nm, which according to Figure 3c in the main article corresponds to a zein concentration of 55 μ g/ml. If the final volume is 6 ml and the zein concentration is 55 μ g/ml, the amount of zein in the real sample is estimated to be 330 μ g. Since the amount of corn was 10 mg, therefore the percentage of zein in the sample is 3.3 %. This result is in good agreement with the reported typical zein amount in corn (3).



Figure 8: Position of the maximum of the UV-vis band in a real sample (red circle) corresponding to a zein concentration of 55 μ g/ml.

2. Characterization techniques.

¹H Nuclear Magnetic Resonance (¹H NMR) spectra were recorded at room temperature on Bruker spectrometers opetaring at 400 MHz or 500 MHz, using CDCl₃ as solvent.

Size-Exclusion Chromatography / Multi-Angle Laser Light Scattering (SEC/MALLS) measurements were performed at 30 °C on an Agilent 1200 system equipped with PLgel 5 μ m Guard and PLgel 5 μ m MIXED-C columns, a differential refractive index (RI) detector (Optilab Rex, Wyatt) and a MALLS detector (MiniDawn Treos, Wyatt). Data analysis was performed with ASTRA Software from Wyatt. THF was used as eluent at a flow rate of 1 ml/min.

Fourier Transform Infra-Red (FTIR) spectroscopy spectra were recorded at room temperature on a JASCO 3600 FTIR spectrometer.

Dynamic Light Scattering (DLS) measurements were carried out at 25 °C in a Zetasizer Nano-ZS apparatus.

Transmission Electron Microscopy (TEM) measurements were performed using a high-resolution transmission electron microscope TECNAI G220 TWIN. The measurements were carried out using an accelerating voltage of 220 kV, under low dose conditions.

Ultraviolet / Visible (UV/Vis) spectroscopy measurements were carried out at 25 °C in an Agilent 8453A apparatus with Peltier thermostatic cell holder, T-controller 89090A.

3. References.

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