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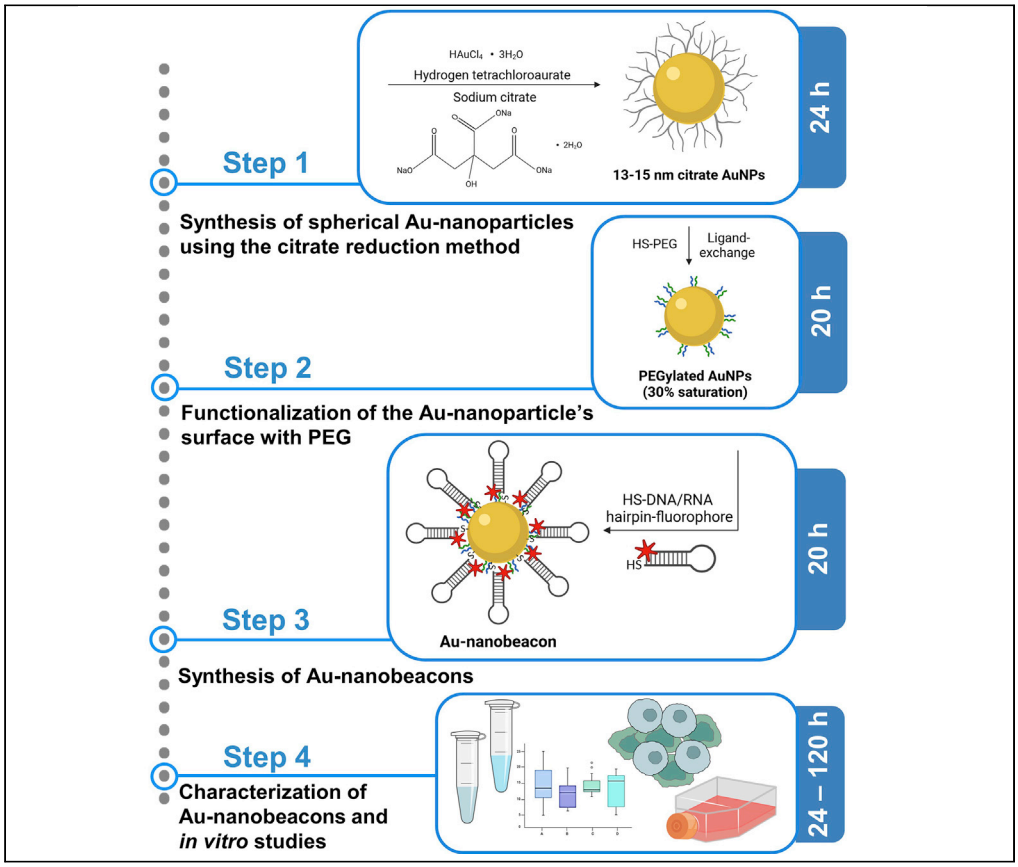
Using gold nanobeacons as a theranostic technique to recognize, detect, and inhibit specific nucleic acids



This protocol describes the synthesis and characterization of gold nanoparticle-based nanobeacons as a theranostic strategy for the recognition, detection, and inhibition of miRNA and mRNA. This system is designed for an *in vitro* evaluation of a sequence's silencing potential and later used for cellular and *in vivo* gene silencing approaches using fluorescence imaging, enhancing theranostic procedures in which nanoparticle-based sensors and inhibitors may provide simultaneous detection of different gene-associated conditions and nanodevices for a real-time monitoring of gene delivery.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Synthesis/functionalization of gold nanoparticles with PEG and DNA/RNA oligonucleotides

Characterization of the nanosystems for the recognition and inhibition of nucleic acids

Detection of nucleic acids using fluorescence imaging

Real-time monitoring of gene delivery using a nanobacon-based delivery system

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Using gold nanobeacons as a theranostic technique to recognize, detect, and inhibit specific nucleic acids

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SUMMARY

This protocol describes the synthesis and characterization of gold nanoparticle-based nanobeacons as a theranostic strategy for the recognition, detection, and inhibition of miRNA and mRNA. This system is designed for an *in vitro* evaluation of a sequence's silencing potential and later used for cellular and *in vivo* gene silencing approaches using fluorescence imaging, enhancing theranostic procedures in which nanoparticle-based sensors and inhibitors may provide simultaneous detection of different gene-associated conditions and nanodevices for a real-time monitoring of gene delivery.

For complete details on the use and execution of this protocol, please refer to Conde et al. (2015, 2013).[1,2]

BEFORE YOU BEGIN

Institutional permissions

All the animal experiments were approved by the Ethical Committee and the Animal Welfare and Ethics Body of the Nova Medical School (ORBEA) and complied with the Animal Research guidelines of Nova Medical School and of the Directorate-General for Food and Veterinary Medicine of Portugal.

Important: Acquiring permissions from the relevant institutions for the realization of animal experiments is necessary. Prepare all the documents and protocols that are required in advance, as the approval processes often take time.

This protocol describes the synthesis and characterization of gold nanobeacons for the detection and silencing of nucleic acids such as miRNA and mRNA and real-time monitoring of gene delivery through fluorescence (Figure 1A). A molecular beacon consists of a stem-loop DNA/RNA single-stranded oligonucleotide that is linked to a fluorophore at the 3′ end and to a quencher at the 5′ end. When closed, this structure promotes the proximity of the fluorophore and the quencher, which causes fluorescence quenching. When a nucleic acid with a sequence that is complementary to the molecular beacon is present, the stem-loop opens and the molecular beacon hybridizes, forming a double-stranded structure. This promotes an increase in the distance between the fluorophore and the quencher, restoring fluorescence emission. Fluorescence emission allows to quantitatively analyze the kinetics of conformational alterations that occur in the molecular beacon in different cellular environmental conditions (e.g., pH, redox, temperature) and to monitor gene delivery in real-time. However, the use of molecular beacons for gene therapy encounters difficulties.[3,4] For

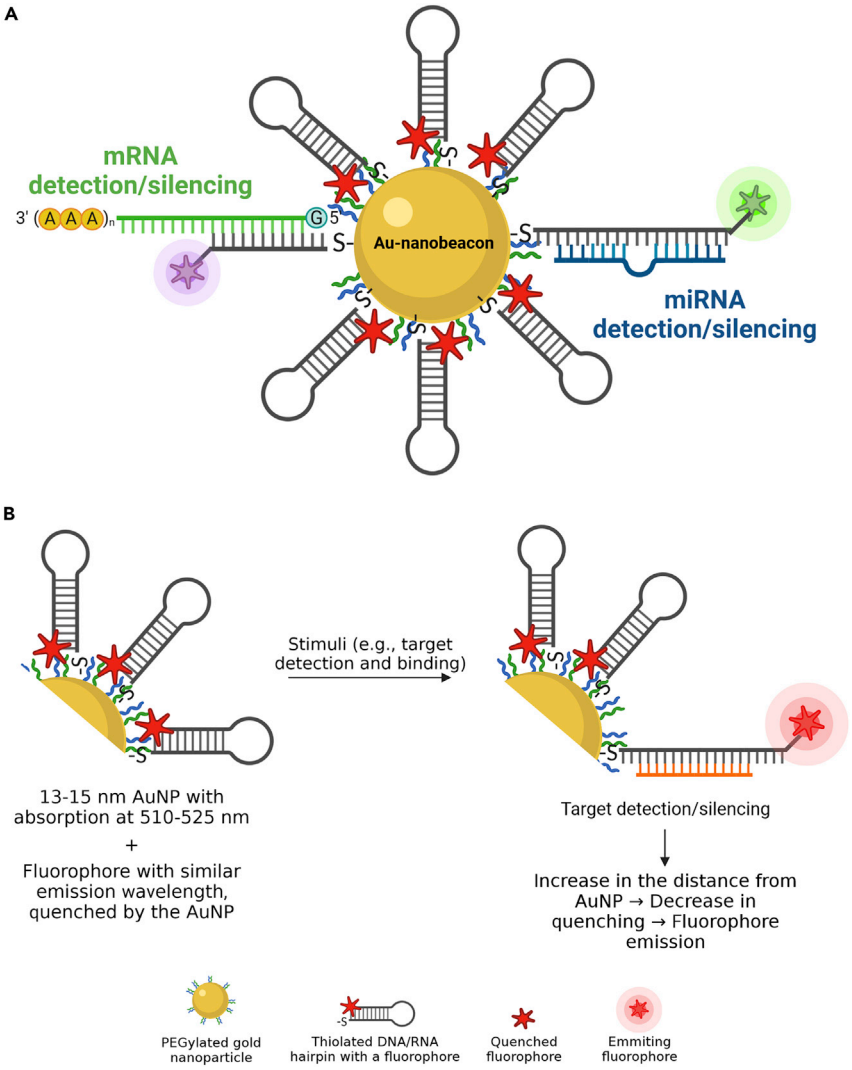

Figure 1. Gold nanobeacons as a theranostic technique to recognize, detect, and inhibit specific nucleic acids

(A and B) Application of gold nanobeacons for nucleic acid detection and silencing (A), and illustration of the process of fluorescence quenching/emission using a gold nanobeacon (B).

instance, nucleic acids are easily degraded by endogenous nucleases and are negatively charged, which affects their therapeutic efficacy and cellular internalization.

Gold nanoparticles have shown to be great delivery vehicles due to the possibility of having their surface coated with different moieties and having different possibilities for molecule release. Moreover, the optical properties observed in gold nanoparticles show they can modulate the fluorescence of molecules in their vicinity and act as quenchers (Figure 1B). Also, conjugation of molecular beacons to gold nanoparticles offers protection against degradation and promotes cellular targeting and uptake, allowing the modulation of gene expression pathways such as miRNA and anti-sense DNA. Thus, gold nanobeacons – gold nanoparticles functionalized with a fluorophore-labeled hairpin DNA/RNA – are an useful theranostic tool to detect gene-specific silencing and inhibition.

Preparation of solutions

Timing: 1–2 h

1. Prepare in advance the following solutions, according to the recipes found in the materials and equipment section below.
   a. DEPC-treated water.
   b. SDS 1%.
   c. 0.5 M phosphate buffer solution, pH=7.0.
   d. 1 M DTT.
   e. Ionic strength solution A.
   f. Ionic strength solution B.
   g. 10 mM phosphate buffer, pH=8.0.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DL-Dithiothreitol (DTT)	Sigma-Aldrich	Cat# D0632
(O-[2-(3-Mercaptopropionylamino)ethyl]-O′-methylpolyethylene glycol, 5000)	Sigma-Aldrich	Cat# 11124
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich	Cat# D5758
Gold(III) chloride trihydrate	Sigma-Aldrich	Cat# G4022
Sodium citrate tribasic dihydrate	Sigma-Aldrich	Cat# S4641
Heat-inactivated fetal bovine serum	Thermo Fisher Scientific (Gibco)	Cat# 10100147
Penicillin-streptomycin (10,000 U/mL)	Thermo Fisher Scientific (Gibco)	Cat# 15140122
ProLong™ Gold Antifade Mountant with DAPI	Thermo Fisher Scientific	Cat# P36941
Hydrochloric acid, 37%	Sigma-Aldrich	Cat# 258148
Nitric acid, 65%–67%	Supelco	Cat# 1.01799
5,5-Dithiobis(2-nitrobenzoic acid) (DTNB)	Sigma-Aldrich	Cat# D8130
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	Cat# L3771
Ethyl acetate	Sigma-Aldrich	Cat# 270989
Sodium phosphate monobasic	Sigma-Aldrich	Cat# S8282
Sodium phosphate dibasic	Sigma-Aldrich	Cat# 71642
Sodium chloride	Sigma-Aldrich	Cat# S7653
Magnesium chloride	Sigma-Aldrich	Cat# 208337
Paraformaldehyde	Sigma-Aldrich	Cat# P6148
RNase, DNase-free, from bovine pancreas	Roche	Cat# 11579681001
DNase I, from bovine pancreas	Roche	Cat# 10104159001
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific (Gibco)	Cat# 25200056
Culture medium (e.g., DMEM)	Thermo Fisher Scientific (Gibco)	Cat# A1896701
Critical commercial assays		
Pierce™ Coomassie (Bradford) Protein Assay Kit	Thermo Scientific	Cat# 23200
PureLink™ RNA Mini Kit	Invitrogen	Cat# 12183025
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor	Applied Biosystems	Cat# 4374966
TaqMan™ Fast Advanced Master Mix	Applied Biosystems	Cat# 4444556
TaqMan probes for genes/miRNAs of interest (e.g., *MRP1*, miR-21, *GAPDH*, RN6UB)	Thermo Scientific	N/A
Experimental models: Cell lines		
Cells of interest (e.g., MDA-MB-231, breast cancer)	ATCC	Cat# HTB-26

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Anti-*MRP1* nanobeacon	Conde et al.[1]	Thiol- 5′tttgcatGGCTACATT CAGATGACACatgcaaa 3′ -Q705
Nonsense nanobeacon (mRNA silencing approach)	Conde et al.[1]	Thiol- 5′ tttgcatTTCTCCGAACGT GTCACGTatgcaaa 3′-Q705
MRP1 target	Conde et al.[1]	5′ GTGTCATCTGAATGTAGCC 3′
Anti-miR-21 Au-nanobeacon (complementary to the mature microRNA-21)	STABVIDA[2]	Thiol- 5′-TTTGCCTCAACATCAGTC TGATAAGCTAGGCAAA 3′ – Cy3
Nonsense nanobeacon (miRNA silencing approach)	STABVIDA[2]	Thiol- 5′-TTTGCCTCAACATCAGT CTGATAAGCTAGGCAAA 3′ – Cy3
miR-21 target	STABVIDA[2]	5′ UAGCUUAUC AGACUGAUGUUGA 3′
Non-complementary target	STABVIDA	Reverse and complementary sequence of the nonsense nanobeacon
Software and algorithms		
ImageJ	https://doi.org/10.1038/nmeth.2089	https://imagej.nih.gov/ij/download.html
Other		
Milli-Q water purification system	Millipore	N/A
24-well microplates, tissue culture treated	Sarstedt	Cat# 83.3922
Spectrophotometer	Shimadzu (UV-2401PC)	N/A
Fluorescence or confocal microscope	Zeiss LSM710	N/A
Nanodrop 2000 Spectrophotometer	Thermo Scientific	Cat# ND-2000
Refrigerated centrifuge	Gyrozen (1696R)	N/A
Heating mantle for 500 mL round flasks	Enzymatic	Cat# MSHM-500-001
Rounded bottom 500 mL two-neck flask	Enzymatic	Cat # BF23-500-001
Magnetic stirrer	Enzymatic	Cat# MAGV-030-005
Condenser	Enzymatic	Cat# CIC3-300-002
Stopper	Enzymatic	Cat# Enzymatic STP3-001-001
pH meter	Mettler Toledo S220	N/A
Illustra NAP-5 columns	GE Healthcare Life Sciences	Cat# GE17-0853-02
Quartz cuvette	Anton Paar	Cat# 163390
96-well plates	Thermo Scientific	Cat# 167008
Microplates for Fluorescence-based Assays, 96-well	Thermo Scientific	Cat# M33089
Microscope glass slides	Bio Optica	Cat# 09-1000MB
Bright-line hemacytometer	Merck	Cat# Z359629
Coverslips with 12 mm diameter and <1.5 mm thickness	VWR	Cat# 631-1577P
Plate reader	SpectraMax i3×	N/A
Quantitative PCR instrument	QuantStudio 5	N/A

MATERIALS AND EQUIPMENT

DEPC-water		
Reagent	Final concentration	Amount
DEPC	0.1% (v/v)	1 mL
Milli-Q water	N/A	999 mL
Total	**N/A**	**1,000 mL**

Shake vigorously after adding DEPC and let the solution rest overnight (16 h) before autoclaving to inactivate DEPC. Store at room temperature (23°C–25°C).

Alternatives: Commercial DNAse/RNAse-free distilled water (Thermo Scientific, Cat# 1097 7049) can be used instead of DEPC-treated water for the nanoparticle synthesis steps and for the preparation of all the solutions.

SDS 1% stock solution		
Reagent	Final concentration	Amount
SDS	1% (w/v)	1.0 g
DEPC-treated water	N/A	100 mL
Total	**N/A**	**100 mL**
Store at room temperature (23°C–25°C).		

Phosphate buffer solution (0.5 M, pH=7.0)		
Reagent	Final concentration	Amount
Na_2HPO_4	1 M	14.1960 g
NaH_2PO_4	1 M	11.9980 g
DEPC-treated water	N/A	200 mL
Total	**N/A**	**200 mL**
Adjust the pH to 7.0 and autoclave. Store at 4°C. Warm to 25°C before use to dissolve any precipitate that forms.		

DTT solution (1 M)		
Reagent	Final concentration	Amount
Dithiothreitol (DTT)	1 M	15.425 g
DEPC-treated water	N/A	100 mL
Total	**N/A**	**100 mL**
Store at room temperature (23°C–25°C).		

⚠ CRITICAL: DTT is a hazardous chemical (hazard statements H302, H315, H319). When manipulating DTT, work in a chemical hood and wear protective equipment (gloves, lab coat and protective glasses). Waste containing DTT should be collected and disposed according to the institute guidelines and regulations.

Phosphate buffer solution (10 mM, pH=8.0)		
Reagent	Final concentration	Amount
Na_2HPO_4	9.32 mM	1.3231 g
NaH_2PO_4	0.68 mM	0.0816 g
DEPC-treated water	N/A	1,000 mL
Total	**N/A**	**1,000 mL**
Adjust the pH to 8.0 and autoclave. Store at 4°C.		

Ionic strength solution A		
Reagent	Final concentration	Amount
SDS	2% (w/v)	2.0 g
Phosphate buffer, pH = 8 (10 mM)	10 mM	100 mL
Total	**N/A**	**100 mL**
Sterilize by filtration (Millex-FG, 0.20 μm, hydrophobic PTFE, 25 mm, Millipore, Cat# SLFGL25BS) and store at 4°C until use. Warm to 25°C before use to dissolve any precipitate that forms.		

Ionic strength solution B		
Reagent	Final concentration	Amount
SDS	0.01% (w/v)	0.01 g
NaCl	1.5 M	8.7660 g
Phosphate buffer, pH = 8 (10 mM)	10 mM	100 mL
Total	**N/A**	**100 mL**

Sterilize by filtration (0.22 μm) and store at 4°C until use. Warm to 25°C before use to dissolve any precipitate that forms.

Phosphate Buffered Saine (PBS) 1×		
Reagent	Final concentration	Amount
NaCl	137 mM	8.0063 g
KCl	2.7 mM	0.2013 g
Na_2HPO_4	10 mM	1.4196 g
KH_2PO_4	2 mM	0.2722 g
Milli-Q water	N/A	1,000 mL
Total	**N/A**	**1,000 mL**

Adjust the pH to 7.4 and autoclave. Store at 4°C.

Alternatives: PBS can also be prepared from tablets (Sigma-Aldrich, Cat# P4417-100TAB) or used as an already prepared commercial solution (Thermo Fisher Scientific, Cat# 10010023).

STEP-BY-STEP METHOD DETAILS

Synthesis of spherical gold nanoparticles using the citrate reduction method

Timing: 24 h

This step provides indications for the synthesis of 14 nm spherical gold nanoparticles using the citrate reduction method (see Figure 2). This size of nanoparticles is used because they can be synthesized with high quality and reproducibility,[5] and the protocol of functionalization with nucleic acids has been well established.[6,7]

1. In a fume hood, prepare *aqua regia*: mix 3:1 concentrated HCl:HNO_3 in an open and large container.

   CAUTION: Be exceptionally careful when preparing and working with *aqua regia* and wear personal protective equipment. *Aqua regia* should be freshly prepared and should not be stored in closed containers, as these may explode. *Aqua regia* should be disposed by carefully diluting and neutralizing.

2. Immerse all the glassware (500 mL two-neck flask, magnetic stir bar, stopper, condenser and Erlenmeyer) in the freshly prepared *aqua regia* for at least 15 min. Afterwards, rinse the glassware with Millipore-filtered water.

   ⚠ CRITICAL: High-quality nanoparticles are essential for the success of the experiment. Care should be taken to make sure that no contamination with other metals is introduced during nanoparticle synthesis.

3. Add 225 mL of 1 mM $HAuCl_4$ (88.61 mg) into the rounded bottom two-neck flask and place the flask in the heating mantle.
4. Place the stirrer inside the flask.
   a. Connect the condenser to one neck of the flask and place the stopper in the other neck.

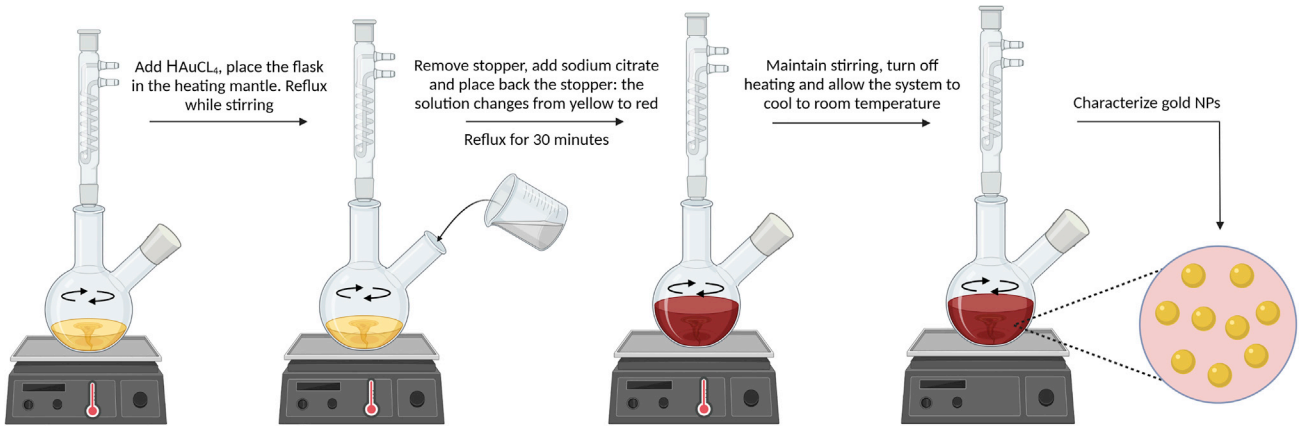

Figure 2. Schematic representation of gold nanoparticle synthesis using the citrate reduction method

b. Turn on the heating mantle to reflux while stirring.

5. When the solution begins to reflux remove the stopper and swiftly add 25 mL of 38.8 mM (285 mg) sodium citrate.
   a. Add the stopper back into the two-neck flask.

   Note: A color change from pale yellow to deep red should be observed in approximately 1 min.

   b. Allow the system to reflux for another 30 min.
6. Maintain stirring and turn off heating.
   a. Allow the system to cool to room temperature (23°C–25°C) protected from light.
   b. Transfer the colloidal solution to a 250 mL amber flask with a ground glass cap.

   Note: The obtained gold nanoparticles should be spherical under transmission electron microscopy (TEM), with a diameter of approximately 14 nm (13–15 nm), a Surface Plasmon Resonance peak at approximately 520 nm, with a correspondent extinction value of 2.33×10^8 $M^{-1}.cm^{-1}$ [8] and the solution should have a burgundy red color.

7. Using a quartz cuvette, record a UV-Visible spectrum (300–900 nm) of the gold nanoparticles.
   a. Determine the concentration using the absorbance at the peak in the Beer-Lambert law ($A = \varepsilon \times l \times c$, in which: A is the NP absorbance at the peak, ε is gold molar absorptivity (2.33×10^8 $M^{-1}.cm^{-1}$), l is the length of light path (1 cm) and c is the concentration). Consider any dilution that is performed previously to the measurement.

   Note: The wavelength (in nm) corresponding to the absorbance at the peak is the Surface Plasmon Resonance peak. Figure 3 shows an example of morphology (3A), size (3B), zeta-potential (3C) and UV-Visible spectra (3D) of the resulting nanoparticles.

   Pause point: The prepared nanoparticles are stable for months when stored in a clean container (preferably glass) at 4°C. Gold nanoparticles should not be frozen, as this will induce particle aggregation and prevent further use. Maximum time for storage – 2 months at 4°C.

Functionalization of the gold nanoparticle's surface with PEG

Timing: 20 h

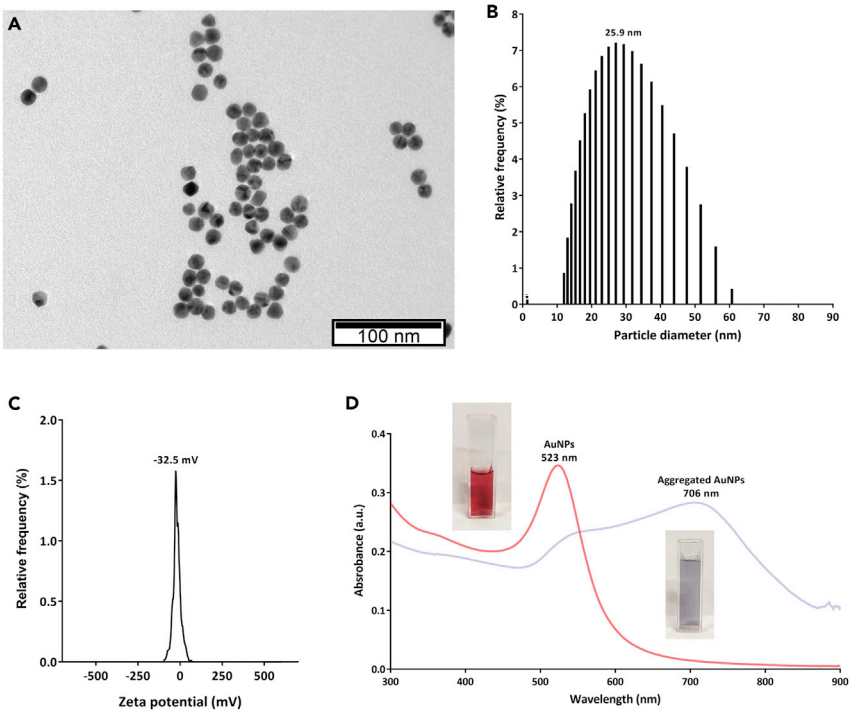

Figure 3. Characterization of gold nanoparticles

(A) Transmission electron microscopy (TEM) image showing the spherical morphology of the nanoparticles.

(B) Size distribution histogram, obtained by dynamic light scattering, showing an average hydrodynamic diameter of 25.9 nm.

(C) Zeta potential histogram, obtained by dynamic light scattering, showing an average surface charge of -32.5 mV.

(D) Absorbance spectra showing the surface plasmon resonance peak of AuNPs (red) and the shift observed upon nanoparticle aggregation (blue; e.g., formed by increasing salt concentration or freezing), and a representative photograph of each type of nanoparticle.

This step provides indications for the functionalization of 14 nm spherical gold nanoparticles with polyethylene glycol (PEG), to improve their stability and biocompatibility (see Figure 4).

8. Mix 41.7 mL of a 12 nM stock solution of citrate-capped gold nanoparticles (final concentration, 10 nM) with 150 μL of a 1 mg/mL stock solution of (O-[2-(3-Mercaptopropionylamino)ethyl]-O′-methylpolyethylene glycol, 5000, Maximum time for storage – 6 months at −20°C) (PEG) (final concentration: 0.003 mg/mL) in an aqueous solution of SDS (final concentration: 0.028%, Maximum time for storage – 3 months at 25°C).
   a. Add DEPC-treated water to reach the desired final volume.
   b. Incubate at room temperature (23°C–25°C) in a rotative shaker for 16 h.

   Note: The PEG powder stock should be dissolved in DMSO to intermediate concentrations, aliquoted and stored at −20°C to maintain the integrity of the thiols and avoid the formation of disulfide bridges. Prepare the working solution of PEG only at the time of functionalization by diluting an aliquot to the concentration of 1 mg/mL in DEPC-treated water.

   Note: The final PEG concentration can be adjusted, depending on the objective. For instance, gold nanoparticles can be previously incubated with different amounts of PEG to estimate which concentration corresponds to a certain percentage of PEG molecules at the nanoparticle's surface, and then that concentration can be used for the functionalization step in a larger scale.

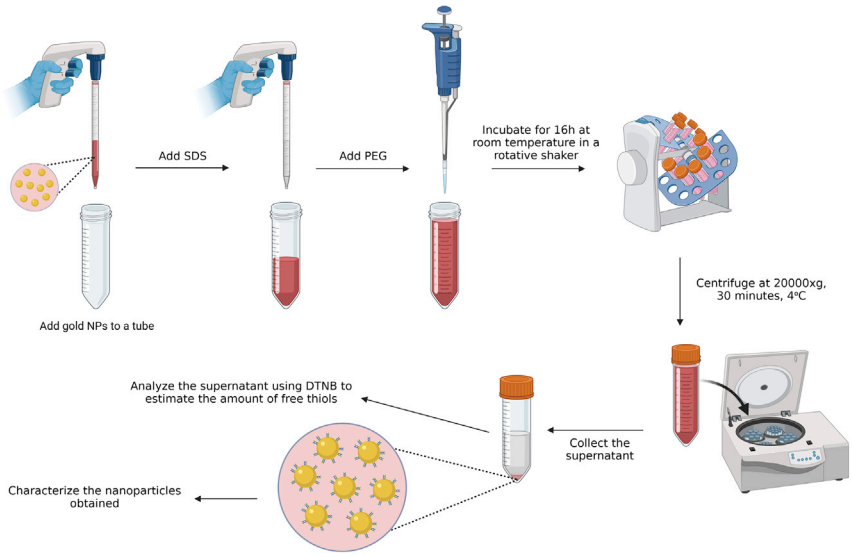

Figure 4. Schematic representation of PEGylated gold nanoparticle synthesis

9. Distribute the volume in centrifuge tubes and centrifuge at 20,000 × *g* for 30 min at 4°C to remove the PEG excess.

   Note: After centrifugation, the tubes should have an oily red deposition at the bottom and a clear supernatant.

   a. Collect 80%–90% of the supernatant and store it at 4°C for subsequent analysis. Keep track of the removed volume.
   b. Resuspend the deposition in DEPC-treated water.

10. Repeat step 9 twice.

    Note: In the end, three supernatants should have been collected.

    a. After the final centrifugation, redisperse the deposition in DEPC-treated water.

    Note: If the supernatant is not clear after the first centrifugation, collect it and centrifuge it again.

11. Using a quartz cuvette, record a UV-Visible spectrum of the PEGylated gold nanoparticles and determine the concentration as described in step 7.

    Note: The wavelength (in nm) corresponding to the absorbance at the peak is the Surface Plasmon Resonance peak.

12. Taking into consideration the concentration of PEG added to the nanoparticles, prepare a calibration curve of PEG, in triplicate, by mixing the appropriate amount of stock solution of PEG for each concentration with 100 μL of phosphate buffer 0.5 M (pH=7).
    a. Add DEPC-treated water up to 300 μL.
    b. For sample preparation, mix, in triplicate, 200 μL of the supernatants retrieved in steps 9 and 10 and mix with 100 μL of phosphate buffer 0.5 M (pH=7).
    c. Add the calibration curve concentrations and the supernatant samples to the wells of a 96-well plate.

d. Add 7 μL of 5 mg/mL 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), to each of these mixtures and incubate for 10 min at room temperature (23°C–25°C).

Note: If other PEG concentrations were used for functionalization, the calibration curve concentrations also need to be adjusted. DTNB is used to estimate the free thiols in the supernatants and should be freshly prepared before use by dissolving the powder in phosphate buffer 0.5 M (pH=7). A yellow color is expected to develop when many free thiols are present, which estimates that many PEG molecules are present. This should be observable at least in the higher concentrations of the calibration curve.

13. Record the absorbance values at 412 nm and an UV-Visible absorption spectrum (290–600 nm) of all samples.
    a. Use the calibration curve to calculate the amount of free thiols (and consequently, of PEG) in the supernatants and subtract this value to the amount initially added to the solution when functionalization started.

Note: In this step it is important to know the total volume of initial solution and the volumes of supernatant collected. This process estimates the number of PEG chains and is used to determine the amount of PEG molecules attached to the AuNPs. To do this, use the equation of a straight line, which in this case is the tendency line that is obtained from the calibration curve data points: $y = mx + b$, in which y is the absorbance at 412 nm, m is the slope of the line, x is the concentration/amount of PEG and b is the intercept.

Pause point: Store the nanoparticles functionalized with PEG at 4°C, protected from light, for 6 months. Save an aliquot of these nanoparticles for later characterization.

Synthesis of gold nanobeacons

Timing: 20 h

This step provides indications for the functionalization of 14 nm PEGylated spherical gold nanoparticles with molecular beacons to develop a theranostic nanoconjugates for recognition, detection and inhibition of specific nucleic acids (see Figure 5).

14. Mix the lyophilized thiolated oligonucleotide with 1 mL of DEPC-treated water.
    a. Collect an aliquot of 100 μL and store it at −20°C for later use.
    b. Add 100 μL of 1 M Dithiothreitol (DTT, Maximum time for storage – 6 months at 4°C) to the remaining 900 μL of oligonucleotide to attain a final concentration of 0.1 M in DTT.
    c. Incubate in a rotative shaker at room temperature (23°C–25°C) for two hours or overnight (16 h) at 4°C.

Note: DTT is used to quantitatively reduce disulfide bonds and to maintain monothiols in a reduced state. At low concentrations, DTT stabilizes enzymes and other proteins that possess free sulfhydryl groups and was shown to restore activity lost by oxidation groups *in vitro*.[9]

15. Extract one volume (500 μL) of thiol-modified oligonucleotide with two volumes (1,000 μL) of ethyl acetate, to remove DTT. Mix thoroughly.
16. Centrifuge at room temperature (23°C–25°C) for 5 min at 20,000 × *g*. Discard the organic phase (upper phase).
17. Repeat steps 15 and 16 twice.
18. After the third extraction, purify the remaining aqueous phase using a desalting NAP-5 column, according to the manufacturer instructions. Use 10 mM phosphate buffer (pH=8, maximum time for storage – 2 months at 4°C) as eluent.

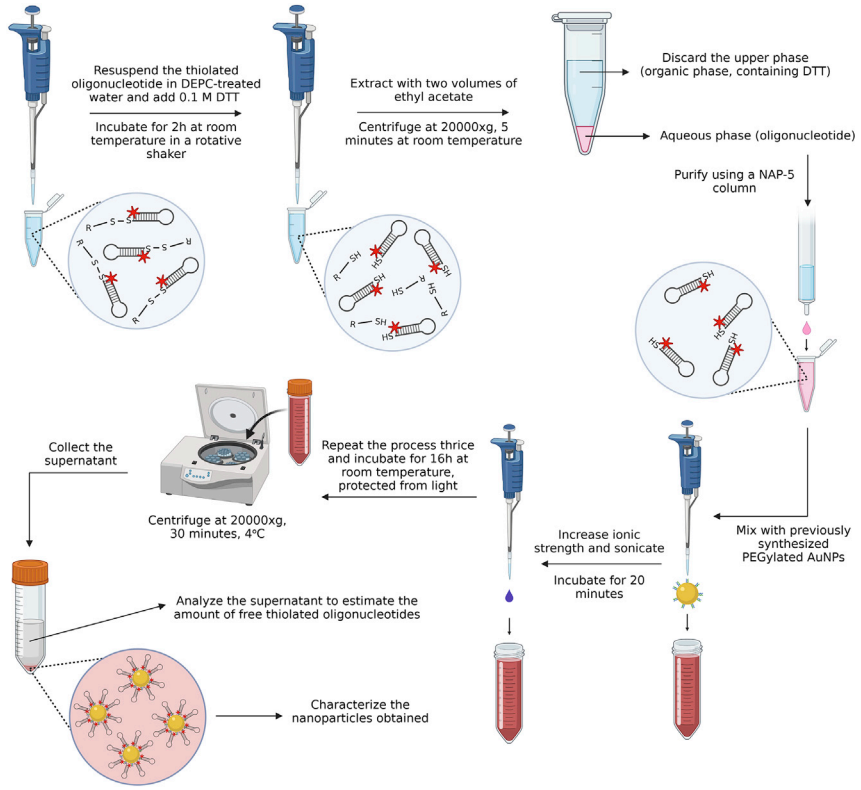

Figure 5. Schematic representation of the synthesis of gold nanobeacons

a. Wash the column three times with 10 mM phosphate buffer (pH=8).
b. Add 500 μL of oligonucleotide solution. Let all the solution pass through the column – the oligonucleotides will be retained in the column and the remaining DTT/ethyl acetate will be washed.
c. Prepare a 1.5 mL Eppendorf for collection and then add 1 mL of 10 mM phosphate buffer (pH=8) to the column to release the oligonucleotide.

Note: This step can be repeated with the remaining 500 μL of DTT/ethyl acetate extracted oligonucleotide, using the same column. To do this, wash the column again and repeat the process.

19. Quantify the purified oligonucleotide by UV-Visible spectroscopy using a Nanodrop and check the spectra for the existence of contaminations with DTT or ethyl acetate.
20. Mix the purified oligonucleotide with the PEGylated AuNPs previously prepared in a 1:100 AuNPs:oligonucleotide ratio.
21. Add ionic strength solution A (10 mM phosphate buffer pH=8, 2% SDS, maximum time for storage – 2 months at 4°C) to achieve a final concentration of 10 mM phosphate buffer (pH=8) and 0.01% (w/v) SDS.
    a. Sonicate for 10 s and incubate for 20 min at room temperature (23°C–25°C) in a rotative shaker.

Note: A conventional ultrasonic bath, 40 kHz, 135 W can be used for the sonication process.

22. Sequentially increase the ionic strength of the solution by adding the respective volume of ionic strength solution B (10 mM phosphate buffer pH=8, 1.5 M NaCl, 0.01% SDS, maximum time for

storage – 2 months at 4°C) up to a final concentration of 10 mM phosphate buffer (pH=8), 0.05 M NaCl and 0.01% (w/v) SDS.

a. Sonicate for 10 s and incubate for 20 min at room temperature (23°C–25°C) in a rotative shaker.

Note: Ionic strength solutions A and B should be stored at 4°C. Before starting the protocol, place them at room temperature until no precipitate is observed. If any precipitate persists, warm the solution with agitation until it disappears.

23. Repeat step 22, thrice, for final concentrations of 0.1, 0.2 and 0.3 M of NaCl.
    a. After the last addition, incubate for 16 h at room temperature (23°C–25°C), in a rotative shaker, protected from light.

Note: Carefully examine the aspect of the nanoparticles while increasing the ionic strength of the solution. NPs may be destabilized by the addition of salt and the color of the solution can be altered if aggregation occurs. An example of the aspect of the nanoparticles and their absorption spectra when aggregated is shown in Figure 3D. See troubleshooting 1.

24. Centrifuge at 4°C for 20 min at 20,000 × *g*.

Note: There should be an oily precipitate at the bottom of the tube.

    a. Collect 80%–90% of the supernatant and store it at 4°C for subsequent analysis. Keep track of the removed volume.
    b. Resuspend the deposition in 10 mM phosphate buffer pH=8.
25. Repeat step 24, twice. In the end, three supernatants should have been collected.
    a. After the final centrifugation, redisperse the deposition in 20%–30% of the initial volume in 10 mM phosphate buffer pH=8.

⚠ CRITICAL: Be careful not to disturb the precipitate while removing the supernatant, as re-dispersing the Au-nanobeacons during this step can result in major losses of Au-nanobeacons and contamination of the supernatant with Au-nanobeacons.

26. Record a UV-Visible spectrum of the Au-nanobeacons and determine the concentration as described in step 7.

Note: The wavelength (in nm) corresponding to the absorbance at the peak is the Surface Plasmon Resonance peak.

27. Prepare a calibration curve, in triplicate, using the oligonucleotide aliquot collected in step 14a.
    a. Mix the appropriate amount of stock solution of fluorescent oligonucleotide with ionic strength solutions A and B to obtain a final volume of 100 μL with a final concentration of 10 mM phosphate buffer (pH=8), 0.3 M NaCl and 0.01% (w/v) SDS.

⚠ CRITICAL: Use at least 5 different concentrations to build the calibration curve to ensure that it is possible to quantify the amount of oligonucleotide that did not bind.

28. Add all the calibration curve concentrations and 100 μL of each supernatant previously collected in steps 24 and 25, in triplicate, to the wells of a microplate for fluorescence-based assays, 96-well.
    a. Record the fluorescence emission of all the samples.

Note: Be sure to use the appropriate excitation and detection wavelengths for the fluorophores in use (i.e., for 6-FAM excite the fluorophore at 495 nm and read the emission at 520 nm).

29. Use the equation of the calibration curve to determine the amount of fluorophore-labeled oligonucleotides that are present in each supernatant.

Note: To do this use the equation of a straight line, which in this case is the tendency line that is obtained from the calibration curve data points: $y = mx + b$, in which y is the emission at the wavelength that corresponds to the chosen fluorophore, m is the slope of the line, x is the concentration and b is the intercept.

    a. Subtract the result to the amount of oligonucleotides initially added in step 20 to obtain the amount of oligonucleotides that are present at the surface of the oligonucleotides. Use this value to ascertain the final AuNP:oligonucleotides ratio.

30. Fully characterize the Au-nanobeacons by determining the hydrodynamic radius using Dynamic Light Scattering and the surface charge by measuring the Zeta Potential.

Note: The size, morphology and functionalization of Au-nanobeacons can be evaluated by staining a sample with 2% phosphotungstic acid (maximum time for storage – 3 months at 25°C) for 60–90 s and analyzing by TEM.

Pause point: Store the Au-nanobeacons at 4°C, protected from light, for 3 months without loss of fluorescence signal or signs of aggregation.

Characterization of gold nanobeacons

Timing: 10 to 120 min, depending on the assay

This step provides a characterization of the nanobeacons in terms of their interactions with the respective targets and in terms of their stability under different conditions and upon different stimuli that can also be found in cells, such as temperature, pH, reductive environments and presence of nucleases.

31. Hybridization specificity.
    a. Mix 1 nM of Au-nanobeacons with complementary and non-complementary targets for a final volume of 100 μL, in triplicate.

Note: To better observe the nanobeacon behavior with different concentrations of target, it can be incubated with different target/non-complementary target concentrations, for instance: a) a lower concentration than the nanobeacon's; b) the same concentration as the nanobeacon's; c) five times the nanobeacon's concentration. The targets depend on the downstream applications of the nanobeacons and can be single-stranded nucleic acid molecules (e.g., miRNAs), double-stranded DNA molecules, for example.

    b. Add the samples to a black 96-well plate and record the emission (fluorescence intensity) of the Au-nanobeacons at 37°C, every two minutes, for 120 min, using a plate reader.

Note: Use the appropriate excitation and emission wavelengths for the fluorophore in use.

    c. As a control, add in other wells the same concentration of Au-nanobeacons alone i.e., not mixed with complementary/non-complementary target.
    d. Plot the results against the variable in study (i.e., amount of complementary/non-complementary target, time).

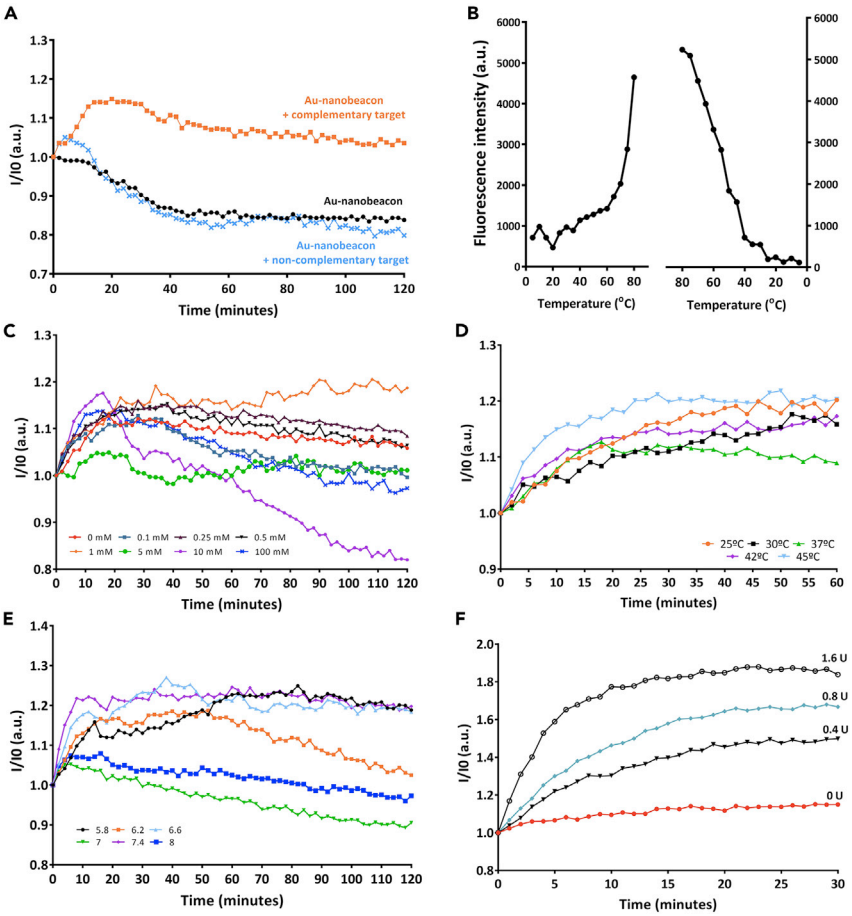

Figure 6. Gold-nanobeacon characterization studies

(A) Target hybridization test.

(B) Hairpin reversible denaturation/renaturation test.

(C) Stability in reductive environments.

(D) Stability in different temperatures.

(E) Stability in different pHs.

(F) Stability with nucleases (i.e., RNase).

Note: An increase in fluorescence is expected to be observed with time in the condition "Au-nanobeacon + complementary target" and no relevant variation should be observed in the condition "Au-nanobeacon + non-complementary target", when compared to Au-nanoconjugates alone. Figure 6A shows an example of the expected results.

⚠ CRITICAL: See troubleshooting 2.

32. Hairpin reversible denaturation/renaturation test.
    a. Incubate 1 nM of the Au-nanobeacons in phosphate buffer 10 mM (pH=8) at increasing temperatures ranging from 5°C to 80°C. Record the emission every two minutes considering the excitation/emission wavelengths of the fluorophore in use.
    b. Incubate 1 nM of the Au-nanobeacons in phosphate buffer 10 mM (pH=8) at decreasing temperatures ranging from 80°C to 5°C. Record the emission every two minutes considering the excitation/emission wavelengths of the fluorophore in use.
    c. Plot the resulting emission values against temperature.

Note: The graph should be similar to denaturing (step 32a) and renaturing (step 32b) DNA profiles, indicating if the Au-nanobeacons can reversibly change their hairpin conformation. An example of these results is shown in Figure 6B.

Note: This test was performed using a qRT-PCR equipment, which allows to design temperature gradients and to record the fluorescence emitted at each temperature.

⚠ CRITICAL: See troubleshooting 3.

33. Stability in reductive environments.
    a. Mix, in triplicate, 1 nM of Au-nanobeacons with different concentrations of glutathione (e.g., 0.05, 0.1, 0.25, 0.5, 1, 5, 10 and 100 mM, maximum time for storage – 2 months at 4°C).
        i. Transfer the samples to a microplate for fluorescence-based assays, 96-well (100 µL/well) and incubate at 37°C for 120 min, acquiring the emission data every 2 min.

    Note: Consider the correct excitation/emission wavelengths for the fluorophore in use.

    b. Repeat the procedure with the same concentrations of DTT.
    c. Plot the emission data results against time. An example of the results is shown in Figure 6C.

    Note: Glutathione is an ideal reagent to test how Au-nanobeacons behave in reducing conditions as it exists in the organism and allows to mimic the endogenous microenvironment and its physiological concentration (1 mM). DTT is used to compare how the Au-nanobeacons react to other types of exogenous stimuli.

    ⚠ CRITICAL: If the emission data of the Au-nanobeacons is altered in the presence of concentrations equal or higher than 5 mM of reducing agents it is probable that the Au-nanobeacons are unstable and will not maintain their integrity in further experiments.

34. Stability in different temperatures.
    a. Prepare, in triplicate, 1 nM of Au-nanobeacons.
    b. Transfer the samples to a microplate for fluorescence-based assays, 96-well (100 µL/well) and incubate at different temperatures (e.g., 25, 30, 37, 40 and 45°C) for 60 min, acquiring the emission data every 2 min.

    Note: Consider the correct excitation/emission wavelengths for the fluorophore in use.

    c. Plot the emission data results against time. An example of the result is shown in Figure 6D.
35. Stability in different pHs.
    a. Mix, in triplicate, 1 nM of Au-nanobeacons with 0.1 M potassium or sodium phosphate buffer solutions of different pHs (e.g., 5.8, 6.0, 6.2, 7.0, 7.4 and 8.0, maximum time for storage – 2 months at 4°C).
    b. Transfer the samples to a microplate for fluorescence-based assays, 96-well (100 µL/well) and incubate at 37°C for 120 min, acquiring the emission data every 2 min.

    Note: Consider the correct excitation/emission wavelengths for the fluorophore in use.

    c. Plot the emission data results against time. An example of the result is shown in Figure 6E.

    Note: Alterations in the pH of the solution can destabilize the fluorophore and alter its emission.

36. Stability with RNAse or DNAse.

a. Mix, in triplicate, 1 nM of Au-nanobeacons with different concentrations of RNAse or DNAse (e.g., 0.5, 1, 2.5 and 5 U, maximum time for storage – 6 months at −20°C).
b. Transfer the samples to a microplate for fluorescence-based assays, 96-well (100 μL/well) and incubate at 37°C for 30 min, acquiring the emission data every minute.

Note: Consider the correct excitation/emission wavelengths for the fluorophore in use.

c. Plot the emission data results against time. An example of the result is shown in Figure 6F.

Note: RNAse is more efficient than DNAse,[10] so lower concentrations may be used when compared to DNAse. However, concentrations may need to be optimized.

In vitro studies

Timing: 24–120 h

This step provides instructions for different assays that are used to study the modulation of crucial gene silencing pathways (antisense DNA and microRNA) by Au-nanobeacons.

37. Silencing *MRP1* expression with Antisense Au-nanobeacons.
    a. In the day before incubation with antisense Au-nanobeacon for *MRP1*, detach cells (e.g., MDA-MB-231, triple-negative breast cancer) with trypsin-EDTA 1×, resuspend them in complete growth medium (e.g., DMEM plus supplements, maximum time for storage – 3 to 4 weeks at 4°C), and pellet by centrifugation at 250×*g* for 5 min at room temperature (23°C–25°C).
    b. Resuspend cells in 10 mL of complete growth medium. Using a Neubauer chamber or hemocytometer for cell counting, calculate the amount of cell suspension needed and seed 1×10^5 cells/well in 24-well plates in 500 μL of the appropriate complete growth medium.
    c. Incubate at 37°C in a humidified 5% CO_2 atmosphere for 24 h.

⚠ CRITICAL: Check the cell density using an inverted optical microscope: cells should be 70%–80% confluent at the time of incubation with Au-nanobeacons. Adjust the seeding density according to the type of cells that are being used, as cells have different growth rates.

⚠ CRITICAL: Usually, in an experiment starting from a confluent culture flask (75 cm^2) and plating 1×10^5 cells per well in 24-well plates, for the equivalent to 50% confluence (surface coverage), use 100 μL of cell suspension plus 400 μL of complete growth medium, antibiotic-free, per well. However, this depends on the cells used and should be optimized.

Note: For confocal microscopy experiments, cells should be seeded on sterile glass slides added to the bottom of the wells (e.g., for 24-well plates, use round coverslips with 12 mm diameter and <1.5 mm thickness). In case the cells do not adhere directly to the coverslips, these can be pre-treated with gelatin (2% w/v, in water) for 15 min at 37°C.

Note: Confocal dishes could be used alternatively to glass slides on 24-well plates, as the latter are easily broken during removal from the plate.

    d. Remove the medium of each well and add the Au-nanobeacons targeting *MRP1* at the chosen concentrations, in complete growth medium, to obtain a final volume of 500 μL/well.
        i. Mix well to ensure even distribution of nanoparticles in the well. Consider preparing different plates to test the effects on different time-points.

e. Incubate at 37°C in a humidified 5% CO_2 atmosphere for 24 h.
f. To evaluate mRNA expression, collect and lyse the cells after 24 h for total RNA extraction (e.g., PureLink™ RNA Mini Kit) and prepare the samples for cDNA conversion (High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor) following the manufacturer's instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0017977_highcap_cDNA_RT_UG.pdf).
    i. Use the cDNA samples to perform RT-qPCR (TaqMan™ Fast Advanced Master Mix, using TaqMan probes for the target mRNA and for an endogenous control, such as GAPDH) following the manufacturer's instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0025706_TaqManFastAdvMM_UG.pdf).
    ii. Evaluate the expression against the endogenous control and the fold-change relatively to the non-treated cells. See troubleshooting 4.
g. To evaluate Au-nanobeacon signal, after 24 h, remove the growth medium and wash cells with PBS 1×.
    i. Fix cells with 4% paraformaldehyde (maximum time for storage – 2 months at 4°C) in PBS1× for 15 min at 37°C.
    ii. Mount the coverslip with cells on a slide with 15–20 μL of ProLong® Gold Antifade Reagent with DAPI, for nuclear staining.
    iii. Image the cells on a fluorescence or confocal microscope taking into account the excitation/emission wavelengths of the dyes used and of the fluorophore in the Au-nanobeacons (e.g., DAPI: Excitation/Emission = 358/461 nm; Quasar705: Excitation/Emission = 690/705 nm). An example of the expected results can be found in Figure 7A. See troubleshooting 5 and 6.

38. microRNA silencing via anti-miR-21 Au-nanobeacons.
    a. Plate cells at a density of 1×10^5 cells/well in 24-well plates.

Note: Cells must be 70%–80% confluent at the time of incubation with Au-nanobeacons and should be grown in complete medium.

    i. Prepare the cells as mentioned in steps 37a to 37c.
    b. After 24 h add 10, 30 and 50 nM (concentration of hairpin oligo functionalized on the nanoparticle's surface calculated in steps 27–30) of Anti-miR Au-nanobeacons to the cells and incubate for 24, 48 and 72 h.
    c. At the different time-points, wash cells with PBS 1×, lyse them and extract total RNA (as mentioned in step 37f) or prepare the samples for fluorescence/confocal microscopy (as mentioned previously in step 37g). An example of the expected results regarding fluorescence signal and miRNA expression can be found in Figures 8A and 8B and in Figures 8D and 8E, respectively.

Note: Although many times the endogenous control is a housekeeping gene, in this case, since the expression target is a miRNA, ideally the endogenous control should also be a miRNA, such as RN6UB.

EXPECTED OUTCOMES

Here we provide a protocol for the synthesis and characterization of gold nanobeacons that can recognize and block essential silencing pathways for *in vitro* and *in vivo* applications, using fluorescence to monitor gene delivery in real-time. With this protocol, the resulting gold nanobeacons can be constructed with high reproducibility and are homogenous, stable, and highly specific for the chosen target. It is expected that after hybridization with the target, the molecular beacon changes conformation, leading to fluorescence emission which signals that the target was detected, and the

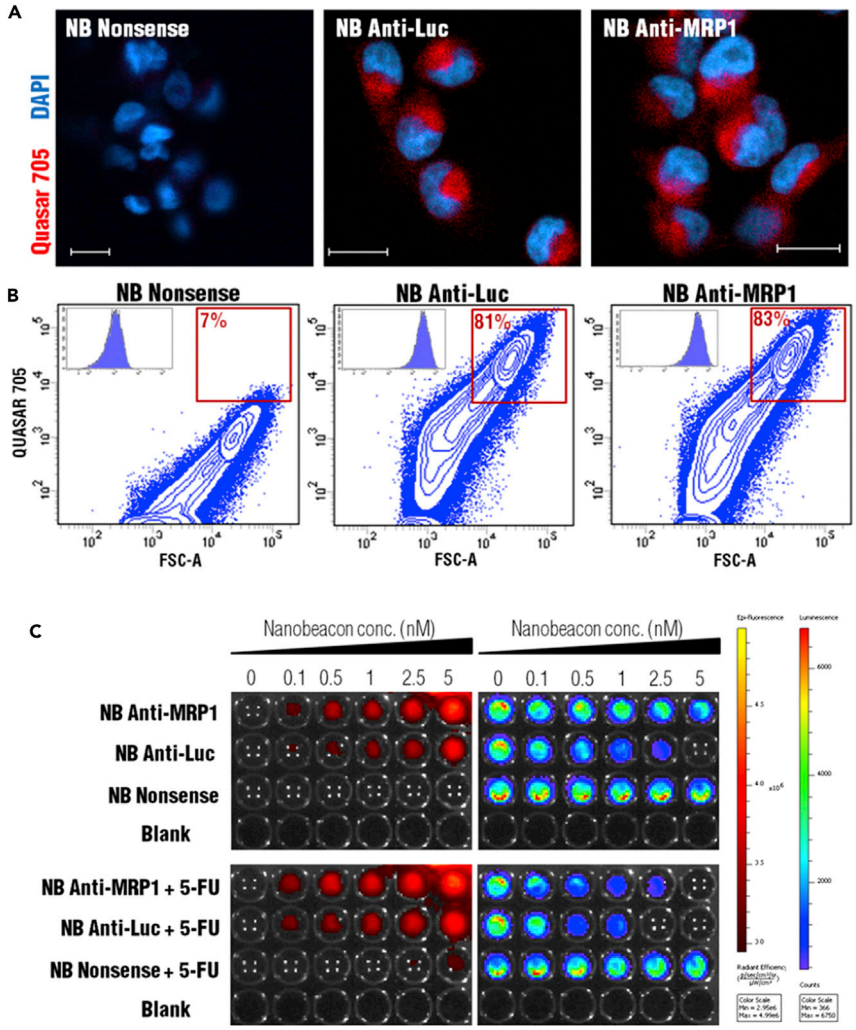

Figure 7. Example of the results obtained for mRNA silencing in resistant MDA-MB-231 cells (triple-negative breast cancer, TNBC)

These cells were incubated with increasing concentrations of 5-fluorouracil (5-FU) to induce resistance, as this chemotherapeutic agent if often used in TNBC patients.

(A) Representative confocal images of resistant MDA-MB-231 cells incubated for 48 h with a nanobeacon nonsense, nanobeacon anti-Luc, and nanobeacon anti-*MRP1* (concentration used: 5 nM; scale bars in the pictures correspond to 20 μm).

(B) Flow cytometry analysis comparing nanobeacon anti-Luc and nanobeacon anti-*MRP1*–treated cells to nanobeacon nonsense.

(C) Fluorescence (in red) and luminescence signals of resistant MDA-MB-231 cells incubated with increasing amounts (0.1–5 nM) of dark-gold nanobeacons loaded without and with 5-FU. This system is a variation from the one described in this protocol. It uses a gold nanoparticle core decorated with a thiol-DNA hairpin that is labeled with a near-infrared dye (Quasar 705) and intercalated with 5-FU, and a thiol-DNA oligonucleotide which is labeled with a black-hole dark quencher (BHQ2). The system works in the same way as the one described in this protocol: when in hairpin configuration, the closeness of the near-infrared dye to the dark quencher promotes to fluorescence quenching. Hybridization of the DNA hairpin to a complementary target (in this case, the *MRP1* mRNA) restores fluorescence emission, as the conformational reorganization causes the distance between the fluorophore and the quencher to increase, yielding a quantitative response. Adapted and reproduced with permission (Conde, PNAS, 2015).[1]

inhibition process is occurring. This should be observable by a decreased expression of the target genes in a certain tissue, allowing to discriminate between healthy and tumor cells, which often have deregulated miRNA and mRNA expressions. The fluorescence signal allows to follow the

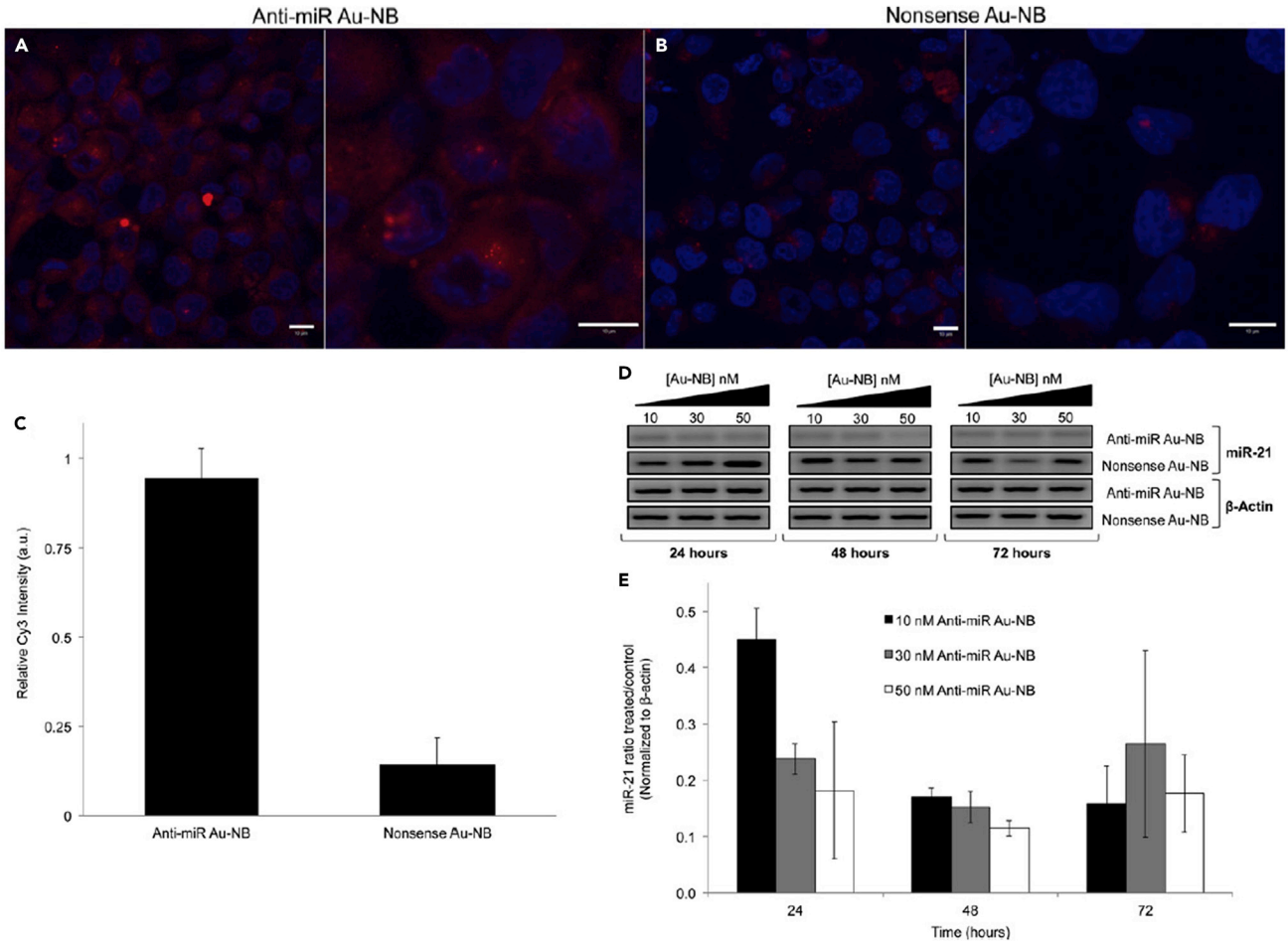

Figure 8. Example of the expected results for the miRNA silencing

(A and B) Au-nanobeacons silencing of miR-21. Confocal imaging (scale bar, 10 μm) shows internalization of 50 nM (A) Anti-miR Au-nanobeacon 50 nM and (B) nonsense Au-nanobeacon. Target (mature miR-21) recognition leads to change of Anti-miR Au-nanobeacon conformation in the cytoplasm with concomitant fluorescence signal (red, Cy3) encircling the cell nuclei (blue, DAPI).

(C) Specificity of target recognition is corroborated by the relative fluorescence intensity of Au-nanobeacons.

(D) Quantitative assessment of miR-21 silencing in colorectal carcinoma cells (HCT-116) induced by 10, 30 and 50 nM of Anti-miR Au-nanobeacon for 24, 48 and 72 h of incubation using b-actin as reference was confirmed by RT-PCR followed by agarose gel electrophoresis.

(E) qRT-PCR analysis of assay versus control normalized for b-actin expression for the same conditions depicted as in (D). Error bars indicate ± s.d. from 3 independent experiments. Reproduced with permission (Conde, Biomaterials, 2013).[2]

inhibition process in real-time and to observe in which cellular and sub-cellular locations the gold nanobeacons tend to accumulate. This approach can be used to detect and inhibit any gene of interest in the tumor or in the tumor-microenvironment cells and can be combined with the delivery of other molecules for a combination therapy strategy. Furthermore, these gold nanobeacons can also be embedded in other matrices, such as a hydrogel, allowing different types of administrations and a controlled release of the therapeutics. Figure 9 shows an example of the outcomes that can be obtained in the *in vivo* experiments using the system described in the protocol, or a similar one. It can be observed that its targeted effect decreases tumor progression, that it can be combined with other types of drugs such as chemotherapeutic agents obtain a synergistic effect and overcome the multidrug resistance often found in cancer patients, with the possibility to be administered through different routes, and to use matrices such as a hydrogel to improve a controlled release in the tumors. We also see how it affects the expression of its main target, in this case *MRP1*, and what happens to downstream targets such as VEGF and EGFR, which are involved in tumor progression by contributing to angiogenesis, shown not only through gene analysis but also in tissue expression.[1]

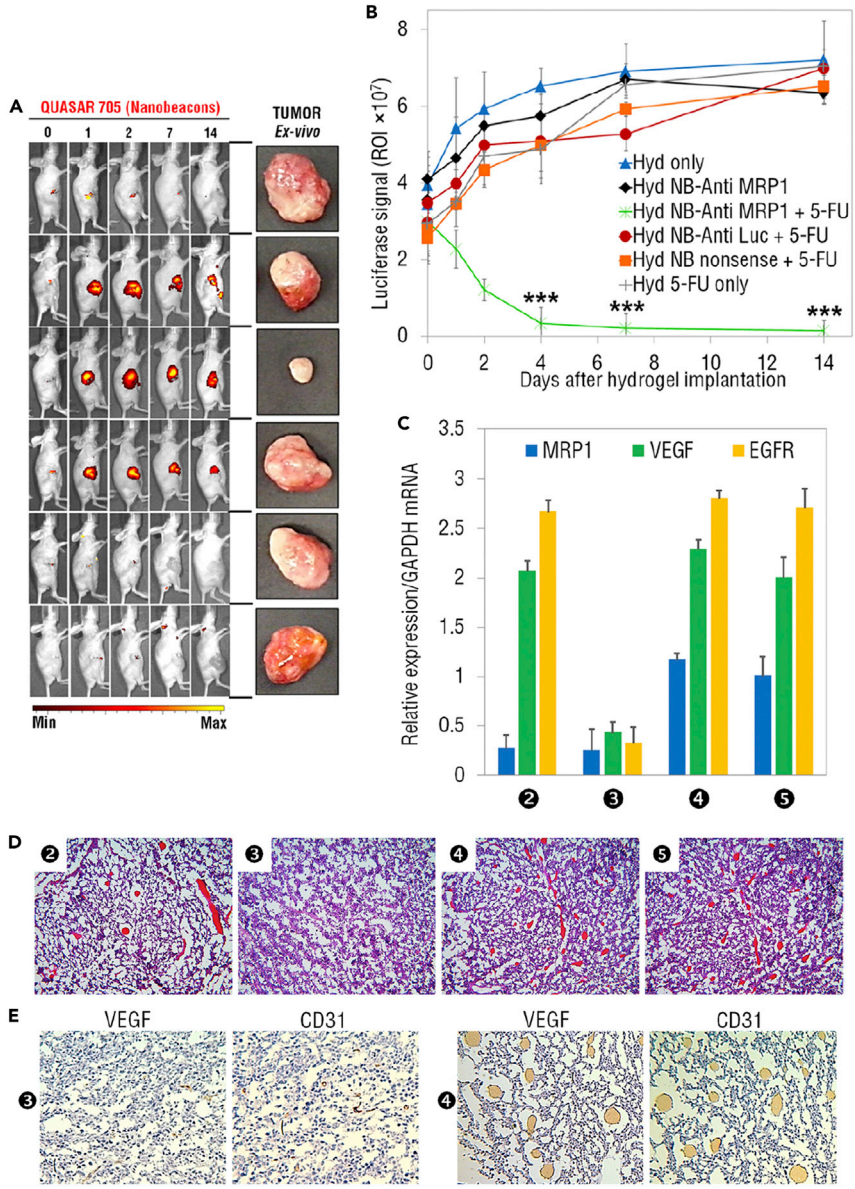

Figure 9. Example of the expected results regarding *in vivo* experiments using a system similar to the one described in this protocol

(A) *Ex vivo* images of breast tumors for each group are also represented.

(B) Evaluation of change in tumor size as a function of time after treatment with nanobeacons (n = 5, ***p < 0.005).

(C) Evaluation of *MRP1*, VEGF, and EGFR expression via real-time PCR of tumors after treatment with nanobeacon anti-*MRP1* (❷), nanobeacon anti-*MRP1* with 5-FU (❸), nanobeacon anti-Luc with 5-FU (❹), and nanobeacon nonsense with 5-FU (❺), using GAPDH as the reference gene. Error bars indicate ± s.d. from at least 3 independent experiments.

(D) H&E stains of treated groups in hydrogels embedded with different nanobeacons, in mice bearing xenografts tumors.

(E) Immunohistochemical evaluation of tumors treated with hydrogels embedded with nanobeacon anti-*MRP1* with 5-FU (❸) and with nanobeacon anti-Luc with 5-FU (❹) for VEGF and vessel density (CD31). Adapted and reproduced with permission (Conde, PNAS, 2015).[1]

LIMITATIONS

The design and construction of this system has limitations, as a full characterization is necessary every time the molecular beacon sequence and fluorophore are altered. When aiming for gene

silencing, it is essential to select the proper target sequence for the molecular beacon, as not all sequences complementary to a certain target miRNA or mRNA are effective. To do this, computational tools can be used based on experimental data (e.g., http://sirna.wi.mit.edu/) or freely available web-based search tool for sequence design. Another limitation is the narrow choice of fluorophores, as this system is based in the properties of gold nanoparticles for the quenching of the fluorophore, so an overlap between their surface plasmon resonance and the emission spectrum of the fluorophore should occur.

TROUBLESHOOTING

Problem 1

Black precipitate forms, solution turns black or aggregation occurs (step 23 of synthesis of gold nanobeacons).

Potential solution

Restart synthesis and decrease ionic strength and/or change the AuNP:oligonucleotide ratio (e.g., 1:150).

Problem 2

Au-nanobeacons do not respond to the presence of a synthetic complementary target (step 31 of characterization of gold nanobeacons).

Potential solution

Discard the Au-nanobeacons and restart synthesis procedure from step 15, change the oligonucleotide sequence (e.g., use a new batch of oligonucleotide) or increase distance to the NP by increasing the number of oligonucleotides in the sequence, taking into consideration that this can affect the quenching effect.

Problem 3

A reversible structure is not observed, Au-nanobeacons do not change conformation (step 32 of characterization of gold nanobeacons).

Potential solution

A problem may have occurred during the synthesis and the Au-nanobeacons should not be used in further experiments. Restart the synthesis, change the oligonucleotide sequence and/or increase distance to the NP.

Problem 4

No silencing effect is detected (step 37f of *in vitro* studies).

Potential solution

Evaluate silencing effect at different time-points (e.g., 24, 48 h, 72 h) and increase Au-nanobeacon concentration.

Problem 5

No *MRP1* signal is detected in cells (step 37g of *in vitro* studies).

Potential solution

Evaluate Au-nanobeacon stability and hybridization with complementary target, increase Au-nanobeacon concentration.

Problem 6

No fluorescence signal is detected (step 37g of *in vitro* studies).

Potential solution

Evaluate silencing effect at different time-points (e.g., 24, 48 h, 72 h) and increase Au-nanobeacon concentration.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, João Conde (joao.conde@nms.unl.pt).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets.

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AUTHOR CONTRIBUTIONS

All authors wrote, reviewed, and approved the final version of the manuscript.

DECLARATION OF INTERESTS

J.C. is a cofounder and shareholder of TargTex S.A. J.C. hold a patent on Theranostic Nanoprobes for Overcoming Cancer Multidrug Resistance and Methods US20160243254A1.

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