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Peptide multifunctionalized gold nanorods decrease toxicity of β-amyloid peptide in a *Caenorhabditis elegans* model of Alzheimer's disease

Francisco Morales-Zavala, PhD[a,b], Hector Arriagada, Biochemist[c], Natalia Hassan, PhD[a,d], Carolina Velasco, Pharmaceutic[a,b], Ana Riveros, PhD[a,b], Alejandra R. Álvarez, PhD[e,f], Alicia N. Minniti, PhD[e], Ximena Rojas-Silva, Chemist[g], Luis L. Muñoz, Chemist[g], Rodrigo Vasquez, Chemist[a,b,h], Katherine Rodriguez, Chemist[h], Macarena Sanchez-Navarro, PhD[i], Ernest Giralt, PhD[i], Eyleen Araya, PhD[h], Rebeca Aldunate, PhD[c,*], Marcelo J. Kogan, PhD[a,b,*]

[a]*Departamento de Química Farmacológica y Toxicológica, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago*
[b]*Advanced Center for Chronic Diseases (ACCDiS), Sergio Livingstone 1007, Independencia, Santiago, Chile*
[c]*Escuela de Biotecnología, Facultad de Ciencias, Universidad Santo Tomás, Ejercito 146, Santiago, Chile*
[d]*Programa Institucional de Fomento a la I+D+I, Universidad Tecnológica Metropolitana, Edificio de Ciencia y Tecnologia, Ignacio Valdivieso 2409, San Joaquin*
[e]*Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Alameda 340, Santiago 8331010, Chile*
[f]*Centro de envejecimiento y regeneración (CARE), Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile*
[g]*Sección de Metrología Química, Comisión Chilena de Energía Nuclear, Nueva Bilbao 12501, La Reina, Santiago, Chile*
[h]*Departamento de Ciencias Quimicas, Facultad de Ciencias Exactas, Universidad Andres Bello, Av. Republica 275, Santiago, Chile*
[i]*Institute for Research in Biomedicine-Barcelona Institute of Science and Technology, Baldiri Reixac 10, 08028 Barcelona, Spain*

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Abstract

The properties of nanometric materials make nanotechnology a promising platform for tackling problems of contemporary medicine. In this work, gold nanorods were synthetized and stabilized with polyethylene glycols and modified with two kinds of peptides. The D1 peptide that recognizes toxic aggregates of Aβ, a peptide involved in Alzheimer's disease (AD); and the Angiopep 2 that can be used to deliver nanorods to the mammalian central nervous system. The nanoconjugates were characterized using absorption spectrophotometry, dynamic light scattering, and transmission electron microscopy, among other techniques. We determined that the nanoconjugate does not affect neuronal viability; it penetrates the cells, and decreases aggregation of Aβ peptide *in vitro*. We also showed that when we apply our nanosystem to a *Caenorhabditis elegans* AD model, the toxicity of aggregated Aβ peptide is decreased. This work may contribute to the development of therapies for AD based on metallic nanoparticles.

Key words: Gold-nanoparticle; Gold-nanorods; Amyloid-beta-peptide; Alzheimer's nanotherapy; Drug-delivery

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the loss of cognitive capacity and memory, impeding the normal development of daily activities. This disease has a significant impact in adults over 65. According to AD International, 46.7 million people suffer from some form of dementia worldwide, and it is projected to reach 131.5 million by 2050.[1] The accumulation of toxic aggregates of amyloid β peptide (Aβ) in the brain (amyloid hypothesis) is one of the most

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*Corresponding authors.
E-mail addresses: pancho.morales@gmail.com (F. Morales-Zavala), hector.arriagada@usach.cl (H. Arriagada), nhassan@utem.cl (N. Hassan), carolinavelasco@ug.uchile.cl (C. Velasco), riveros.ana@gmail.com (A. Riveros), aalvarez@bio.puc.cl (A.R. Álvarez), aminniti@bio.puc.cl (A.N. Minniti), xrojas@cchen.cl (X. Rojas-Silva), lmunoz@cchen.cl (L.L. Muñoz), rvasquez1982@gmail.com (R. Vasquez), katherinerd24@gmail.com (K. Rodriguez), macarena.sanchez@irbbarcelona.org (M. Sanchez-Navarro), ernest.giralt@irbbarcelona.org (E. Giralt), eyleen.araya@unab.cl (E. Araya), rebecaaldunatem@gmail.com (R. Aldunate), mkogan@ciq.uchile.cl (M.J. Kogan).

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important factors in the neuropathology of this disease.[2] This peptide aggregates, generating a range of oligomeric species, fibers and amyloid plaques, which produce oxidative stress and neurotoxicity.[3–5]

Gold nanoparticles (GNPs) are nanomaterials that can be used in medicine because they have a Localized Surface Plasmon (LSP).[6] Gold nanorods (GNRs) have the LSP band in the infrared region, also called the biological window,[7] which is especially relevant for biological systems. This attribute makes them good candidates for developing hyperthermia therapies and drug delivery. Their easy synthesis and photothermal effect can be used to release its load at a distance.[7,8] Moreover, GNPs can be functionalized in very versatile ways with thiols, dithiols, amines, carboxylates, peptides and antibodies to reach different selective targets.

It has been described that naked gold nanospheres (GNSs) affect the aggregation of Aβ, particularly when they are negatively charged.[9] In our laboratory, we have been studying the use of GNRs and GNSs functionalized with the CLPFFD peptide (GNSs-CLPFFD/GNRs-CLPFFD) in the disassembly of Aβ aggregates *in vitro* and also in the reduction of their toxicity.[7,10,11] However, we also demonstrated that only a small percentage of the injected nanoparticles reaches the rat brain.[8] The low bioavailability of nanoparticles in the central nervous system (CNS) is mainly due to the presence of the highly impermeable blood brain barrier. Also, the contact of GNPs with plasma proteins promotes their capture by the reticuloendothelial system (RES) and leads to their accumulation in liver and spleen contributing to lowering availability to the CNS. The low bioavailability of nanoconjugates in the brain has made it very difficult to assess their effectiveness in AD. One strategy to reduce GNPs capping with plasma proteins is to coat them with biocompatible polymers such as polyethylene glycol (PEG), which is hydrophilic and reduces the interaction with plasma proteins. Furthermore, the PEG coating changes the GNPs surface charge, decreasing zeta potential (pZ) to near neutral levels, which also reduces toxicity and increases the colloidal stability due to steric effects.[12]

We have designed a new nanoconjugate using GNRs functionalized with two kinds of peptides in order to function as a drug delivery device for therapeutic peptides. We used the peptides Angiopep 2 (Ang2) and D1. Ang2 (TFFYGGSRGKRNNFKTEEY) is a peptide designed from the Kunitz domain that binds the LRP1 receptor in the Blood Brain Barrier (BBB), therefore improving the delivery of the nanoconjugate to the CNS.[13,14] The D1 peptide (qshyrhispaqv) was selected from a phage library and is made of amino acids of the D series, with a Kd in the submicromolar range for aggregated Aβ peptide, an affinity approximately a thousand times higher than the previously used CLPFFD peptide.[15] This peptide works as a β sheet breaker and was discovered by mirror image phage display methodology.[15] This peptide has been tested before and is a promising candidate for AD therapy.[16,17] GNRs were modified with the peptides Ang2 and D1 (GNR-D1/Ang2).

In order to evaluate the effectiveness of these nanoconjugates *in vivo* we used tr*ansgenic C. elegans* that express the human $A\beta_{1\text{-}42}$ peptide in muscle cells.[18–21] This model offers the possibility to easily evaluate our nanoconjugates *in vivo* in a large number of animals. Although *C. elegans* present barriers for the entry of some molecules, they are not as strong as in rats or mice, which makes it a good model to evaluate the effects of drugs.[22] Moreover, this is an ideal model for testing experimental drugs before moving into vertebrate animal models.

C. elegans has been used successfully to study many aspects of amyloidogenic diseases such as AD and Sporadic Inclusion Body Myositis (IBM).[20,21,23–26,37] The accumulation and aggregation of Aβ peptide have been proposed to have a crucial role in the development of IBM, similar to what occurs in AD. Muscle expression of Aβ causes locomotor impairments in *C. elegans.*[23,25–27] These phenotypic alterations can be efficiently analyzed and quantified, and can also be correlated with the time course of Aβ aggregation.[25,27]

C. elegans offers many advantages for *in vivo* assays (large number of animals can be tested in short periods of time; they have simple anatomy, can be genetically manipulated; there are mutant and transgenic strains available, *etc.*); however it has only started to be used in the nanoparticle field.[28–30] To the best of our knowledge, this is the first work where GNRs are tested in *C. elegans* in the field of Alzheimer's disease.

In this paper we show the development of a drug delivery nanosystem based on gold nanorods functionalized with PEG and peptides for the treatment of AD, with possible applications in photothermal therapy. In this study we demonstrate that the GNRs-D1/Ang2 do not affect neuron viability and that they prevent Aβ peptide aggregation *in vitro*. Furthermore we show for first time the potential use of peptide functionalized gold nanoparticles for decreasing the toxic effects of Aβ peptide in an animal model of amyloidogenic diseases.

Methods

GNRs-CTAB synthesis

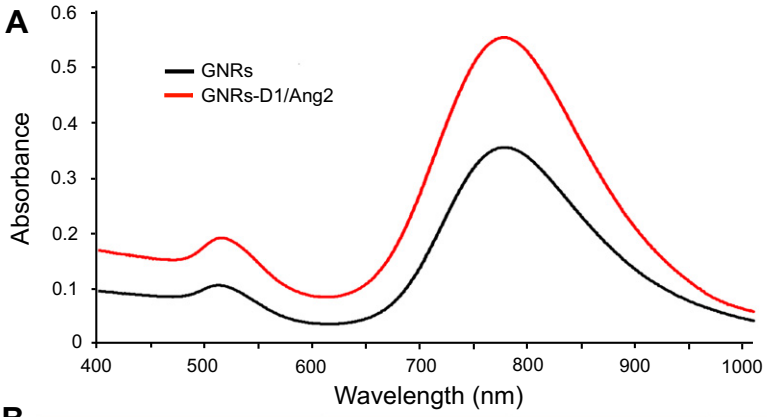
GNRs-CTAB were synthesized using the seed-mediated approach.[7,31,32] For a complete description see the Supplementary Methods section.

GNRs PEGylation and ligand conjugation

PEGylation was performed as described by Huang et al.[33] For a complete description see Supplementary Methods.

In vitro GNRs-D1/Ang2 amyloid fibril assay

$A\beta_{1\text{-}42}$ was purchased from r-Peptide (GA 30622, USA). Aβ was dissolved in water, aliquoted, lyophilized and stored in glass vials at −20 °C until used. To obtain mature Aβ fibrils, the aliquots were treated with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) for 30 min to obtain the monomeric Aβ form. Aliquots were then lyophilized and resuspended in GNRs-D1/Ang2 solution (0.5 nM approximately). The final Aβ concentration was 20 μM. The samples were incubated for 2 days at 37 °C with mechanical shaking.



B

Surface modification	Dh transversal (nm)	Dh longitudinal (nm)	pZ (mV)
CTAB	3±0.03	62±1	53±7
Peptides D1/Ang2	11±0.4	97±5	-10±2

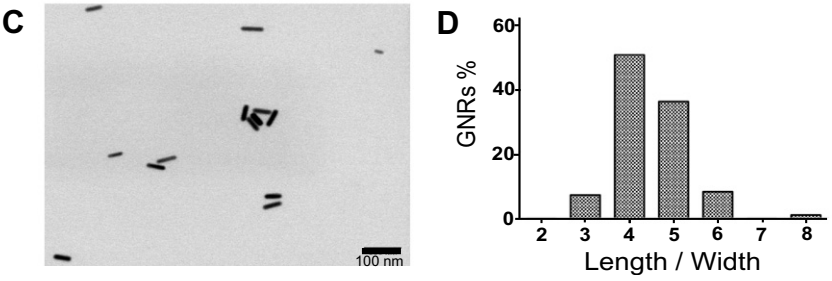


Figure 1. GNRs and GNRs-D1/Ang2 characterization. (**A**) UV–Vis–NIR spectra of GNRs, (**B**) Size Distribution of GNRs obtained by Dynamic Light Scattering (**C**) Scanning Electron Micrograph of GNRs and (**D**) Aspect ratio length/width distribution obtained from 100 particles.

Nematode strains and culture

Nematodes were cultured at 20 °C under standard laboratory conditions on agar plates seeded with *Escherichia coli* (OP50).[34,35] Worm strain CL2120 was maintained at 20 °C. For a description of strains used see Supplementary Methods. Nematode strains used in this work were obtained from the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

Treatment of C. elegans with GNRs-D1/Ang2

Synchronized nematodes were cultured at 20 °C on regular nematode growth medium (NGM). For GNRs-D1/Ang2 treatment the worms were washed from the plates with M9 buffer,[18,26,36] and precipitated by gentle centrifugation. The worms were incubated in 1.5 nM GNRs-D1/Ang2 in M9 buffer for 4 h. For complete details see Supplementary Methods.

Gold quantification in C. elegans treated with GNRs-D1/Ang2

The nematodes were lyophilized and gold content was determined by neutron activation analysis (NAA) to establish gold concentration. The control group was also measured using the same protocol.

Motility assays in liquid medium

Individual 3 and 5-day-old adult animals (between 15 and 25 animals per experiment) were placed on a 30 µl drop of M9 buffer. After a 2 min recovery period each individual worm was recorded for 1.0 min and the turns per minute counted using the WormLab software MBF Bioscience. WormLab is a system for imaging, tracking, and analyzing *C. elegans* and other worms.

Thioflavine-S (ThS) staining

ThS staining was performed as described previously.[27,36,38] For details see Supplementary Methods.

Image fluorescence analyses

Digital quantification of areas positive for ThS fluorescence was done using the ImageJ software. For microscopy details and quantification of Aβ deposits see Supplementary Methods.

Results

Synthesis and characterization of GNRs-D1/Ang2

GNRs-D1/Ang2 were synthetized by the seed-mediated growth method,[32] as we describe in the Methods section. All GNRs

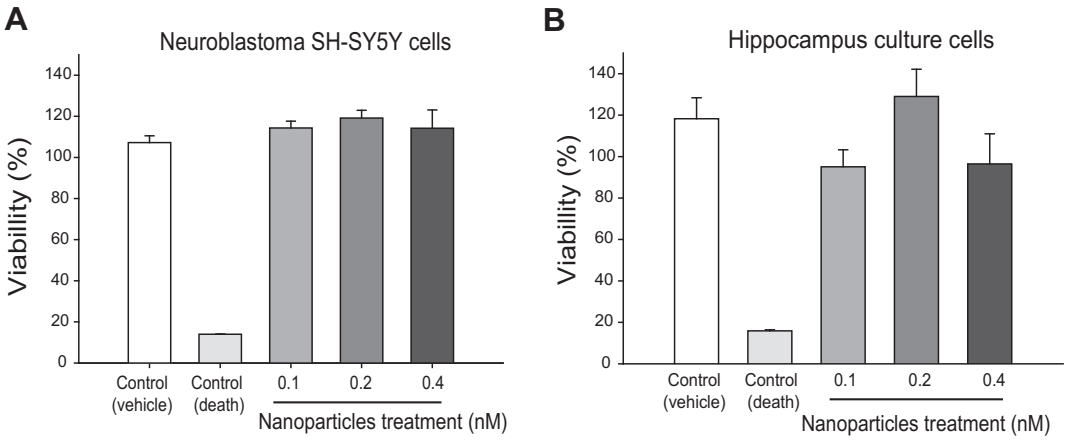


Figure 2. Cell viability assay. Neuronal hippocampal primary culture (left) and SH-SY5Y cell line (right) incubated with GNRs-D1/Ang2 for 24 h at 37 °C. Control medium corresponds to the cells in the culture medium without GNRs-D1/Ang2; the death control corresponds to cells incubated with 23% SDS. The assays were performed 3 times in quintuplicate.

exhibited the characteristic two plasmonic bands of light absorption at 520 nm (longitudinal) and 770 nm (transversal) (Figure 1, *A*).[39] As expected, the hydrodynamic diameter (Dh) and zeta potential (pZ) change from the naked GNRs to the functionalized GNRs. For the functionalized GNRs there is a 20 nm increment in the hydrodynamic diameter and the pZ changes from a high positive value (+53 mV) to a negative value (−10 mV) (Figure 1, *B*). Rod shape and size distribution was confirmed by STEM (Figure 1, *C*); with an average aspect ratio (length/width) of 4 (Figure 1, *D*). On the other hand, to study the stability of GNRs-D1/Ang2 we incubated the nanoparticles with cell culture medium (10% fetal bovine serum albumin) and we observed no significant changes in the plasmonic band. This result indicates that the nanosytstem does not aggregate (supplementary Methods and Supplementary Figure S1).

The number of peptide/nanoparticles was determined by amino acid analysis and quantification of gold in the GNRs samples as described in the Supplementary Methods section. The ratio of peptides/nanoparticle for GNR-D1/Ang2 was 439 ± 23 for D1 and 173 ± 36 for Ang2.

Cell viability and cellular internalization

Considering the potential use of the GNRs-D1/Ang2 for the treatment of neurodegenerative disorders we used primary hippocampal neurons[40] and SH-SY5Y cells to test GNRs-Ang2/D1 cytotoxicity. After a 24-h incubation with the GNRs-Ang2/D1, we did not observe a decrease in cell viability using the MTS test (Figure 2). Additionally, in order to establish whether GNRs-D1/Ang2 were incorporated into both cellular models, we measured the gold content in the cells using neutron activation analysis (NAA). GNRs-D1/Ang2 were internalized in both cell types; however, in primary hippocampal neurons the incorporation was lower (approximately 0.07 μg after a 24 h incubation) than in the neuroblastoma SH-SY5Y cell line (approximately 0.6 μg after a 24 h incubation) (Figure 3, *A* and *B*).

On the other hand, the cell penetration capacity of GNRs (GNRs-D1/Ang2, GNRs-D1, GNRs-Ang2 and GNRs-PEG labeled with Alexa-647) was evaluated using a monolayer of the immortalized mouse brain endothelial cell line bEnd.3. These cells were incubated with the nanosystems (0.05 nM) for 2 h. After this incubation time, the cellular incorporation was assessed by flow cytometry. After a 2-h incubation, we observed differences in the fluorescence-positive populations for each modified GNR (Figure 3, *C*). The cells treated with GNRs-D1-Alexa Fluor 647 showed the lowest fluorescence. The Ang2 and D1/Ang2 modifications show the highest fluorescence with very similar percentages of positive cells. This result indicates that Ang2 improves GNRs-D1 cell penetration (Figure 3, *C*) and that PEGs do not influence nanorod incorporation into the cell.

GNR-D1/Ang2 effects on Aβ fibers growth in vitro

In order to evaluate the inhibition of Aβ fibers growth *in vitro* we incubated 20 μM Aβ peptide with GNRs-D1/Ang2 for 48 h at 37 °C under mechanical stirring. The formation of fibrils was measured using the Thioflavine-T fluorescence assay (ThT). In this assay, the amount of fibrils in suspension can be quantified by measuring the intensity of the fluorescence signal, which is proportional to the amount and/or length of the fibrils being formed.[17] We incubated the Aβ peptide (20 μM) with different concentrations of GNRs-D1/Ang2 (0.2, 0.5 and 1 nM) and with different concentrations of D1 peptide equivalents of D1 peptide in GNRs-D1/Ang2 (*e.g.* 0.2 μM is equivalent to the concentration of D1 peptide present on 0.5 nm GNRs-D1/Ang2). Figure 4, *A* shows the percentage of fluorescence intensity of the samples treated with GNRs-D1/Ang2 and an equivalent concentration of D1. We observed an inhibition of about 40% in fibril formation after treatment with GNR-D1/Ang2 0.5 nM and also at the other assayed concentrations (Figure 4, *B*). The samples treated with D1 peptide (0.2 μM) (Figure 4, *C*) do not show statistically significant differences with the Aβ control; only a trend that may indicate increased aggregation. However, an inhibitory effect on Aβ aggregation is observed at 1 μM D1 (Figure 4, *C*).

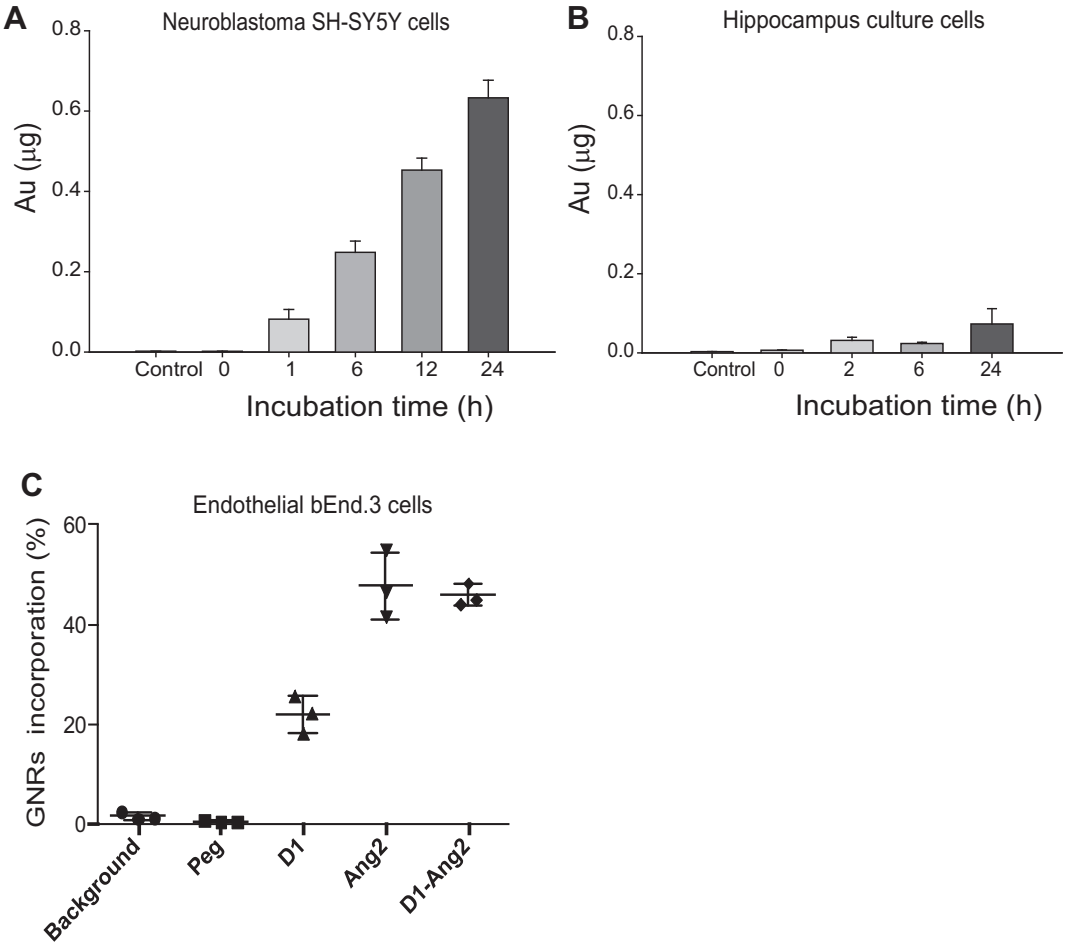


Figure 3. Gold content quantification. Gold content of SH-SY5Ycells (**A**), primary hippocampal neurons (**B**), incubated with GNRs-D1/Ang2 0.4 nM, for different times at 37 °C. We used 60,000 cells per well and the gold content was determined by NAA in CCHEN. The experiments were performed three times in triplicate. (**C**) Percentage of the cell population positive for the incorporation of modified GNRs, evaluated by flow cytometry. BEnd.3 cells were incubated for 2 h with the different modified GNRs, at a concentration of 0.05 nM. The GNRs were marked with Alexa 647 for detection. The assay was performed in triplicate.

Effect of GNRs-D1/Ang2 on a C. elegans model of amyloidogenic diseases

In order to evaluate the effects of GNRs-D1/Ang2 *in vivo* we used a transgenic strain of *C. elegans* that expresses the human amyloid-β peptide in muscle cells. This model has been used to study the time-course of Aβ aggregation *in vivo*[27,39] and how the aggregation is modulated by physiological factors and by exposure to different elements such as drugs and natural agents.[41,42]

First, we evaluated the toxicity of GNRs-D1/Ang2 in this animal model. We treated adult *C. elegans* for 5 days (from the L4 stage to day 5 of adulthood) with GNRs-D1/Ang2 as described in the Methods section. During this period we did not observe any signs of toxicity. There was no lethality of the treated adult worms or the progeny they produced during this period.

Subsequently, we tested if the GNRs-D1/Ang2 were incorporated into the worms' tissues. For this we evaluated the gold content in the treated worms and untreated controls, as described in the Methods section. Gold was only detected in worm samples that had been treated with GNRs-D1/Ang2 (1.6 ± 0.8 μg Au/mg CL2120), confirming that the GNRs-D1/Ang2 are able to access the animal's tissues.

In this animal model, the Aβ peptide aggregates in muscle cells during the worms' lifetime. Aβ expression also causes motor impairments that can be quantified as the reduction in the number of turns per minute the worms make during swimming. We evaluated these two parameters: number and size of Aβ aggregates and motor capacity in worms that had been treated with GNRs and with the D1 peptide as control. We used the D1 control at 0.5 μM, equivalent to the amount of the D1 peptide present on 1.5 nM GNR-D1/Ang2 (0.6 μM of D1).

GNRs-D1/Ang2 affects Aβ aggregation in vivo

Figure 5, *A* (left panels) shows 3-day-old control worms and worms treated with GNRs-D1/Ang2 or D1 peptide stained with ThS (Thioflavine S) to visualize Aβ aggregates. Panels on the right show the corresponding image analyses of the aggregates using the ImageJ software as described in the Methods section. Figure 5, *B*, *C* and *D* shows the quantification of the Aβ

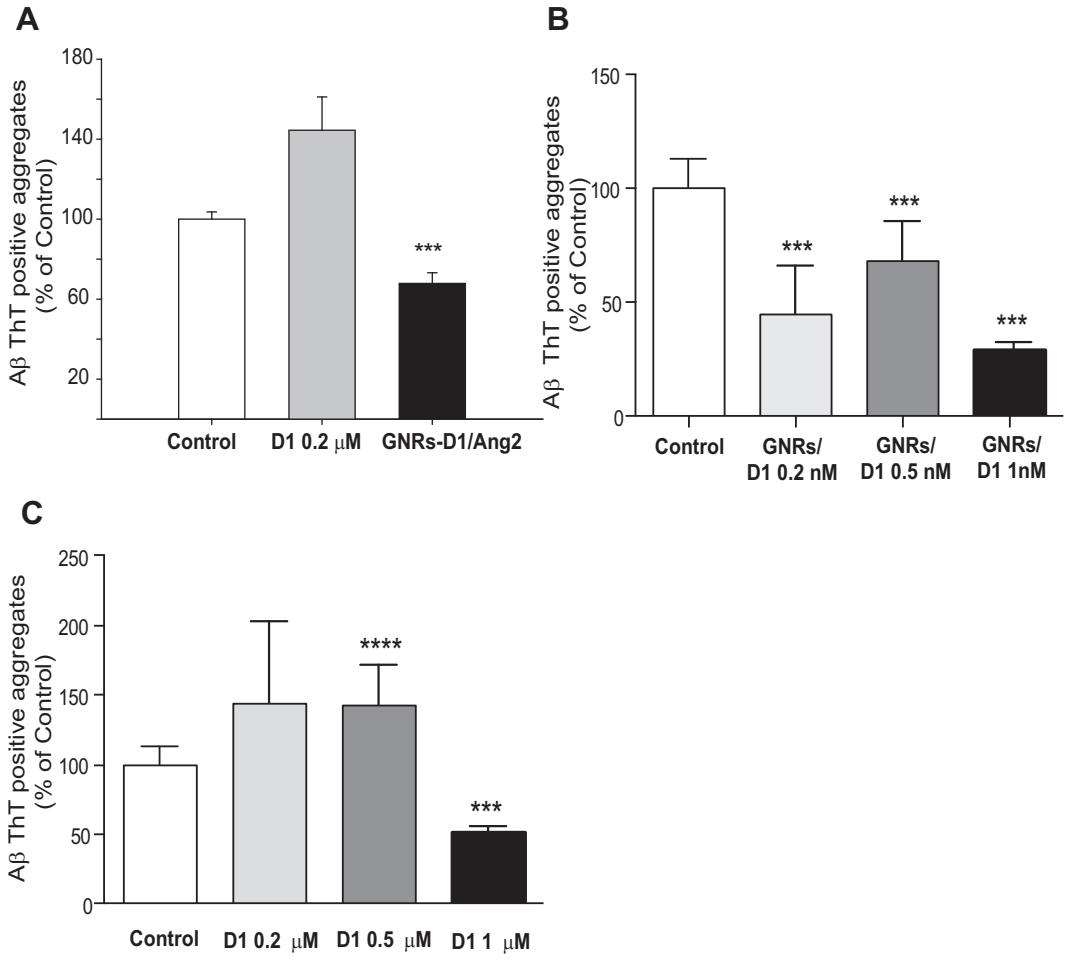


Figure 4. Thioflavine-T *in vitro* assay. (**A**) Fluorescence intensity of Aβ fibrils in the presence of 0.5 nM GNRs-D1/Ang2 and D1 peptide 0.2 μM. The results are expressed as percentage with respect to the intensity of free Aβ peptide. Note: taking into account the number of peptide molecules of D1 per nanoparticle the equivalent concentration of D1 in the sample of GNRs-D1/Ang2 is 0.2 μM. (**B**) Thioflavine-T *in vitro* assay. Fluorescence intensity of Aβ fibrils in the presence of 0.2, 0.5 and 1 nM GNRs-D1/Ang2. The results are expressed as percentage with respect to the intensity of free Aβ peptide. (**C**) Thioflavine-T *in vitro* assay. Fluorescence intensity of Aβ fibrils in the presence of D1 peptide 0.2, 0.5 and 1 μM. The results are expressed as percentage with respect to the intensity of free Aβ peptide. Student's *t* test was used for statistical analysis. ***$P \leq 0.001$.

aggregates: number of aggregates, mean aggregate size, and the percent of ThS positive area normalized with respect to the total area of the worm's head. These quantifications show that the treatment with GNRs-D1/Ang2 induces a trend towards a decrease in the number of Aβ aggregates in young 3-day-old worms (Figure 5, *B*). These aggregates are nevertheless bigger (Figure 5, *C*). However, when we normalized the total area of aggregates with respect to the total area of the head we observed that GNRs-D1/Ang2 decrease the formation of aggregates significantly (Figure 5, *D*). This effect is only observed in young worms, because by day 5 of adulthood no significant differences can be detected in number, size or normalized area of Aβ aggregates between treated and untreated worms. Interestingly, treatment with 1.0 μM D1 peptide is sufficient to decrease the number of aggregates and their total area with respect to the head area (Figure 5, *B*, *C* and *D*). Similarly to the effect seen with GNRs-D1/Ang2, D1 peptide shows an effect only in 3-day-old worms (3-day treatment from the L4 stage).

GNRs-D1/Ang2 improves motility in C. elegans Aβ transgenics

C. elegans Aβ transgenics show motility impairments that start at day 1 of adulthood and become more pronounced as worms grow older[25,26,42] (Figure 6, *A*). Transgenic Aβ worms that are treated with GNRs-D1/Ang2 show moderate but significant motility improvement by day 5 of adulthood (5 day treatment from L4 stage), while the motor capacity of younger animals remains unchanged (Figure 6, *B*). However, when the worms are treated with the D1 peptide, motor capacity is already improved in 3 day-old adults and the improvement is maintained in older worms (Figure 6, *C* see Supplementary Movies). Treatments with GNRs-D1/Ang2 and D1 significantly improve

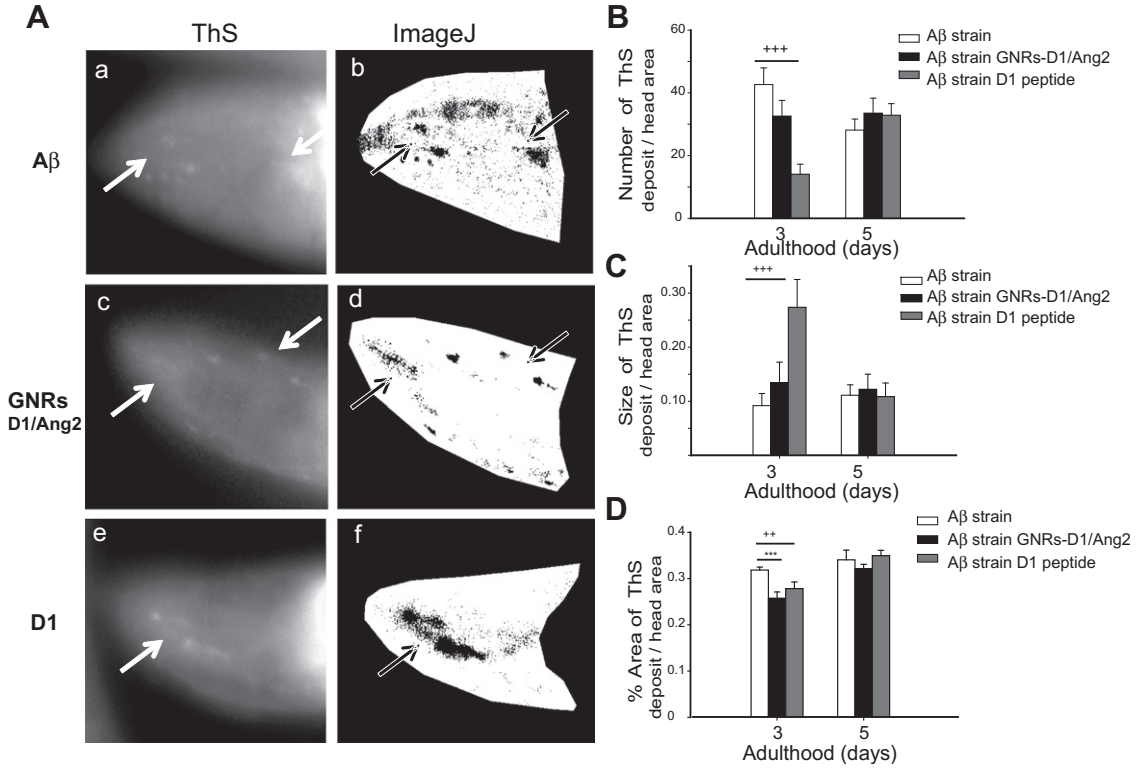


Figure 5. GNRs-D1/Ang2 modulate Aβ aggregation *in vivo*. (**A**) The left panel shows fluorescence images of head of ThS stained 3-day-old Aβ worms (400×). The images show amyloid aggregates in the head muscles. The right panel shows the corresponding ImageJ analyses of Aβ aggregates (arrows). **a** and **b** are representative of an untreated worm, **c** and **d** show a worm treated with 1.5 nM GNR-D1/Ang2, and **e** and **f** show a worm treated with 1 μM D1 peptide. (**B**) The graph shows the quantification of the total number of ThS positive amyloid aggregates in the head area. Data are means ± SEM from n ≥ 30 worms per treatment. (**C**) Quantification of the size of amyloid deposits in the head area. Data are means ± SEM from n ≥ 30 worms per treatment. (**D**) The graph shows the percentage of head area covered by ThS positive aggregates. Data are means ± SEM from n ≥ 30 worms per treatment. Student's *t* test was used for all statistical analysis. $^{+++}P \leq 0.001$, $^{++}P \leq 0.01$. The graphs are the result from at least three independent experiments.

overall motility in Aβ transgenic worms but do not recover wild type motor capacity completely at the assayed dosages (Figure 6, *D*).

Discussion

In this work we developed a new gold nanorod based nanosystem for the delivery of therapeutic peptides for the treatment of Alzheimer's Disease We tested the anti-amyloidogenic capacity of the nanoconjugate in a *C. elegans* AD model. Treatment with our nanosystem caused behavioral improvement in the animals. This is the first time that therapeutic gold nanoparticles are shown to improve Aβ-related conditions in an *in vivo* model.

GNRs were synthesized using the seed-mediated growth method described by Nikoobakht et al. Subsequently; two types of polyethylene glycols (PEGs, O-methylated and carboxylated) were chemisorbed on the surface of GNRs to activate the carboxylic groups for the binding of D1 and Ang2 peptides *via* their free amino groups. The O-methylated PEGs contribute to the colloidal stabilization of GNRs while the PEG-COOH allows the conjugation with the peptides Ang2 and D1. The GNRs were characterized at different stages of modification using several techniques: molecular absorption spectrophotometry, dynamic light scattering (DLS), Zeta potential and STEM. The size, shape, pZ and maximum spectra of molecular absorption spectrophotometry of the GNRs obtained are consistent with previous work from our research group.[7]

After the chemisorption of both types of PEGs on the surface of the GNRs we observed a pZ variation from a positive to a negative absolute value. This variation agrees with what has been reported in the literature.[43] This result could be explained by a replacement of CTAB surfactant that has a positive charge because of the presence of HS-PEG-COOH (negatively charged at pH = 7).

Subsequently, carboxylic acids were activated with the reaction between ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysulfosuccinimide (NHS) for the binding of peptides D1 and Ang2.[33] The characterization of DLS and pZ shows an increase in the hydrodynamic diameter (Dh) associated with the addition of molecules on the GNRs surface; and an increase in the surface charge, which is in agreement with the charges of both peptides (at pH 7 is +1.2 for D1 and +2 for Ang2). The presence of the peptides on the surface of GNRs-Ang2/D1 was confirmed by amino acid analyses.

On the other hand, the MTS assays in the cellular models showed no effects on neuronal viability. Therefore, the CTAB

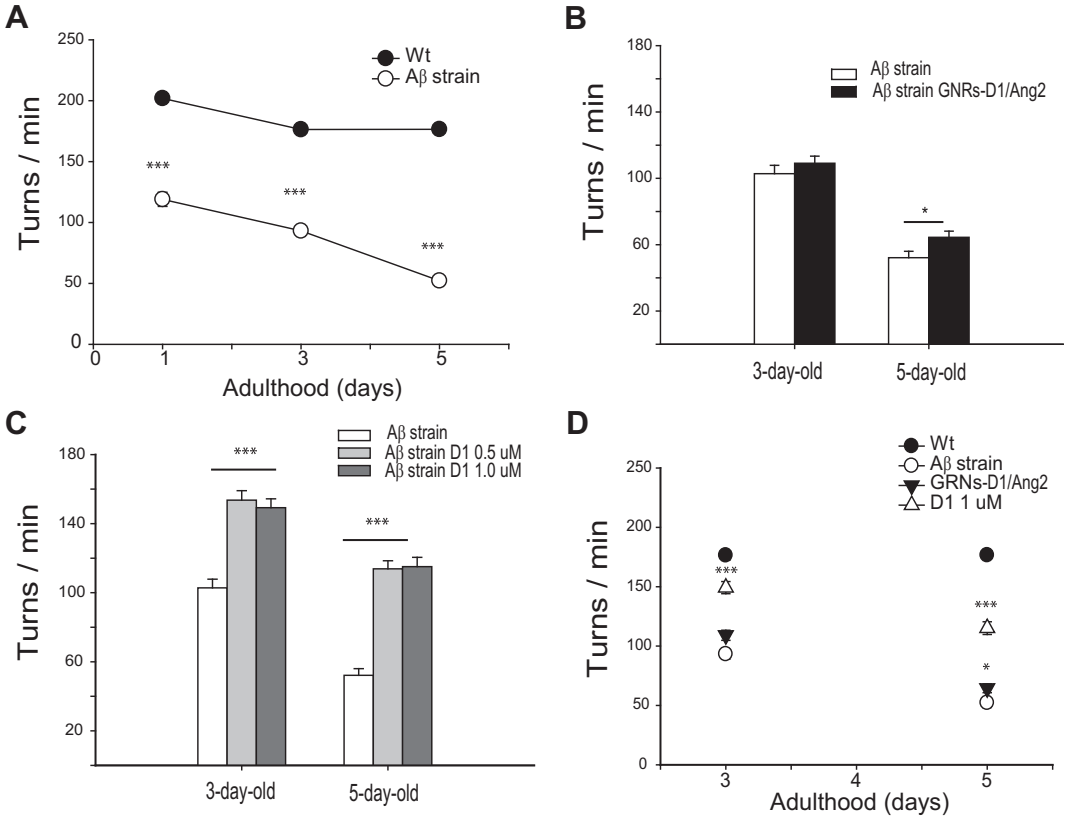


Figure 6. GNRs-D1/Ang2 improve motility in Aβ transgenic worms. (**A**) Quantification of movement in liquid medium (turns per minute) of wild type and Aβ transgenic worms from day 1 to day 5 of adulthood. During this life stage Aβ worms show strong motility impairments. The graph is the result from at least five independent experiments. Data are means ± SEM from n ≥ 60 worms per age. Differences are statistically significant: ***$P \leq 0.001$. (**B**) Aβ transgenic worms (strain CL2120) were treated with 1.5 nM GNR-D1/Ang2 from day 1 of adulthood. Measurements were performed at days 3 and 5. The graph shows that 1.5 nM GNR-D1/Ang2 treatments improve locomotor capacity in 5-day-old worms. The graph is the result from at least three independent experiments. Data are means ± SEM from n ≥ 30 worms per age. Differences are statistically significant: *$P \leq 0.05$. (**C**) Aβ transgenic worms (strain CL2120) were treated with 2 concentrations of D1 peptide (0.5 μM and 1 μM) from day 1 of adulthood. Measurements were performed at days 3 and 5. The graph shows that D1 treatment improves locomotor capacity in 3 and 5 day old worms. The graph is the result from at least three independent experiments. Data are means ± SEM from n ≥ 30 worms per age. Differences are statistically significant: ***$P \leq 0.001$. (**D**) The graph compares motor capacity in wild type, Aβ transgenic worms and Aβ transgenic worms treated with 1.5 nM GNR-D1/Ang2 and D1 peptide.

had probably been largely displaced since it was reported that cell toxicity is associated with its presence on the surface of GNRs. A possible explanation for the decrease in toxicity is that CTAB molecules were replaced by thiolated PEGs, as has been described previously for other functionalized gold nanorods.[7] These results are very relevant considering the possible future applications of this type of nanoparticles in the treatment and diagnostic of neurodegerative diseases and other disorders.

Regarding GNRs-D1/Ang2 internalization, the difference between primary culture cell and the neuroblastoma cell line is remarkable. In the case of SH-SY5Y neuroblastoma cell line, although GNRs-D1/Ang2 were effectively internalized into the cells, we did not observe any deleterious effects on cell viability. However, the results obtained in cultured hippocampal neurons are more relevant. Considering Aβ as a putative extracellular target[44] for AD treatment, the low internalization of GNRs-Ang2/D1 into neurons is a desirable result since this situation would favor a higher local concentration of the peptide functionalized nanoparticles extracellularly where the amyloid plaques form. Moreover, the presence of the nanoparticles in the extracellular media could favor their elimination from the central nervous system by the glymphatic system.[45] On the other hand, in endothelial cells bEnd.3 we demonstrated that the presence of Ang2 improves endothelial cell penetration of GNRs, in agreement with the use of gold nanospheres functionalized with Ang2 for a controlled release of doxorubicine in the central nervous system for treatment of glioma.[46]

We also evaluated the effect of GNRs-D1/Ang2 and the D1 peptide on Aβ fiber formation *in vitro*. An increment of fluorescence intensity can be attributed to the growth of Aβ fibers or to an increase in the number of fibers. For D1 peptide (0.2 μM) we observed an increment in the fluorescence signal that was not statistically significant. However, there is an inhibitory effect on Aβ aggregation with 1 μM D1 in agreement with earlier reports.[17] Furthermore, the incubation of Aβ with a solution of 0.5 nM of GNR-D1/Ang2 (equivalent to 0.2 μM of D1) produced a decrease in Thioflavine signal. This behavior can be attributed to a synergistic nucleation effect between D1 and

the nanoparticle. As is described in the literature, negative charge gold nanoparticles can inhibit the amyloidogenic process.[9]

In order to examine the functionality of GNRs-Ang2/D1 *in vivo* we tested their effect on a transgenic *C. elegans* model of amyloidogenic diseases.

As had been previously reported with gold nanoparticles in *C. elegans,*[30] and similar to what we observed in the cell culture experiments, treatment with GNRs does not cause lethality in this animal model. The GNRs and free D1 peptide are probably taken by the worms through their digestive system (since they have an impermeable cuticle) and are incorporated by the different tissues as measured by gold content in the animals.

Our *C. elegans* model of the amylodogenic disease AD[20,21,24,47] allows us to test the effect of different compounds and other agents on Aβ aggregation and on its toxic effects on the animal's behavior.[24] We can easily visualize Aβ aggregation by staining whole worms with ThS.[25,27,42] The time course of Aβ aggregation correlates with the onset of locomotory impairments in the worms. Our results from the treatment of adult worms with GNRs-D1/Ang2 show that there is a decrease in the number of Aβ aggregates that can be detected during the third day after starting the treatment. At this time (3-day old adult) Aβ reaches its maximum aggregation in our model. After this, the number of aggregates and the total aggregate area remain constant.[25] In our assays the effects of GNRs-D1/Ang2 observed in earlier stages do not last in older worms. It is possible that the capacity of GNRs-D1/Ang2 and D1 for disassembling Aβ aggregates could only be visualized during early stages, before the system reaches its equilibrium between Aβ species. The effect in the number of aggregates observed with GNRs-D1/Ang2 is potentiated when the treatment is performed with the free D1 peptide. This result is probably due to increased bioavailability of D1 that would allow a more efficient interaction with Aβ. It appears that, given the small size and anatomical simplicity of *C. elegans*, the peptide can be easily incorporated into the tissues.

The transgenic AD strain, which expresses the human $A\beta_{1-42}$ peptide from the embryo stage, accumulates aggregates and other Aβ species that cause motility impairments already during the first day of adulthood. Motility normally decreases as the worms age; however, there is no significant variation between day 1 and day 5 of adulthood.[25] In contrast, the Aβ worms show significant and progressive decrease in motor capacity as Aβ accumulates in the form of aggregates and other toxic species (Figure 5, *A*). Even though our experimental strategy of treatment with GNRs-D1/Ang2 allowed us to detect a decrease of Aβ aggregates in 3-day-old adults (third day of treatment), the improvement in motility capacity is detected in day 5. This may be related to the availability of D1 peptide (bound to GNRs) for interaction with Aβ, since treatment with free D1 is more efficient in increasing swimming capacity already in 3-day-old adults. This difference in availability of the D1 peptide could be due to its concentration and/or orientation that can be more limited in the case of GNRs-D1/Ang2 binding.

In this paper we present a first approach to test the effect of gold nanorods *in vivo* using a *C. elegans* model of AD. However, further analyses are necessary in order to elucidate the mechanism of interaction between GNRs-D1/Ang2 and D1 *in vivo* and how they modulate Aβ aggregation. The use of this nanosystem for treatment and diagnosis of AD and other amyloidogenic diseases could improve the stability and the delivery of active drugs through the complex blood brain barrier. Moreover, these gold nanostructures show potential uses in photothermal therapy and detection due to their plasmonic properties.

In summary it is possible to envision that this strategy could offer new alternatives for the treatment of AD and other amyloidogenic neurodegenerative diseases.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2017.06.013.

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