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Angiogenin-mimetic peptide functionalised gold nanoparticles for cancer therapy applications

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ABSTRACT

Gold nanoparticles (AuNPs) have unique anti-angiogenic properties and have been applied in a variety of cancers studies. 'Naked' gold nanoparticles directly bound to angiogenic factors have been demonstrated to inhibit growth factor-mediated signalling in vitro and vascular endothelial growth factor-induced angiogenesis in vivo. Angiogenin (Ang) is a protein physiologically constituent the human plasma but also a pathological marker of different cancer types.

Herein, the peptide $Ang_{60–68}$, encompassing the putative cellular binding site of the protein Ang, has been synthesized and characterized in the interaction with spherical AuNPs of 12 nm of diameter. The Ang mimicking activity of the peptide was assessed in terms of cell cytoskeleton rearrangements visualized by staining of actin, which is an important target of the whole protein. The hybrid assemblies obtained by physical adsorption of the peptide molecules at the surface of the gold nanoparticles were scrutinised by UV–visible spectroscopy, to characterize with titration experiments the changes in the plasmonic properties of AuNPs as well as the peptide spectral features. The latter were obtained by using the fluorescent analogous peptide, Fam-$Ang_{59–68}$, incorporating the carboxyfluorescein (Fam) moiety, through an amidic bond of the N-terminal residue. The hydrodynamic size of the peptide-Au systems were determined by dynamic light scattering (DLS) analyses. Proof-of work experiments with human neuroblastoma cells line demonstrated the non-toxicity of the Ang-mimicking peptide functionalised gold nanoparticles. Moreover, laser scanning confocal microscopy (LSM) experiments showed the localization of the peptide-nanoparticles at the cell membrane and their sub-cellular distribution. These data reveal a promising new platform for imaging and therapeutic activities in cancer therapy.

1. Introduction

Nowadays metal nanoparticles have various applications in medical healthcare owing to their various biological properties such as anti-inflammation, anti-cancer, anti-angiogenesis, and light-triggered nanotheranostic performances [1–3]. In particular, gold nanoparticles (AuNPs), have been demonstrated to inhibit the action of vascular endothelial growth factor (VEGF), one the most important pro-angiogenic factors, in the human umbilical vein endothelial cell migration and tube formation [4,5].

Angiogenin (Ang), is another potent angiogenic factor [6], normally present in the human plasma [7] and strongly overexpressed in various tumours [8–10]. Angiogenin binds with high affinity endothelial cell receptors and, with lower affinity, to extracellular matrix components [11].

A promising strategy to selectively interact with cellular receptors involved in the regulation of angiogenesis, and henceforth the abnormal extra-vascularization in tumours, is the anchoring of biologically active peptides to AuNPs [1,12,13]. In general, the design phase of such peptides is of critical importance and a major approach developed to accomplish this aim is the synthesis of a wide variety of cyclic peptides and/or peptidomimetics [14–16], also in model experiments to test their interaction with the gold surface [17].

In the specific case of angiogenesis, the peptide-functionalised nanoparticles are expected to alter the balance between naturally secreted pro- and antiangiogenic factors, under various biological conditions, without causing toxicity. To this respect, a peptide sequence able to mimic the biological activity of the corresponding whole protein is very useful, in terms of improved pharmacokinetic properties and lower production costs [18].

Considerable evidence suggests that the biological action of angiogenin is mediated through a putative cellular receptor [19–21]. A dual-site model is postulated in which both the catalytic site and the

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cell-binding site are required for the organogenic activity of Ang [22]. The Ang residues from 60 to 68 were identified as a part of a cell-surface receptor binding site, a sequence that is distinct from the catalytic site [22,23]. The interaction of Ang with this putative cell-surface receptor is considered one of the necessary steps in the onset of its angiogenic activity and it is copper dependent [11,24,25]. It is known, in fact, that copper is involved in the regulation of the biological activity of Ang since it modulates and enhances the binding to the endothelial cell [11].

In the present work we designed, synthesized and characterized a hybrid peptide-AuNP assembly by the use of Ang_{60-68}, which encompasses the cellular binding domain of angiogenin [26,27]. To the best of our knowledge, this is the first time that an angiogenin fragment is used for the functionalisation of AuNPs.

To validate the selected peptide sequence in the mimetic role of the whole protein, its effect on the actin organization in human neuroblastoma cells was scrutinised. Indeed, Ang binds to its specific receptor, a 170 kDa cell surface unknown protein, as found on the HUVEC cells membrane [28], but another recognised important target of Ang is actin [29]. Angiogenin binding to actin, followed by dissociation of the Ang-actin complex from the cell surface and subsequent activation of tissue-type plasminogen activator/plasmin, are likely steps involved in the processes of endothelial cell invasion and angiogenesis [30].

In general, among the large number of proteins that can bind to actin and take part in the nucleating, capping, stabilising, severing, bundling, and mechanical movement of actin filaments, Ang has a recognised relevant role as it may cause changes in the cell cytoskeleton, by inhibiting polymerization of G-actin and changing the physical properties of F-actin [31]. Recently, we demonstrated that the Ang-mimetic fragments Ang(1–17) and its acetylated form have different roles in actin organization at the cell membrane of neuroblastoma cells, in the presence or not of copper ions [32].

Here we assess the Ang_{60-68} as first example of angiogenin-mimetic peptide for the functionalisation of gold nanoparticles in potential theranostic applications.

The immobilization by the physical adsorption approach of the Ang_{60-68} peptide and its fluorescent carboxyfluorescein (Fam)-labelled analogous, Fam-Ang_{59-68}, was scrutinised by the changes of the gold nanoparticles plasmonic properties as well as of the hydrodynamic size. Preliminary cellular tests of cell viability and confocal microscopy imaging validated these hybrid peptide-AuNPs as non-toxic nanoplatforms able to be internalized by neuroblastoma cells.

2. Materials and methods

2.1. Chemicals

Gold(III) chloride trihydrate and sodium citrate dihydrate for the Au nanoparticle synthesis were purchased from Sigma-Aldrich. Ultrapure milliQ water was used (18.2 mΩ·cm at 25 °C, Millipore). Glassware was cleaned by soaking in aqua regia (HCl:HNO_3, 1:3 volume ratio) and thorough rinsing with water. Phosphate buffer saline (PBS) solution was prepared with 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride (pH 7.4, at 25 °C) and diluted 10× in milliQ water. GIBCO® Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F-12) media for cell cultures were purchased from Sigma-Aldrich.

2.2. Peptide synthesis

The peptide encompassing the amino acid sequence Ac-KNGNPHSEN-NH_2, Ang_{60-68} (molecular weight, MW, of 1105.5 g/mol, isoelectric pH, pI, of 11.38 [33]) was synthesized with the N-terminus blocked by acetylation and the C-terminus amidated as previously reported [27]. The fluorescent peptide analogous, Fam-Ang_{59-68}, was labelled with 5,6-carboxyfluorescein (Fam) through the side chain of an amidated additional lysine residue (purchased from CASLO, Lyngby, Denmark).

2.3. Preparation of AuNPs and AuNP/peptide assemblies

Gold nanoparticles were synthesized by a modified Turkevich's method of chemical reduction of the chloroauric acid by the reducing agent trisodium citrate [34]. Briefly, gold(III) chloride hydrate ($HAuCl_4 \cdot xH_2O$) was dissolved in 20 mL of water in a beaker on a stirring hot plate at the final concentration of 1 mM; 2 mL of a 1% solution of trisodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) was quickly added to the rapidly-stirred boiling solution. The gold sol gradually forms as the citrate reduces the gold(III). As soon as the solution turned deep red, the beaker was removed from heat, roughly 10 min of elapsing time. This preparation yields about 12 nm diameter particles with plasmonic peak at 518 nm. The actual concentration of synthesized AuNP was typically of 20 nM, as estimated by UV–visible spectra according to $\varepsilon = 1.9 \times 10^8\ M^{-1}\ cm^{-1}$, calculated by the equation: $\ln \varepsilon = k \ln D + a$, where ε is the extinction coefficient, in $M^{-1}\ cm^{-1}$, D is the core diameter of the nanoparticles, and k = 3.32111, a = 10.80505 [35].

2.4. UV–visible spectroscopic and dynamic light scattering (DLS) analyses

UV/Vis spectroscopy was performed on the aqueous dispersions in quartz cuvettes with 1 cm optical path length on a Jasco spectrometer. Dynamic light scattering and ζ-potential measurements were carried out by using a NanoPartica SZ-100 apparatus equipped with a 514 nm laser (Horiba-Scientific). Z-average sizes were collected at 25 °C and analysed from three sequential measurements. Samples were not filtered before measurements. At least 5 measurements were made and data were averaged.

2.5. Cell cultures

Human SH-SY5Y neuroblastoma cells were cultured in DMEM-F12 supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 100 U penicillin/0.1 mg/mL streptomycin and maintained at 37 °C in a humid atmosphere in the presence of 5% CO_2.

2.5.1. Cell viability assays of SH-SY5Y cells

Cells were seeded at a density of 3×10^3 cells/well or 5×10^3 cells/well in 96 multi-well plates (Corning™ Falcon™ Polystyrene Microplates, Fisher Scientific). After 12 h, the cells were treated with fresh growth medium containing Ang_{60-68} (2 μM) or FAM-Ang_{59-68} (2 μM) in the absence or the presence of AuNP (2 nM). Following the treatment period of 48 h, cell proliferation was determined using the MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay kit; Promega) according to manufacturer's instruction. The absorbance of formazan at 490 nm was measured in a colorimetric assay with an automated plate reader (Victor Wallac 2, Perkin Elmer). Data were expressed as the percentage of untreated cells proliferation. Experiments were performed in triplicate.

2.5.2. Confocal microscopy imaging

Cell were plated in glass bottom glass bottom dishes (WillCo-dish®, Willco Wells, B.V.) with 22 mm of glass diameter and incubated at 37 °C in a humid atmosphere in the presence of 5% CO_2 until the 80% of confluence was reached. For actin staining experiments, cells were treated for 1 h with Fam-Ang_{59-68} at the concentration of 1×10^{-5} M. After the incubation time, cells were washed with phosphate buffer saline solution (10 mM PBS, 37 °C, pH = 7.4), fixed with high purity 4% formaldehyde in PBS (pH = 7.3) and stained with the nuclear dye DAPI (ThermoFisher). Afterwards, cells were permeabilized with 0.5% Triton X-100 and stained with a high-affinity F-actin probe, conjugated to green-fluorescent Alexa Fluor® 488 dye (ActinGreen™ 488 ReadyProbes® Reagent, TermoFisher). For the cellular internalization

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