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Visualization of hybrid gold-loaded polymeric nanoparticles in cells using scanning electron microscopy

Edurne Luque-Michel [a, b], Victor Sebastian [c, d], Boguslaw Szczupak [e, 1], Edurne Imbuluzqueta [a, b], Jordi Llop [e], María J. Blanco Prieto [a, b, *]

[a] Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy and Nutrition, University of Navarra, Pamplona, Spain
[b] IdiSNA, Fundación Instituto de Investigación Sanitaria de Navarra, Recinto del Complejo Hospitalario de Navarra, Pamplona, Spain
[c] Department of Chemical Engineering and Environmental Technology, University of Zaragoza, Zaragoza, Spain
[d] Networking Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, 28029 Madrid, Spain
[e] Molecular Imaging Unit, CIC biomaGUNE, Paseo Miramón 182, 20009 San Sebastián, Guipúzcoa, Spain

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ABSTRACT

Nanotechnology is growing quickly, with great advances in the area of nanomedicine. Opening the door to personalized medicine, a considerable number of nanosystems have been synthetized for the diagnosis, treatment and monitoring of diseases. Specifically, gold nanoparticles (AuNPs) have been shown to be good contrast agents. However, they have a limited surface area for the transport of active molecules. In this paper, polymeric nanoparticles encapsulating AuNPs have been synthetized by the double emulsion method (w/o/w) and solvent evaporation technique. This approach opens up the possibility of encapsulating hydrophilic and/or lipophilic thermostable biomolecules. The nanoparticles could be monitored in macrophage cells by simple scanning electron microscopy (SEM). Nevertheless, a micro computed tomography (micro-CT) study revealed that they would not be detected in future *in vivo* studies. In short, this paper explains the difficulty of obtaining nanovehicles that are trackable from early investigation stages to their clinical use, and discusses the controversy surrounding the concentration of AuNPs needed to obtain enough X-ray attenuation with safe doses.

1. Introduction

It is well known that nanotechnology has great potential in the area of biomedicine [1]. Specifically in cancer, nanotechnology promises to resolve the issues of low tumor accumulation and toxicity associated with traditional chemotherapy. In fact, the key advantage of nanobiotechnology is the accumulation of nanovehicles in the tumor mass where the drugs incorporated are released. The transport of active compounds could change not only their biodistribution but also their pharmacokinetic and toxicological characteristics. Nanomedicine thus introduces new neoplasm treatments with lower and more efficient doses, which also could overcome the problem of drug resistance. Additionally, the imaging capacity of nanobiotechnology in cancer diagnosis is also an important issue. Some inorganic nanosystems have shown unique chemical, physical and optical properties at the nanometer scale. These properties make them able to detect tumors at early stages, which is essential to have a good prognosis. At present, various types of nanosystems are being investigated to explore their potential in cancer diagnosis, including gold nanoparticles (AuNPs), quantum dots or superparamagnetic iron oxide nanoparticles (SPION) [2]. The combination of nanosystems as drug delivery systems and, at the same time, as imaging agents to detect tumors, is known as theragnosis. This represents a great advance in the management of this heterogeneous disease because it permits individualized antineoplastic therapies with the possibility of localizing, typifying and monitoring the tumor in real-treatment time. It is also exciting to follow simultaneously the drug and its transporter to understand better how the nanosystems release the drug and are distributed throughout the body [3]. It is worth mentioning that one major problem in the interpretation of nanosystem behavior is that, normally, it may be possible to

* Corresponding author. Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy and Nutrition, University of Navarra, Pamplona, Spain.
E-mail address: mjblanco@unav.es (M.J. Blanco Prieto).
[1] Present Address: Department of Telecommunications and Teleinformatics, Wroclaw University of Science and Technology, Wybrzeze Wyspianskiego 27, 50-370 Wroclaw, Poland.

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quantify the amount of drug that reaches each organ, but it is very difficult to know if the drug reaches them inside the nanoparticles (NPs) or it was released from the NP before.

AuNPs have become tools in cancer diagnosis and therapeutics owing to their surface chemistry, relatively low short-term toxicity, high atomic number and high X-ray absorption coefficient [4]. Moreover, these NPs are simple to prepare and have easy surface functionalization [5]. Several imaging techniques can be used to detect them [6], for instance, with the use of AuNPs the contrast between normal and cancerous tissue can be enhanced using X-ray based computed tomography (CT), one of the leading radiology technologies applied in hospitals nowadays [7]. In fact, AuNPs have revealed higher contrast and longer imaging times than the iodinated contrast agents used in clinical practice [2,7]. Apart from this, due to the high atomic number of Au, AuNPs are also good contrast agents for use with the transmission electron microscope (TEM) [8] and scanning electron microscope (SEM) [9] which makes these nanosystems very interesting at early stages of research. These techniques can help us to understand the behavior of nanosystems in different situations, such as their internalization by cells, or the way they cross biological barriers.

Several methods have been developed for the synthesis of theragnostic NPs. In general, the “one for all” approach, where metallic NPs act as imaging contrast agent and drug transporter at the same time, has a limited drug loading capacity [2,10]. This is worsened by the fact that the surfaces of Au nanostructures are often covered with different materials [11]. In order to overcome this limitation, in this work the “all in one” approach was used. AuNPs were internalized inside poly(lactic-*co*-glycolic acid) (PLGA) NPs allowing the encapsulation of several types of thermostable biomolecules (hydrophilic or/and lipophilic) within this polymeric NP. In this work, AuNPs were encapsulated into polymeric NPs by the double emulsion method (w/o/w) and solvent evaporation technique [8]. Their internalization inside macrophages by SEM was investigated, and a proof of concept of their X-ray attenuation capacity by micro-CT was obtained.

2. Materials and methods

2.1. Materials

For Au-PLGA NPs synthesis, the polymer PLGA 50:50 (MW 38000–54000 Da, Resomer® RG 504) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Sodium cholate, chloroauric acid ($HAuCl_4 \cdot 3H_2O$), sodium citrate tribasic dehydrate ≥ 99% ($Na_3C_6H_5O_7 \cdot 2H_2O$), taurocholic acid sodium salt hydrate ≥ 95% (TC), ethyl acetate ACS reagent and phosphotungstic acid hydrate, as a contrast agent for microscopy, were purchased from Sigma Aldrich (St Louis, MO, USA).

For cellular studies, RPMI Medium 1640 (1X), 0.25% trypsin-EDTA (1X), penicillin-streptomycin (Pen Srep), fetal bovine serum (Heat Inactivated FBS) and Collagen I rat tail 3 mg/mL were purchased from Gibco (Invitrogen Inc. Carlsbad, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Barcelona, Spain). Finally, Dulbecco's Phosphate Buffered Saline–0.0095 M without Ca and Mg (Biowhittaker DPBS) was purchased from Lonza (Veviers, Belgium).

2.2. Au-PLGA NPs synthesis and characterization

Au-PLGA NPs were synthetized by an *in situ* reduction method using a double-emulsion w/o/w as described previously [8]. For this purpose, the precursors forming the AuNP, gold chloride (III) hydrate and tribasic sodium citrate dihydrate were used as the internal aqueous phase in a 1: 5 ratio. The internal aqueous phase was poured onto the organic phase composed of 50 mg of PLGA dissolved in 1 mL of ethyl acetate. The two phases were mixed by sonication at 15 Watts for 15 s. The formed w/o emulsion was poured onto the external aqueous phase (2 mL of a 1% aqueous solution of sodium taurocholate) and sonicated under the same conditions. The obtained w/o/w emulsion was poured into 10 mL of a 0.3% aqueous solution of sodium taurocholate and capped. The temperature was then increased to 40 °C for 20 min to allow the formation of AuNP in the internal aqueous phase of the w/o/w emulsion. After 3 h at room temperature to allow solvent evaporation, the nanoparticles were collected by centrifugation and washed. Blank NPs were synthesized following the previous procedure but without adding the AuNPs precursors. The formulations obtained were lyophilized using mannitol (25.0%; w/w with respect PLGA) or glucose (12.5%) as cryoprotectants.

Au-PLGA NPs were characterized with respect to the size, surface charge, morphology and surface plasmon resonance (SPR). The average particle size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) and the surface charge (zeta potential) by laser Doppler electrophoresis using a ZetaSizer Nano ZS analyzer system (Malvern Instruments, UK). The morphology was evaluated at the LMA-INA facilities by an environmental Scanning Electron Microscope Quanta™ FEG-250 (FEI, Hillsboro, Oregon, USA) with an accelerating voltage of 30 kV and by a TEM FEI™ Tecnai T20 at 200 kV. A 5 μL suspension of stained PLGA NPs was pipetted onto a TEM copper grid having a continuous carbon film. Samples were let to evaporate completely and then analyzed. The typical SPR peak of AuNPs was measured using a UV-visible Agilent 8453 spectrophotometer.

2.3. Cellular internalization assay of Au-PLGA NPs

The J774 cell line (ATCC TIB-67) was maintained in RPMI 1640 medium supplemented with glutamax®, 10% of heat-inactivated fetal bovine serum and 1% of penicillin-streptomycin solution. Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere and passaged twice a week at 80% of confluence.

To visualize the internalization of the nanosystems, J774 cells were grown and treated with Au-PLGA NPs on a metallic insert. This insert had an area of growth of 0.38 cm^2 which was collagened before seeding 10000 cells. After 12 h, they were treated with 1 mg/mL of Au-PLGA NPs (theoretically 0.27 mM of Au) or PLGA NPs (blank NPs). Cells were incubated with the treatments at 37 °C in a humidified 5% CO_2 atmosphere for 24 h. Afterwards, they were fixed with para-formaldehyde 4% (for 15 min at RT) and prepared to be analyzed by FEG SEM, using secondary and backscattered electrons. The presence of AuNPs inside the macrophage cells was confirmed by an elemental analysis with the environmental FEG SEM which is equipped with an energy-dispersive X-ray spectrometer (EDX).

2.4. Viability assay

To test the toxicity of Au-PLGA NPs, 10000 cells per well were seeded in a 96 well plate. The cytotoxic activity of 1 mg/mL of Au-PLGA NPs was analyzed in triplicate with the cellular proliferation test MTT [12]. After 24 h of treatment, the cells were washed three times with PBS and 100 μL of MTT reactant (0.50 mg/mL in culture medium) were added. After 2 h of incubation at 37 °C in a humidified 5% CO_2 atmosphere, all reactant was removed, 100 μL of DMSO were added to each well and the plate was read at $\lambda = 540$ nm using an iEMS reader (Labsystems, Finland). This experiment was repeated three times on different days.

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