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● *Original Contribution*

STUDY OF THP-1 MACROPHAGE VIABILITY AFTER SONODYNAMIC THERAPY USING METHYL ESTER OF 5-AMINOLEVULINIC ACID GOLD NANOPARTICLES

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Abstract—Sonodynamic therapy (SDT) is emerging as new atherosclerosis treatment. The use of gold nanoparticles (AuNPs) as the vehicle for a sensitizer delivery improves reactive oxygen species formation. In this study, methyl ester of aminolevulinic acid (MALA) gold nanoparticles (MALA:AuNPs) functionalized with polyethylene glycol (PEG) were synthesized by photoreduction and characterized by ultraviolet/visible optical absorption, zeta potential and electron microscopy. The reactive oxygen species generation induced by ultrasound irradiation of MALA:AuNPs solutions was studied by observing the decrease in the 1,3-diphenylisobenzofuran emission band. The potential use of MALA:AuNPs as sensitizer for sonodynamic therapy was investigated on THP-1 macrophages. The cytotoxicity test was also described. The findings suggested that ultrasound combined with MALA:AuNPs provides impressive results in *in vitro* studies. Sonodynamic therapy with MALA:AuNPs through 2 minutes of ultrasound exposure (1 MHz and 1 W/cm^2) culminated with total macrophage reduction. Thus, sonodynamic therapy combined with MALA:AuNPs has potential as a treatment for atherosclerosis. (E-mail: lccourrol@gmail.com)

Key Words: **Sonodynamic therapy, Atherosclerosis, Methyl ester of aminolevulinic acid, Gold nanoparticles.**

INTRODUCTION

The role of the macrophage is of major importance in atherosclerotic lesion development and thrombogenicity. Macrophages participate in lipoprotein uptake and accumulation, giving rise to foam cells. Accumulation of foam cells contributes to lipid storage and atherosclerotic plaque growth (Wennink et al. 2017). Macrophages occupying the atherosclerotic plaque have a decreased ability to migrate, which leads to failure of inflammation resolution and to further progression of the lesion into complicated atherosclerotic plaque (Haka et al. 2012). Hence, reduction of macrophages from plaques represents a new strategy for the treatment of atherosclerosis.

Sonodynamic therapy (SDT) has emerged as a promising alternative to traditional atherosclerosis treatments, as it is able to focus ultrasound waves on plaques and locally activate sonosensitizers that have accumulated in the macrophage (Li et al. 2017; Mehrad and Farhoudi 2016; Mehrad et al. 2017; Trendowski 2014). SDT can induce macrophage apoptosis and appears to be a versatile therapy (Cheng et al. 2013; Li et al. 2017; Mehrad et al. 2017; Wang et al. 2013; Zheng et al. 2014). Several studies have reported positive results regarding the use of SDT to induce macrophage apoptosis. Three elements are crucial to SDT: low-intensity ultrasound, a sonosensitizer and molecular oxygen. SDT involves the administration of sonosensitizer molecules that bind to the macrophages and. subsequently under ultrasound irradiation, produce reactive oxygen species (ROS) that chemically destroy macrophages (Wood and Sehgal 2015). The significant advantage of SDT is the deep penetration of ultrasound in soft tissue (Trendowski 2014).

Cheng et al. (2013) observed effects of 5-aminolevulinic acid-mediated sonodynamic therapy (ALA-SDT) on macrophages. ALA is a naturally occurring compound, the early intermediate in the heme biosynthesis pathway. For therapeutic purposes, ALA is administered topically or systemically and penetrates into cells, where it is metabolized to the active sensitizer protoporphyrin IX (PpIX). ALA-SDT exhibits synergistic apoptotic

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effects on THP-1 macrophages, involving excessive intracellular ROS generation and mitochondrial membrane potential loss (Cheng et al. 2013). Therefore, ALA-SDT is a potential treatment for atherosclerosis. This effect occurs because inflammatory cells contain elevated levels of PpIX. PpIX preferentially accumulates in THP-1 macrophages after the administration of ALA.

The methyl ester of ALA (methyl-ALA or MALA) is more stable and penetrates deeply into the tissues than ALA because of its increased lipophilicity (Peng et al. 2001). Compared with the parental ALA, MALA has been found to produce higher intracellular PpIX concentrations at lower doses of photosensitizer, resulting in a similar phototoxic effect (Manivasager et al. 2006; Wakui et al. 2010).

A promising approach for improving SDT efficiency is the use of gold nanoparticles as carriers of sonosensitizers such as MALA. It is known that nanoparticles provoke cell cytotoxicity by inducing the production of ROS. Wen (2013) proposed that the combination of gold nanoparticles (AuNPs) and ultrasound could be a promising strategy for future medical applications. Brazzale et al. (2016) described the role of targeted AuNPs as sonosensitizers. The gold nanoparticles exhibit plasmon resonance, a resonant phenomenon where light induces collective oscillations of conductive metal electrons at the nanoparticle surface, tunable to different wavelengths by varying the nanoparticle size (Voliani 2013). The AuNPs are very suitable for imaging (magnetic resonance imaging, tomography, *etc.*) and photothermal therapy (Louis and Pluchery 2012). The nanoparticles are prepared using various physical methods, such as photoreduction and sonification, and chemical methods, such as co-precipitation and sol−gel technique (Gupta and Kumar 2008).

In this study, MALA gold nanoparticles (MALA:AuNPs) functionalized with polyethylene glycol (PEG) were synthesized by photoreduction and administered to THP-1 macrophages to evaluate their toxicity and applications in SDT. The results indicated that the use of AuNPs as the vehicle for MALA delivery and represents a promising approach to improving MALA-SDT results.

METHODS

The MALA:AuNPs were synthesized and characterized. The toxicity of these nanoparticles in THP-1 macrophages was determined. PpIX fluorescence spectra of macrophages were obtained. ROS generation was observed using a diphenylisobenzofuran (DPBF) probe, and SDT was performed. Figure 1a is the flow diagram of the experimental study.

MALA:AuNP synthesis

To synthesize MALA:AuNPs, 15 mg of tetrachloroauric acid ($HAuCl_4$), 45 mg of 5-aminolevulinic acid methyl ester hydrochloride and 100 mg of PEG were added to 100 mL of distilled water at 20°C and stirred vigorously for 5 min. Ten milliliters of the resulting solution was exposed to a 300-W Cermax xenon lamp for 5 min. After irradiation, the pH of the solution was adjusted to 7.0. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

MALA:AuNP characterization

Ultraviolet−visible spectra of the gold nanoparticles were measured with the UV-2600 spectrophotometer (Shimatzu Japan) using 1-cm quartz cells.

Transmission electron microscopy (TEM) was performed with a Jeol (Zeiss, Germany) transmission electron microscope, with images captured by a MegaView III camera and processed with the software ITEM (Universal TEM Imaging Platform (Olympus Soft Imaging Solutions GmbH, Germany). For this, a drop of MALA:AuNPs dispersed in distilled water was placed onto a carbon-coated copper grid. The excess liquid was removed using a paper wick, and the deposit was dried in air prior to imaging.

The hydrodynamic size and zeta potential distribution of the synthesized MALA:AuNPs were determined using a Nano-ZS90 (Malvern) instrument (5-mW HeNe laser at 632 nm).

Reactive oxygen species generation

In a quartz cuvette, 1.0 mL of MALA:AuNPs (30 nM) and 40 μL of DPBF (4 μM) were irradiated with therapeutic ultrasound (Sonic Compact 1−3 MHz; HTM, Amparo, São Paulo, Brazil; diameter = 3.5 cm, continuous wave, resonance frequency = 1 or 3 MHz and intensity = 1 or 2 W/cm^2). The transducer was positioned leaning against the cuvette. Irradiations were performed in two different ways: (i) by fixing irradiation time (5 min) and varying frequencies and intensities (spatial-peak temporal average intensity [I_{SPTA}]) from 1 MHz and 2W/cm^2, to 3 MHz and 2 W/cm^2, to 3 MHz and 1 W/cm^2 and to 1 MHz and 1 W/cm^2; and (ii) by fixing frequency at 1 MHz and intensity at 1 W/cm^2 and varying irradiation duration from 0 to 10 min. DPBF fluorescence was measured immediately after ultrasound irradiation by exciting samples at 422 nm with a Fluorolog 3 fluorimeter (Horiba Jobin Yvon, Irvine, CA, USA).

Cell culture

Human monocytic leukemia THP-1 cells (Sigma Aldrich) were cultured in RPMI-1640 medium. Cells were seeded at 5000 cells per well in 96-well plates and incubated at 37°C in a humidified atmosphere of 5%

CO_2 in 95% air. THP-1 cells were treated with 75 ng/mL phorbol myristate acetate (PMA, Sigma-Aldrich Co.) for 48 h to induce differentiation of the cells into macrophages. After differentiation, non-attached cells were removed by aspiration, and the adherent macrophages were washed three times with RPMI-1640 medium and then incubated in cell culture medium at 37˚C.

Cell viability assay

Cytotoxicity evaluation of MALA:AuNPs was performed using the MTS assay. THP-1 cells were incubated with MALA and MALA:AuNPs for 24 hours in the dark. A similar quantity of MilliQ water or NaCl was added to the control cells (cc) to observe the influence of the vehicle. The volume in each well in 96-well plate was kept constant. Studied concentrations are described in Table 1. The cells were washed several times with phosphate-buffered saline (pH 7.2−7.6) and plate centrifugation, to remove any residual extract color that could interfere with the colorimetric assay. The MALA-free cells were finally suspended in 100 μL medium and assayed for viability using the colorimetric MTS assay kit based on the CellTiter 96 Aqueous One Solution (Promega, Madison, WI, USA), which measures mitochondrial function; the latter correlates with cell viability. In this procedure, the cells were incubated with fresh medium containing MTS reagent for 2 h before measurements at an absorbance of 490 nm. The effect of particles on cell viability was expressed as percentage of inhibition of cell growth relative to the control. Results were statistically compared (analysis of variance and Bonferroni post-test) with negative (cc + NaCl 0.9%) or positive (latex extract, 0.5g/L in culture medium, 24 h) controls. Percentage cell survival was calculated after background absorbance correction and blank absorbance subtraction as follows: % Cell viability = 100 × experimental well absorbance/untreated control well absorbance. In this experiment, n = 4 for the latex, NaCl and control groups, and n = 9 for the 30, 60, 90 and 120 nM MALA groups.

Table 1. Groups and solution concentrations of MALA and MALA:AuNPs

Group		MALA concentration (nM)
THP-1 (CC)	100 μL RPMI-1640	
THP-1 (CC + H_2O)	10 μL of Milli-Q water in 90 μL serum-free RPMI-1640	
THP-1 (CC + NaCl)	10 μL of NaCl in 90 μL serum-free RPMI-1640	
MALA	40 μL of MALA in 60 μL serum-free RPMI-1640	120
MALA:AuNPs	10 μL of MALA:AuNPs in 90 μL serum-free RPMI-1640	30
MALA:AuNPs	20 μL of MALA:AuNPs in 80 μL serum-free RPMI-1640	60
MALA:AuNPs	30 μL of MALA:AuNPs in 70 μL serum-free RPMI-1640	90
MALA:AuNPs	40 μL of MALA:AuNPs in 60 μL serum-free RPMI-1640	120

MALA:AuNPs = methyl ester of 5-aminolevulinic acid gold nanoparticles.

PpIX extraction of cells

Around 30,000 cells and 75 ng/mL PMA were added to each well of a 96-well plate. After 4 or 24 h, the cells were incubated with MALA or MALA:AuNPs, and the macrophages were divided into the groups described in Table 1.

The contents of each well were collected and added to tubes containing 3 vol of acetone. These solutions were then centrifuged for 15 min at 4000 rpm, and the supernatants were analyzed with the Fluorolog 3 fluorimeter. Samples were excited at 400 nm, and emission spectra were measured between 415 and 785 nm. The experiment was performed in triplicate.

SDT procedures

The protocol described above was used to prepare cells, and after differentiation, macrophages were incubated with culture medium containing MALA (120 nM) or MALA:AuNPs (30 nM) for 24 h.

The transducer of the Sonic Compact was placed below the plate containing cells (Fig. 1b). A fine layer of sterile ultrasound gel was used between the transducer and plate. Cell groups were irradiated separately for 2 min. The attenuation coefficient in the plate (polystyrene) was ~0.31 dB/mm (Mažeika et al. 2010). A digital thermometer was used to measure temperature in the plate during ultrasound irradiation. Temperature increased <0.5˚C during 2 min irradiation.

After SDT the cells were washed with phosphate-buffered saline and incubated with fresh medium containing MTS reagent for 4 h before measurements at 490 nm. In this experiment, n = 9 for the cc, cc + US, MALA + SDT and MALA:AuNPs + SDT groups.

Statistical analysis

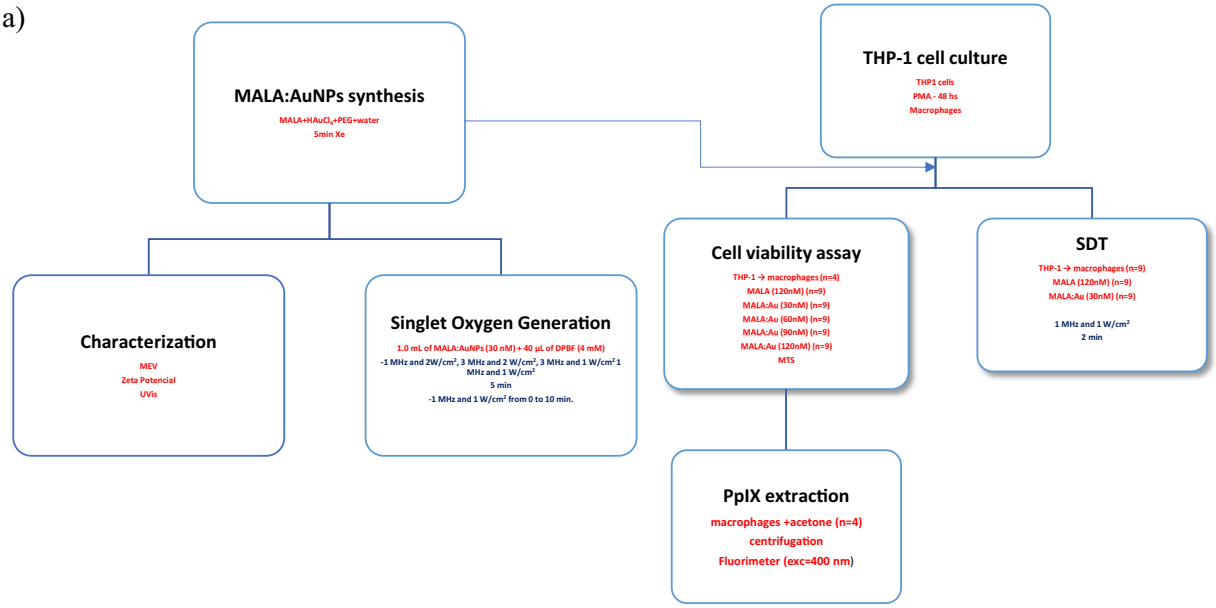
Data were presented as the mean ± standard deviation (SD), unless otherwise specified. Differences <0.05 ($p < 0.05$) were considered statistically significant.

RESULTS

MALA:AuNP characterization

The absorbance spectrum of PEGylated MALA:AuNPs solution is provided in Figure 2a. The absorption

a)



b)

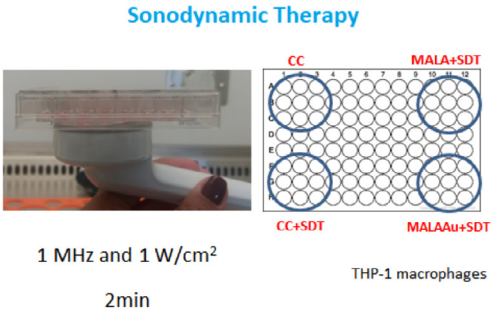


Fig. 1. (a) Flow diagram of experimental study. (b) Schematic diagram of the SDT setup. The transducer (diameter: 3.5 cm, continuous wave, resonance frequency: 1.0 MHz, intensity 1 W/cm^2) was placed at the bottom of the plate, and a thin layer of sterile ultrasound gel was used to make contact between the transducer and the 96-well plate. The circular irradiated area comprised approximately nine wells. The transducer was moved to irradiate the groups distributed on the plate: cc + SDT (THP-1 macrophages in RPMI medium), MALA + SDT (THP-1 macrophages incubated with MALA 120 nM) and MALA:AuNPs + SDT (THP-1 macrophages incubated with MALA:AuNPs 30 nM). MALA = methyl ester of 5-aminolevulinic acid; AuNPs = gold nanoparticles; SDT = sonodynamic therapy; cc = control cells; DBPF = 1.3-diphenylisobenzofuran; PMA = phorbol myristate acetate; PEG = polyethylene glycol.

peak at approximately 522 nm, caused by the surface plasmon resonance (SPR) band, indicates the presence of gold nanoparticles.

The transmission electron microscopy image for MALA:AuNPs, shown in Figure 2b, reveals nearly spherical particles ranging from 10 to 30 nm. An average particle size of ~18 nm was calculated after statistical analysis. On the other hand, small size (~26 nm) and a polydispersity index of 0.577 were measured by dynamic light scattering (see Fig. 2c). MALA:AuNPs had a negative zeta potential (−23.4 ± 0.98 mV).

ROS generation

The production of ROS induced by ultrasound irradiation was evaluated by measuring DPBF. DPBF is a fluorescent molecule that possesses a highly specific reactivity to singlet oxygen $^1O_2$, forming an endoperoxide that decomposes to give 1,2-dibenzoylbenzene (Carloni et al. 1993). Figure 3a illustrates a decreasing

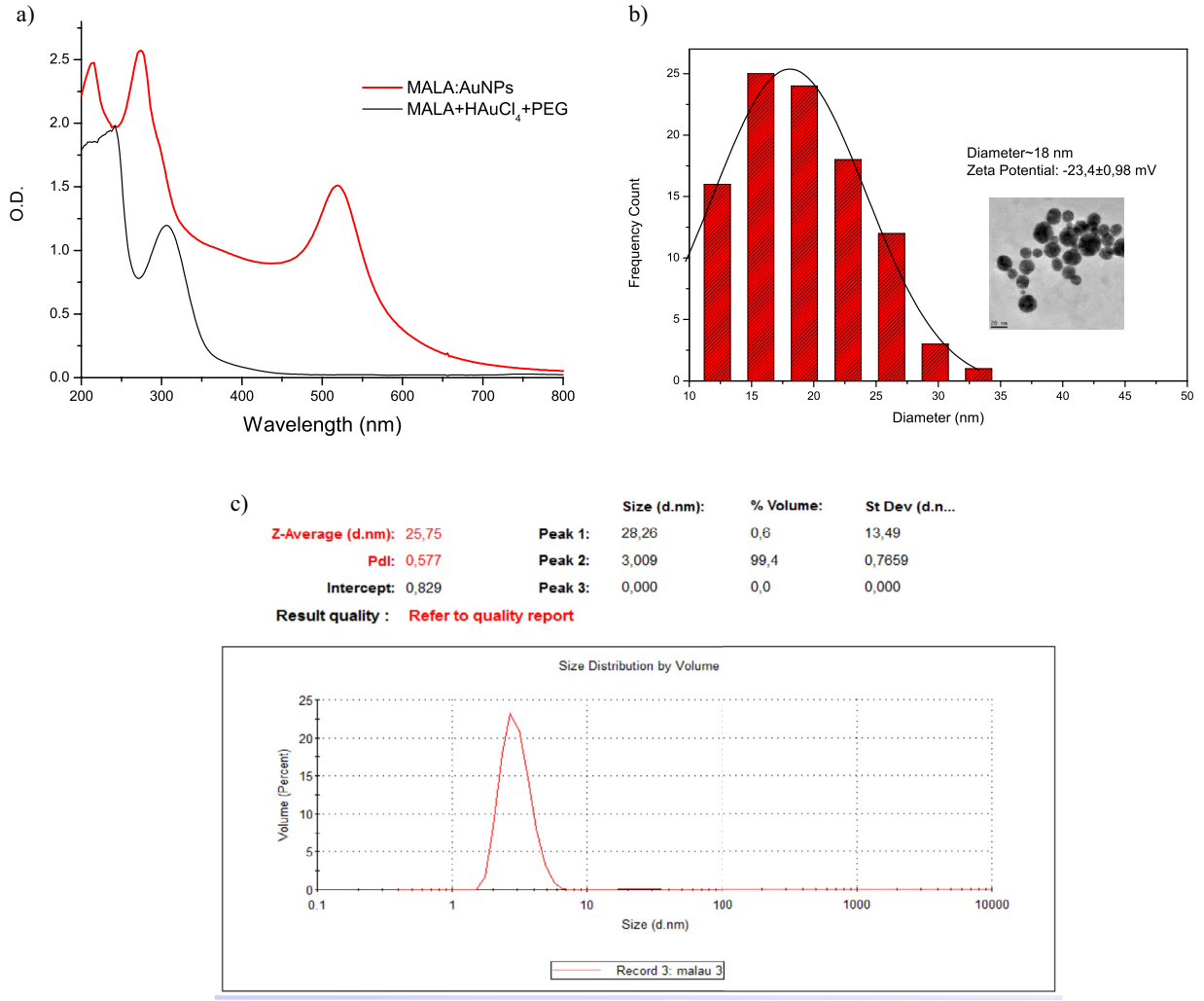


Fig. 2. (a) Absorption spectra of PEGylated MALA:AuNPs synthesized with illumination for 5 min by a Xe lamp and adjusted pH ~ 7.0. (b) Size distribution and transmission electron microscopy image of synthesized MALA:AuNPs. (c) Dynamic light scattering measurement of MALA:AuNPs (size distribution by intensity). MALA = methyl ester of 5-aminolevulinic acid; AuNPs = gold nanoparticles; PEG = polyethylene glycol; O.D. = optical density.

course of DPBF fluorescence in the presence of MALA:AuNPs exposed to 5 min of ultrasound irradiation varying in frequency and intensity. The settings that promoted higher DPBF photobleaching were 1 MHz and 1 W/cm^2 and were used in the SDT experiments. The time dependence of DPBF in the presence of nanoparticle photobleaching promoted by ultrasound irradiation, caused by the generation of ROS, is illustrated in Figure 3b.

Effects of MALA:AuNPs on THP-1 cells

A cell viability assay was used to determine the toxicity of MALA:AuNPs. In the MTS assay, only cells that were viable after 24 h of exposure to the nanoparticles were capable of metabolizing a dye efficiently, and the purple-colored precipitate, which was dissolved in a detergent, was analyzed spectrophotometrically. Figure 4 illustrates the number of viable THP-1 cells as a function of MALA:AuNP concentration. Percentage cell viability was calculated as follows: % cell viability = 100 − % cell cytotoxicity. Percentage cell cytotoxicity = 100 × (experimental well absorbance − negative control well absorbance)/(positive control well absorbance − negative control well absorbance). All calculations were performed after background absorbance correction and blank absorbance subtraction. The half-maximal inhibitory concentration (IC_{50}) value was ~34.8 nM.

Twenty-four hours post-treatment, MALA:AuNP cells exhibited excellent viability in concentrations <35 nM. For this reason, we chose 30 nM as the

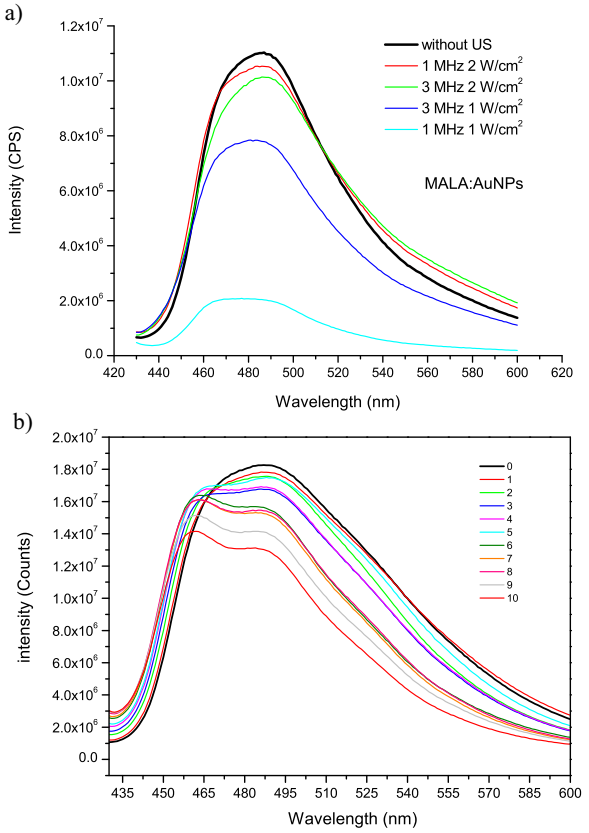


Fig. 3. (a) DPBF (4 μM) fluorescence and DPBF fluorescence in the presence of MALA:AuNP solution and 5 min of 1 MHz/2 W/cm^2, 3MHz/2 W/cm^2, 3 MHz/1 W/cm^2 and 3 MHz/2 W/cm^2 ultrasound irradiation. Excitation: 422 nm. (b) DPBF (4 μM) photobleaching in the presence of MALA:AuNPs and 1 MHz/1 W/cm^2 ultrasound irradiation from 0 to 10 min. MALA = methyl ester of 5-aminolevulinic acid; AuNPs = gold nanoparticles; DBPF = 1.3-diphenylisobenzofuran.

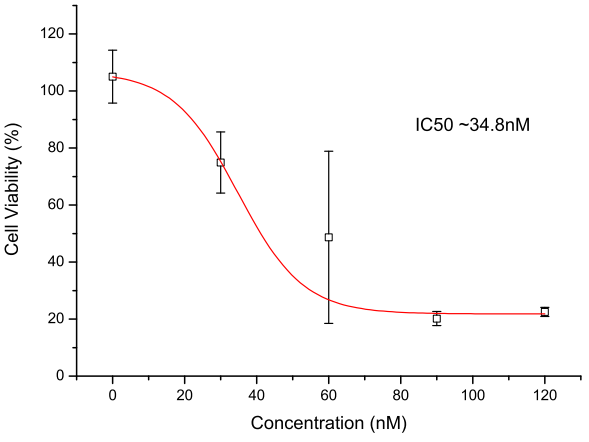


Fig. 4. Reduction of THP-1 cell viability in response to increasing MALA:AuNP concentrations. MALA = methyl ester of 5-aminolevulinic

concentration for the singlet oxygen generation and SDT procedures.

PpIX fluorescence

Protoporphyrin IX was extracted with acetone from THP-1 cells exposed to nanoparticles (30 and 60 nM), and fluorescence intensities were measured by exciting the samples at 400 nm. The results were compared with the PpIX fluorescence intensities obtained for cells incubated with MALA (120 nM). The results are illustrated in Figure 5. There was an increase in the emission signal around 630 and 700 nm, characteristic of PpIX, with incubation of cells with MALA and MALA:AuNPs for periods of 4 h (Fig. 5a) and 24 h (Fig. 5b), when compared with the control group (cells in culture medium). The results suggest that MALA was incorporated by the gold nanoparticles and a quick conversion in endogenous porphyrins occurred, which led to the accumulation of PpIX. Figure 5c and d illustrate the fluorescence intensity obtained for MALA at the higher concentration of 120 nM, after incubation for 4 and 24 h, respectively. After 24 h of incubation with 60 nM MALA:AuNPs, the PPIX fluorescence intensity was smaller than that for 30 nM. This must be due to a reduction in cell viability with MALA:AuNPs at high concentration.

Cell viability after ultrasound irradiation

Macrophages viability after 2 min of ultrasonic irradiation (continuous wave, 1 MHz and 1 W/cm^2) is illustrated in Figure 6. Although ultrasound did not significantly influence cell viability for the control and MALA groups, MALA:AuNP treatment reduced cell viability. Statistically significant differences ($p < 0.05$) were obtained on comparing the control group with MALA:AuNP-treated cells. In this case, cell viability decreased to ~0%. MALA:AuNPs exhibited greater cytotoxicity against THP-1 cells than MALA alone.

In the same figure, it is observed that addition of 10 μL of water to control cells or 10 μL of NaCl to negative controls did not affect cell viability after 2 min of ultrasound irradiation. These indicate that the cell apoptosis promoted by SDT observed in the cells incubated with MALA:AuNPs was not the result of ultrasound exposure in a hypotonic medium.

DISCUSSION

Macrophages can be found in all phases of atherosclerosis and are the main contributors to atherosclerotic plaque development, progression and destabilization. Anti-inflammatory therapeutic medications that result in reduced plaque macrophage content are of great interest. Unlike highly proliferating cancer cells, macrophages are specialized phagocytic cells expressing scavenger

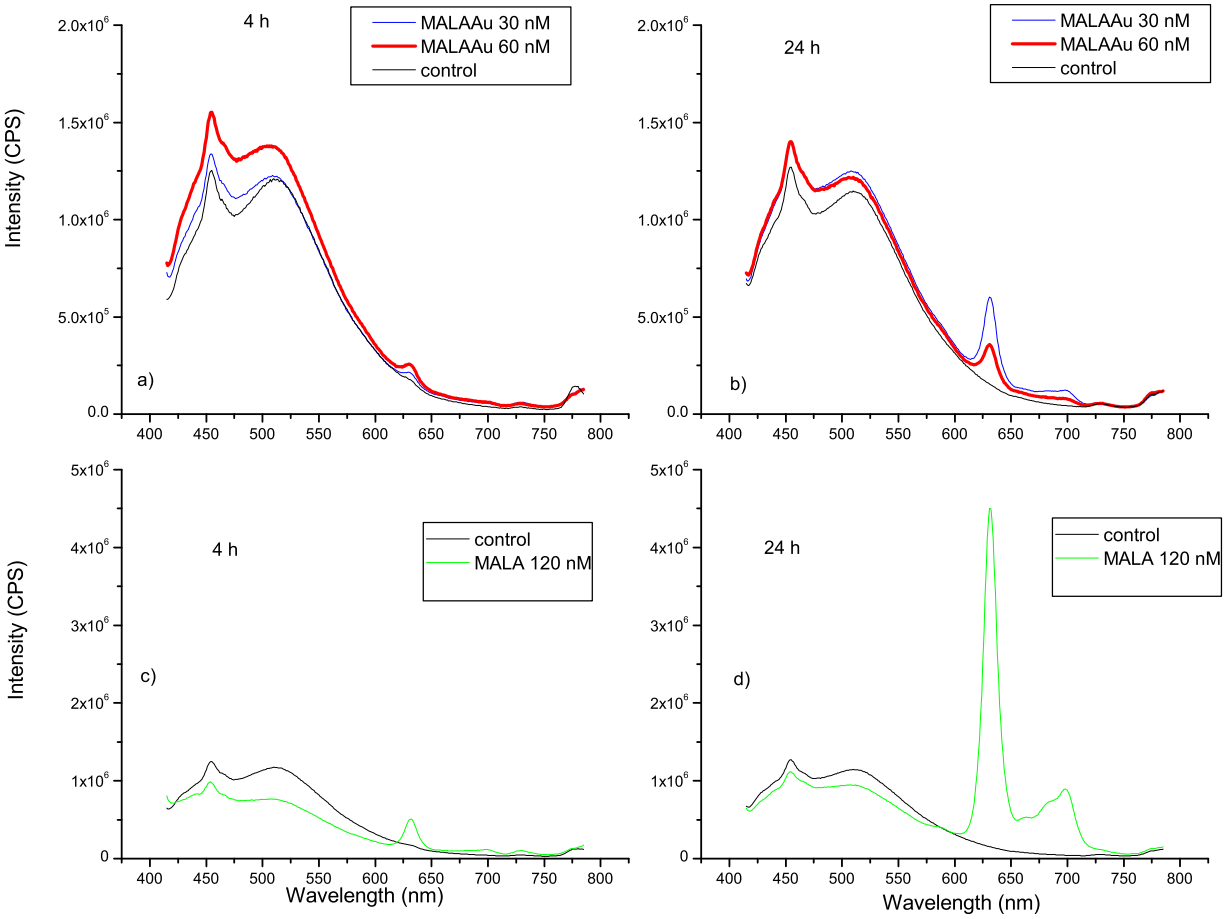


Fig. 5. Fluorescence of protoporphyrin IX extracted of THP-1 cells incubated with MALA gold nanoparticles for (a) 4 h and (b) 24 h and with MALA for (c) 4 h and (d) 24 h. MALA = methyl ester of 5-aminolevulinic acid.

receptors able to bind a diverse array of foreign or endogenous molecules such as PpIX (Liu and Hamblin 2005). Previous studies have reported accumulation of PpIX in atherosclerotic plaque (Guo et al. 2013; Peng et al. 2011).

Precursor substances like ALA and MALA are metabolized to PpIX (Manivasager et al. 2006; Wakui et al. 2010). Tian et al. (2016) reported reduction of THP-1 macrophages *in vitro* with sonodynamic therapy mediated by ALA. The generation of reactive oxygen species from ultrasound-activated PpIX is considered to be responsible for the sonodynamic damage of macrophages (Cheng et al. 2013). Benito et al. (2013) reported the use of AuNPs as the vehicle for ALA delivery, demonstrating that the immobilization of photosensitizer on the particle surface is better for reactive oxygen species formation (Benito et al. 2013).

Before study of the potential use of ~18-nm spherical MALA:AuNPs (Fig. 2) as a sensitizer for sonodynamic therapy of THP-1 macrophages, the interaction of synthesized nanoparticles with ultrasound was investigated. The results illustrated in Figure 3 indicate that MALA:AuNPs can release ROS in the presence of ultrasound. Comparison of different ultrasound irradiation parameters (Fig. 3a) revealed the following tendency: ultrasound pulses with a frequency of 1 MHz and intensity of 1 W/cm^2 promoted a marked decrease in DPBF emission intensity, indicating elevated singlet oxygen generation. These parameters were considered appropriate for use in the SDT assay.

It was also important verify which MALA:AuNP concentrations are not toxic to THP-1 macrophages. Various cell lines can react differently after incubation with nanoparticles. Biochemical and genetic changes and oxidative stress may be the main factors causing cytotoxicity (Tang et al. 2015). The cytotoxicity of gold nanoparticles strongly depends on several significant parameters such as size, shape and surface chemistry. Investigations revealed that 4- to 28-nm Au nanospheres aggregate in high concentrations and, at long intervals of incubation, may increase toxicity (Wozniak et al. 2017).

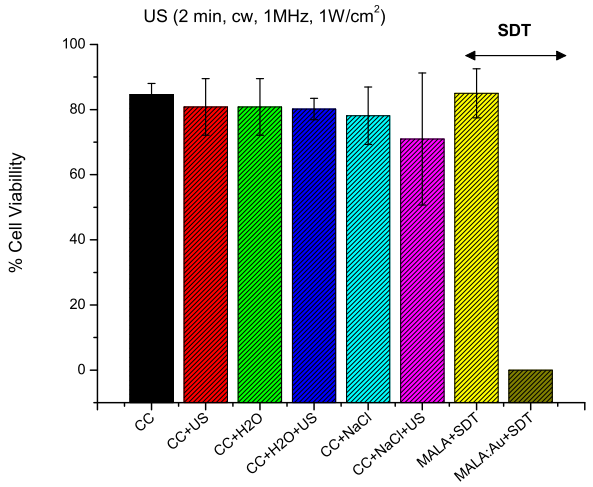


Fig. 6. Viability of THP-1 cells. The cc, the THP-1 macrophages incubated in RPMI medium for 24 h in the dark and the macrophages incubated with water or NaCl (cc + H_2O and cc + NaCl) are compared with groups subjected to 2 min of US (continuous wave, 1 MHz, 1 W/cm^2): cc + US, cc + H_2O + US and CC + NaCl + US, MALA + SDT (134 nM) and MALA:AuNPs + SDT (32 nM). MALA = methyl ester of 5-aminolevulinic acid; AuNPs = gold

Spherical structures are highly efficient in internalization and delivery of drugs and nucleic acids, as well as in targeted therapy. The use of gold nanoparticles for transporting drugs, proteins and genetic materials is a well-established practice. Figure 4 indicates that exposure of THP-1 macrophages to nanoparticles may cause toxicity depending on the MALA:AuNPs concentration. On the basis of this study, the concentration of 30 nM was considered not significantly toxic to macrophages and could be used in SDT studies.

To verify if gold nanoparticles could be internalized by macrophages and if MALA:AuNPs can act as carriers that allow delivery of MALA through the cell membrane, PpIX was extracted from macrophages incubated and not incubated with MALA:AuNPs. Figure 5 illustrates an increase in the fluorescence intensity of PpIX caused by MALA:AuNPs, indicating stable encapsulation of MALA into gold nanoparticles and further conversion to fluorescent PpIX on photo-exposure. These results indicate that MALA:AuNPs could be used as an atherosclerosis-selective therapeutic agent. Figure 6 confirms that the combination of radiation (emitted by a transducer placed below the plate containing the cells) at 1 MHz and 1 W/cm^2 for 2 min, without a significant increase in temperature, and MALA gold nanoparticles induced the total reduction of macrophages.

The non-thermal interaction of ultrasound with an aqueous environment is always accompanied by cavitation, which results in the formation of microscopic air bubbles (Pang et al. 2016). At an appropriate ultrasound intensity, bubbles are produced and implode quickly, generating light by concentration of energy, which induces further reactions within and surrounding the bubbles and gold nanoparticles (Umemura et al. 1996). This process culminates with photoexcitation of molecular oxygen caused by the well-known local electric field enhancement near metal nanoparticles by the plasmon electrons. Consequently, ROS are generated. An alternative scenario, however, would be that the gold particles serve as nucleation centers for bubble formation, and bubble collapse causes the thermal generation of ROS. Simultaneously, when the PpIX induced by MALA:AuNPs was exposed to 1 MHz/1 W/cm^2 ultrasound, it was activated from ground state to an excited state. On returning to the ground state, it released energy, which was transferred to oxygen to produce ROS, such as singlet oxygen and free radicals. So, both nanoparticles and PpIX are responsible for total reduction of macrophages.

It is worth noting that MALA:AuNPs have low cytotoxicity and no inhibitory effect and are reactive only after being exposed to ultrasonic irradiation. Therefore, the risk of any systemic effects is greatly reduced.

Further testing is necessary to understand the mechanisms underlying the total reduction of macrophages, and it would be interesting to assess whether the treatment could also have effects on healthy cells in areas that are not involved in the growth of atherosclerotic plaques.

CONCLUSIONS

Methyl ester aminolevulinic acid gold nanoparticles were synthesized by photoreduction using MALA the as reducing and capping agent. The toxicity of nanoparticles was tested in THP-1 cells that had differentiated into macrophages, and the half-maximal inhibitory concentration obtained was ~34.8 nM. PpIX was extracted from macrophages after incubation periods of 4 and 24 h, and the increase in fluorescence intensity confirmed that MALA acts as a capping agent and induces the production of PpIX in the cells. This result indicates that MALA:AuNPs can be delivered to the macrophages of the atherosclerotic plate and induce its apoptosis through a PpIX sonosensitizer effect. In the present study, singlet oxygen was increased following MALA:AuNP irradiation using the lower-frequency ultrasound (continuous wave, 1 MHz and 1 W/cm^2). SDT applied for only 2 min promoted total reduction of macrophages, indicating it is a useful and promising sonosensitizer for atherosclerosis.

The combination of MALA:AuNPs and SDT is a promising alternative for atherosclerosis therapy because of its distinct advantages, including its non-invasive

nature, high selectivity for pathological sites and low systemic toxicity.

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REFERENCES

Benito M, Martin V, Blanco MD, Teijon JM, Gomez C. Cooperative effect of 5-aminolevulinic acid and gold nanoparticles for photodynamic therapy of cancer. J Pharm Sci 2013;102:2760–2769.

Brazzale C, Canaparo R, Racca L, Foglietta F, Durando G, Fantozzi R, Caliceti P, Salmaso S, Serpe L. Enhanced selective sonosensitizing efficacy of ultrasound-based anticancer treatment by targeted gold nanoparticles. Nanomedicine 2016;11:18.

Carloni P, Damiani E, Greci L, Stipa P, Tanfani F, Tartaglini E, Wozniak M. On the use of 1,3-diphenylisobenzofuran (DPBF): Reactions with carbon and oxygen-centered radicals in model and natural systems. Res Chem Intermed 1993;19:395–405.

Cheng JL, Sun X, Guo SY, Cao W, Chen HB, Jin YH, Li B, Li QN, Wang H, Wang Z, Zhou Q, Wang P, Zhang ZG, Cao WW, Tian Y. Effects of 5-aminolevulinic acid-mediated sonodynamic therapy on macrophages. Int J Nanomed 2013;8:669–676.

Guo SY, Sun X, Cheng JL, Xu HB, Dan JH, Shen J, Zhou Q, Zhang Y, Meng LL, Cao WW, Tian Y. Apoptosis of THP-1 macrophages induced by protoporphyrin IX-mediated sonodynamic therapy. Int J Nanomed 2013;8:2239–2246.

Gupta R, Kumar A. Bioactive materials for biomedical applications using sol−gel technology. Biomed Mater 2008;2:034005.

Haka AS, Potteaux S, Fraser H, Randolph GJ, Maxfield FR. Quantitative analysis of monocyte subpopulations in murine atherosclerotic plaques by multiphoton microscopy. Plos One 2012;7(9):e44823.

Li QS, Kang J, Xiong XJ, Liu Y, Cao WW, Li YN. Protoporphyrin IX-mediated sonodynamic therapy promotes autophagy in vascular smooth muscle cells. Oncol Lett 2017;14:2097–2102.

Liu Q, Hamblin MR. Macrophage-targeted photodynamic therapy: Scavenger receptor expression and activation state. Int J Immunopathol Pharmacol 2005;18:391–402.

Louis C, Pluchery O. Gold nanoparticles for physics, chemistry and biology. London/Singapore: Imperial College Press; 2012.

Manivasager V, Yee KKL, Heng PWS, Soo KC, Olivo M. A study comparing endogenous protoporphyrin IX induced by 5-ALA and ALA-methyl ester with exogenous PpIX and PpIX dimethyl ester in photodynamic diagnosis of human nasopharyngeal carcinoma xenografts. Int J Oncol 2006;29:997–1002.

Mažeika L, Šliteris R, Vladišauskas A. Measurement of velocity and attenuation for ultrasonic longitudinal waves in the polyethylene samples. Ultragarsas 2010;65:12–15.

Mehrad H, Farhoudi M. Investigation of protoporphyrin IX-mediated sonodynamic therapy on intermediate stage atherosclerosis using a new computerized B-mode ultrasound analyzing method. Atherosclerosis 2016;252 E192−E92.

Mehrad H, Farhoudi M, Foletti A. Protoporphyrin IX-loaded microbubbles-mediated sonodynamic therapy reduce foam cells in the early atherosclerosis. Atherosclerosis 2017;263 E152−E152.

Pang X, Xu CS, Jiang Y, Xiao QC, Leung AW. Natural products in the discovery of novel sonosensitizers. Pharmacol Ther 2016;162:144–151.

Peng Q, Warloe T, Moan J, Godal A, Apricena F, Giercksky KE, Nesland JM. Antitumor effect of 5-aminolevulinic acid-mediated photodynamic therapy can be enhanced by the use of a low dose of photofrin in human tumor xenografts. Cancer Res 2001;61:5824–5832.

Peng C, Li Y, Liang H, Cheng J, Li Q, Sun X, Li Z, Wang F, Guo Y, Tian Z, Yang L, Tian Y, Zhang Z, Cao W. Detection and photodynamic therapy of inflamed atherosclerotic plaques in the carotid artery of rabbits. J Photochem Photobiol B Biol 2011;102:26–31.

Tang Y, Shen YF, Huang LB, Lv GJ, Lei CH, Fan XY, Lin FX, Zhang YX, Wu LH, Yang YJ. In vitro cytotoxicity of gold nanorods in A549 cells. Environ Toxicol Pharmacol 2015;39:871–878.

Tian F, Yao JT, Yan M, Sun X, Wang W, Gao WW, Tian Z, Guo SY, Dong ZX, Li BC, Gao TL, Shan P, Liu B, Wang HY, Cheng JL, Gao QP, Zhang ZG, Cao WW, Tian Y. 5-Aminolevulinic acid-mediated sonodynamic therapy inhibits RIPK1/RIPK3-dependent necroptosis in THP-1-derived foam cells. Sci Rep 2016;6:21992.

Trendowski M. The promise of sonodynamic therapy. Cancer Metastasis Rev 2014;33:143–160.

Umemura S, Kawabata K, Sasaki K, Yumita N, Umemura K, Nishigaki R. Recent advances in sonodynamic approach to cancer therapy. Ultrason Sonochem 1996;3:S187–S191.

Voliani V. Update on gold nanoparticles: From cathedral windows to nanomedicine. EBSCO ebook academic collection. Shrewsbury, UK: Smithers Rapra; 2013.

Wakui M, Yokoyama Y, Wang H, Shigeto T, Futagami M, Mizunuma H. Efficacy of a methyl ester of 5-aminolevulinic acid in photodynamic therapy for ovarian cancers. J Cancer Res Clin Oncol 2010;136:1143–1150.

Wang FP, Gao QP, Guo SY, Cheng JL, Sun X, Li QN, Wang TY, Zhang ZG, Cao WW, Tian Y. The sonodynamic effect of curcumin on THP-1 cell-derived macrophages. Biomed Research Int 2013;2013:737264.

Wen DS. Nanoparticle-related heat transfer phenomenon and its application in biomedical fields. Heat Transfer Eng 2013;34:1171–1179.

Wennink JWH, Liu Y, Makinen PI, Setaro F, de la Escosura A, Bourajjaj M, Lappalainen JP, Holappa LP, van den Dikkenberg JB, al Fartousi M, Trohopoulos PN, Yla-Herttuala S, Torres T, Hennink WE, van Nostrum CF. Macrophage selective photodynamic therapy by meta-tetra(hydroxyphenyl) chlorin loaded polymeric micelles: A possible treatment for cardiovascular diseases. Eur J Pharm Sci 2017;107:112–125.

Wood AKW, Sehgal CM. A review of low-intensity ultrasound for cancer therapy. Ultrasound Med Biol 2015;41:905–928.

Wozniak A, Malankowska A, Nowaczyk G, Grzeskowiak BF, Tusnio K, Slomski R, Zaleska-Medynska A, Jurga S. Size and shape-dependent cytotoxicity profile of gold nanoparticles for biomedical applications. J Mater Sci Mater Med 2017;28:11.

Zheng LB, Sun XY, Zhu X, Lv FX, Zhong ZY, Zhang F, Guo WH, Cao WW, Yang LM, Tian Y. Apoptosis of THP-1 derived macrophages induced by sonodynamic therapy using a new sonosensitizer hydroxyl acetylated curcumin. Plos One 2014;9(3):e93133.