

Improvement of the inhibitory effect of xanthenes on NO production by encapsulation in PLGA nanocapsules

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Abstract

For the first time the inhibitory effect of xanthone and 3-methoxyxanthone on nitric oxide (NO) production by IFN- γ /LPS activated J774 macrophage cell line is reported. A remarkable improvement of this effect promoted by encapsulation of these compounds in nanocapsules of poly (DL-lactide-co-glycolide) (PLGA) is also demonstrated. A weak inhibitory effect of 3.6% on NO production by activated macrophages was observed for xanthone at the highest studied concentration (100 μ M). This effect was slightly higher for 3-methoxyxanthone at the same concentration, producing a reduction of 16.5% on NO production. In contrast, equivalent concentrations of xanthone and 3-methoxyxanthone incorporated in nanocapsules produced a significant decrease on NO production of 91.8 and 80.0%, respectively. Empty nanocapsules also exhibited a slight NO inhibitory activity, which may be due to the presence of soybean lecithin in the composition of the nanosystems. The viability of the macrophages was not affected either by free or nanoencapsulated xanthenes. Fluorescence microscopy analysis confirmed that a phagocytic process was involved in the macrophage uptake of xanthone- and 3-methoxyxanthone-loaded PLGA nanocapsules. Phagocytosis might be the main mechanism responsible for the enhancement of the intracellular delivery of both compounds and consequently for the improvement of their biological effect.

Keywords: Xanthone, 3-methoxyxanthone, nanocapsules, PLGA, nitric oxide, J774 macrophages

Introduction

Nitric oxide (NO) is a gas produced by constitutive and inducible isoforms of NO synthase (NOS) from L-arginine and molecular oxygen in a variety of cells (Gross and Wollin 1995, Aramaki 2000). Constitutive NOS isoforms are expressed in endothelial and neuronal cells and are calcium- and calmodulin-dependent (Kolb and Kolb-Bachofen 1992, Aramaki 2000). NO produced by these NOS acts as an intracellular messenger. In contrast, the inducible NOS isoform (iNOS) is not dependent on calcium or calmodulin and its expression is induced by

immunological stimuli in virtually all nucleated mammalian cells (Gross and Wollin 1995). Activation of macrophages with some cytokines and/or with lipopolysaccharide (LPS) induces the production of large quantities of NO generated by iNOS (Lowenstein et al. 1993, Gross and Wollin 1995). NO regulates several important physiological processes, namely the maintenance of normal blood pressure, neuronal mediation and inflammatory responses and also acts as a defensive agent by damaging pathogenic DNA (Kuo and Schoeder 1995). However, excessive and unregulated NO synthesis has been implicated as causal or contributing to

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pathophysiological conditions, including many lethal and debilitating diseases of humans, such as vascular shock, stroke, diabetes, arthritis and chronic inflammation (Gross and Wollin 1995).

Xanthenes (= 9*H*-xanthen-9-ones) are heterocyclic compounds based on the dibenzo- γ -pirone nucleus (Figure 1) including natural, semi synthetic and totally synthetic structures. Xanthenes have extensive biological activities such as hepatoprotection, anti-inflammation, immunomodulation, growth inhibition of human cancer cell lines and modulation of protein kinase C (PKC) (Fernandes et al. 1995, Lin et al. 1996, Pinto and Nascimento 1997, Gonzalez et al. 1999, Peres et al. 2000, Pedro et al. 2002, Saraiva et al. 2002a,b, Pinto and Sousa 2003, Saraiva et al. 2003). Nevertheless, studies on reduction of NO production by xanthone derivatives are scarce. Some data are reported only for mangiferin, which is a C-glucosyl-xanthone (García et al. 2002, Leiro et al. 2003).

Xanthone and 3-methoxyxanthone (Figure 1) were already evaluated in our group concerning different biological activities, namely inhibitory activity of monoamine oxidase (Thull et al. 1993), immunomodulatory (Pinto and Nascimento 1997), PKC modulation (Saraiva et al. 2002b) and growth inhibitory activity of human cancer cell lines (Pedro et al. 2002). In the present work, the inhibitory effect of these xanthenes on the production of NO by the murine macrophage cell line J774 is reported for the first time. In order to enhance the intracellular delivery of the xanthenes and thus to improve their biological activity, both compounds were encapsulated in biodegradable nanocapsules of poly (DL-lactide-co-glycolide) (PLGA). In fact, nanoencapsulation is known to increase the intracellular delivery of several drugs into phagocytic cells (Morin et al. 1994, Wang and Zhang 2001). Nanocapsules can be easily obtained by interfacial deposition of the polymer around an oily core in which lipid-soluble drugs can be dissolved, as we have described previously (Teixeira et al. 2004). PLGA has been selected for nanocapsule preparation, since polyesters, including poly (lactic acid), poly (glycolic acid) and their copolymers, have emerged as the most widely studied class of polymers for pharmaceutical use due to their biocompatibility and biodegradability characteristics (Jain et al. 1998).

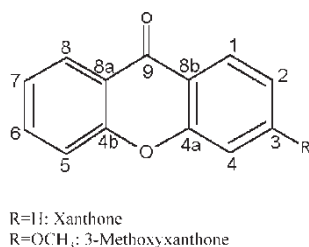


Figure 1. Chemical structure of xanthenes.

The main goal of the present study was to evaluate *in vitro* the inhibitory effect of xanthone and 3-methoxyxanthone on NO production by the activated macrophage cell line J774 as well as to improve that effect through the encapsulation of the compounds in PLGA nanosystems.

Materials and methods

Chemicals and reagents

Xanthone, PLGA (50:50) MW 50,000–75,000, *Pluronic F-68* and soybean lecithin (40% purity by thin-layer chromatography) were purchased from Sigma Chemical Co. (St Louis, USA). 3-Methoxyxanthone was synthesized in our laboratory by alkaline cyclization of 2-hydroxy-2',4-dimethoxybenzophenone as previously described (Fernandes et al. 1998). *Myritol 318* (caprylic/capric acid triglyceride) was kindly supplied by Henkel (Lisbon, Portugal). Foetal bovine serum (FBS) and RPMI-1640 were purchased from Gibco Invitrogen Co. (Barcelona, Spain). Other chemicals were of analytical grade and unless otherwise indicated were purchased from Sigma.

Preparation and characterization of nanocapsules

Nanocapsules containing either xanthone or 3-methoxyxanthone were prepared as previously described (Teixeira et al. 2004). Briefly, about 50 mg of PLGA and 100 mg of soybean lecithin were dissolved in 10 ml of acetone. Xanthone (7.2 mg) or 3-methoxyxanthone (16.8 mg) was dissolved in 0.6 ml of *Myritol 318* and the obtained solution was added to the acetonic solution. The final solution was poured into 20 ml of an aqueous solution of *Pluronic F-68* 0.5% (w/v) under moderate stirring, leading to the formation of nanocapsules. Acetone was then removed under vacuum and the colloidal dispersion of nanocapsules was concentrated to 5 ml by evaporation under reduced pressure. Non-encapsulated xanthenes (either xanthone or 3-methoxyxanthone) were separated by ultrafiltration/centrifugation (centrifugal filter devices Centricon YM-50, Millipore®, Bedford, USA) at 4000*g* for 2 h (Beckman UL-80 ultracentrifuge, Albertville, USA).

Empty nanocapsules were prepared according to the same procedure but omitting xanthenes in the organic phase. Nanoemulsion (NE) was prepared in the same way as nanocapsules, but omitting the polymer and the xanthenes. Nanospheres (NS) were obtained omitting lecithin, *Myritol 318* and xanthenes. Lecithin dispersion (LD) was prepared omitting polymer, *Myritol 318* and xanthenes. *Myritol 318* aqueous dispersion (MD) was obtained omitting polymer, lecithin and xanthenes. Freshly prepared formulations were used in all experiments.

Mean particle size of xanthone- and 3-methoxy-xanthone-loaded nanocapsules and empty nanocapsules, determined by photon correlation spectroscopy (Zetasizer 5000, Malvern Instruments, Malvern, UK) was 273 ± 18 , 271 ± 16 and 261 ± 17 nm, respectively.

The amount of xanthone and 3-methoxyxanthone in nanocapsules was determined following dissolution of aliquots of nanocapsule dispersions in acetonitrile by a previously validated HPLC method (Teixeira et al. 2003). Incorporation efficiency was higher than 77% for nanocapsule formulations containing either xanthone or 3-methoxyxanthone.

NO production assay

The murine J774 macrophage cell line was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS serum, 2 mM glutamine and 50 μ g/ml of gentamicin (designated thereafter as culture medium) at 37°C in a 5% CO₂ humidified atmosphere. Cells were added to 96-well flat-bottom microplates at a density of 0.5×10^6 cells/ml and allowed to adhere for 2 h. To induce iNOS, culture medium was replaced by fresh medium containing LPS (1 μ g/ml) and IFN- γ (100 U/ml). Aqueous dispersions of nanocapsules containing xanthone or 3-methoxyxanthone as well as solutions of xanthone or 3-methoxyxanthone in DMSO were diluted with culture medium (concentrations ranging from 2 to 100 μ M) and tested. Equivalent concentrations of empty nanocapsules were also tested. Final concentrations of dimethylsulfoxide (DMSO) showed no interference with the tested biological activity. Sample dilutions were added together with stimulus and their effect on NO production was evaluated after 24 h of incubation at 37°C, quantifying nitrite accumulation in cell culture supernatant by the Griess reaction (Green et al. 1982).

NO scavenging assay

Sodium nitroprusside (5 mM) in PBS was mixed with the different samples (xanthone- and 3-methoxy-xanthone-loaded nanocapsules, empty nanocapsules as well as xanthone and 3-methoxyxanthone solutions in DMSO) and with ethanol/PBS (1:9) and incubated for 150 min at 25°C in 96-well flat-bottom microplates. After incubation, Griess reagent (1% w/v sulphanilamide and 0.1% w/v naphthylethylenediamide in 5% v/v phosphoric acid) was added and allowed to react during 10 min at 25°C and the absorbance was measured at 550 nm. Controls consisted of ethanol/PBS (for 0% of nitrite production) and sodium nitroprusside (for 100% of nitrite production). The eventual interference of the samples with the Griess reagent was also evaluated, using the same procedure but omitting the sodium nitroprusside. Scavenging

activity, determined in terms of percentage of nitrite formation, was present when the percentage of nitrite formed in the presence of the sample was less than 70% of the sodium nitroprusside control (De las Heras 1997).

Macrophage viability

To evaluate the eventual toxicity of both xanthenes and the respective nanocapsule formulations on J774 macrophages the MTT-assay was used (Mossman 1983). This assay measures the ability of viable cells to reduce the tetrazolium salt of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Briefly, macrophages plated in 96-well flat-bottom microplates were activated with IFN- γ /LPS and exposed for 24 h to serial concentrations of the different samples. Following this incubation period, the MTT solution (1 mg/ml) was added. After 4 h of incubation, formazan was solubilized overnight at 37°C in a sodium lauryl sulfate/*N,N*-dimethylformamide (SDS/DMF) solution (20% SDS in a 50% solution of DMF, pH 4.7). Absorbance of the coloured solution was measured at 550 nm. Results were expressed, as percentage of viable cells compared to control cells incubated with fresh culture medium (100% viability). Cell toxicity was considered when the viability of the exposed cells was less than 70%.

Uptake of nanocapsules by J774 macrophage cell line

The fluorescence exhibited by xanthone and 3-methoxyxanthone was used to study the cellular uptake of the respective nanosystems by fluorescence microscopy. Macrophages were plated at a density of 0.5×10^6 cells/ml onto glass cover slips in 6-well plates and allowed to adhere for 2 h. Then macrophages were activated with IFN- γ /LPS, as described above and incubated with 100 μ M of each nanocapsule formulation for 4 h at 4°C or at 37°C. After incubation, cells were washed three times with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were observed with an Axiovert 200M fluorescence microscope (Carl Zeiss, Germany) equipped with a filter set standard for DAPI. The images were acquired with an AxioCam Camera (Carl Zeiss, Germany) at different focus positions with intervals of 0.5 mm and deconvolved with Axiovision LE 4.1 (Carl Zeiss, Germany).

Statistics

Results are presented as mean values \pm SEM of at least three experiments. Paired *t*-test was used to evaluate the statistical significance of differences.

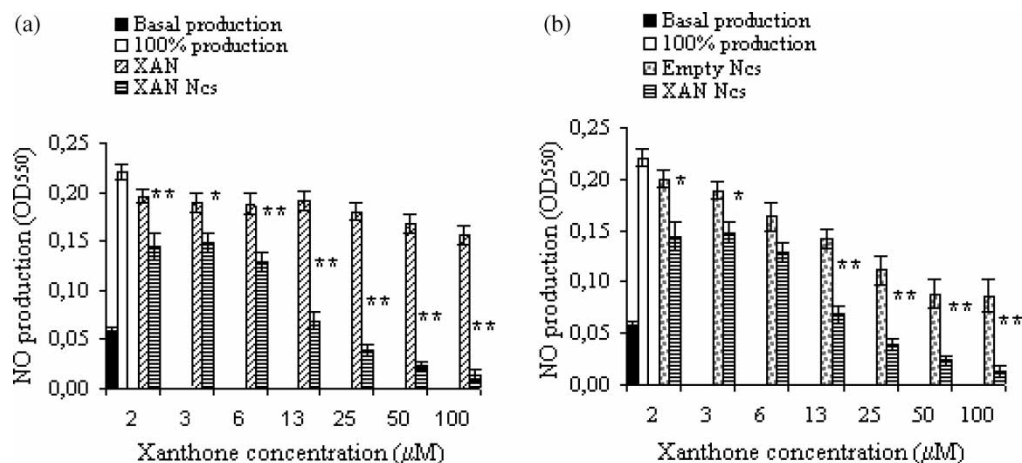


Figure 2. Effect of xanthone (XAN) and xanthone-loaded nanocapsules (XAN Ncs) (2a) and of empty nanocapsules (Empty Ncs) and xanthone-loaded nanocapsules (XAN Ncs) (2b) on NO production by IFN- γ /LPS stimulated J774 macrophages. Results show mean values \pm SEM ($n = 5$). * $P < 0.05$, ** $P < 0.01$.

P values < 0.05 or < 0.01 were considered statistically significant. Statistic calculations were performed with SPSS for Windows (Release 11.5).

Results and discussion

Effect of free and encapsulated xanthenes on NO production

The effect of xanthone, 3-methoxyxanthone and the respective nanocapsule formulations on NO production by the IFN- γ /LPS activated murine macrophage cell line J774 was evaluated (Figures 2 and 3). As shown in Figure 2a, NO production was weakly affected by the presence of free xanthone. In fact, even at the highest studied concentration (100 μ M) the inhibition of NO production was only 3.6%. In contrast, an equivalent concentration of xanthone

incorporated in nanocapsules produced a significant decrease on NO production (91.8%; $P < 0.01$). Results also showed that for all tested concentrations, xanthone-loaded nanocapsules were always associated with a significant decrease ($P < 0.05$) of NO production when compared with free xanthone.

Comparing Figures 2a and 3a, 3-methoxyxanthone showed a higher inhibitory effect on NO production than xanthone. In fact, a 100 μ M concentration of 3-methoxyxanthone caused a 16.5% inhibition of NO production. Moreover, the incorporation in nanocapsules of an equivalent concentration of 3-methoxyxanthone caused a decrease of 80%, showing that, as it was observed for xanthone, nanoencapsulation of this compound also leads to a significant ($P < 0.01$) improvement of its NO inhibitory effect.

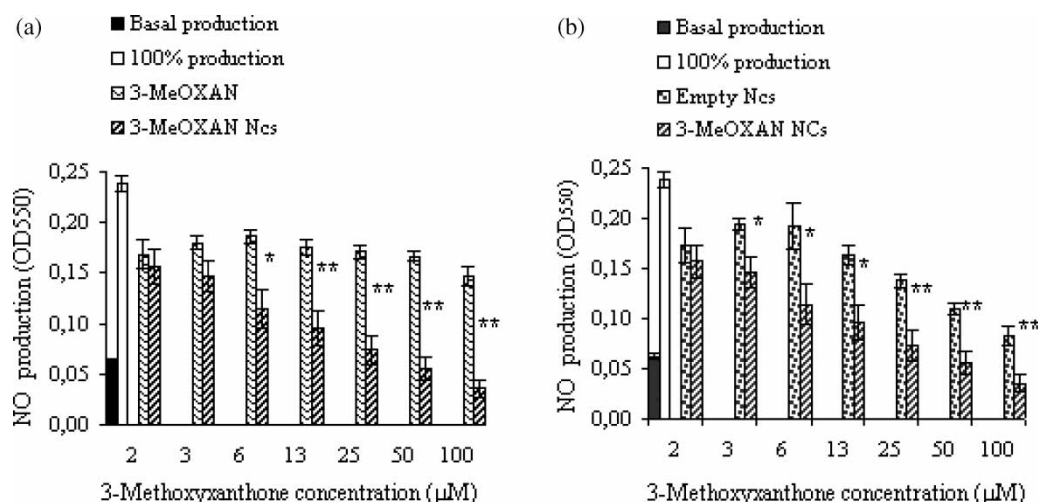


Figure 3. Effect of 3-methoxyxanthone (3-MeOXAN) and 3-methoxyxanthone-loaded nanocapsules (3-MeOXAN Ncs) (3a) and of empty nanocapsules (Empty Ncs) and 3-methoxyxanthone-loaded nanocapsules (3-MeOXAN Ncs) (3b) on NO production by IFN- γ /LPS stimulated J774 macrophages. Results show mean values \pm SEM ($n = 5$). * $P < 0.05$, ** $P < 0.01$.

Results from Figures 2b and 3b show that empty nanocapsules also produced an inhibitory effect on NO production. Nevertheless, the comparison of the inhibitory activities of empty and xanthenes-loaded nanocapsules showed that these latter were always associated with a significantly higher NO inhibitory effect ($P < 0.05$). Furthermore, the difference between the effects of nanocapsules containing xanthenes and empty nanocapsules was significantly higher ($P < 0.05$) than the effect of free xanthenes. Thus, an additive effect due to the presence of the xanthenes was accompanied by an effect due to the nanoencapsulation of the compounds.

NO inhibitory activity detected for all tested products was not due to a toxic effect, since the J774 activated macrophages exposed to the different samples always showed cell viability higher than 70%. A direct scavenging activity of NO either by the tested xanthonic compounds or by the nanocapsule formulations was also excluded since no NO scavenging activity was observed (data not shown).

In order to investigate which nanocapsule excipients were responsible for the NO inhibitory effect produced by empty nanocapsules, the following formulations were prepared: nanoemulsion (NE), nanospheres (NS), lecithin dispersion (LD), *Myritol 318* dispersion (MD) and *Pluronic F-68* solution (PS) (0.5% w/v). Each tested preparation had the same concentration of the different excipients present in xanthone-loaded nanocapsules, i.e. theoretical xanthone concentration equivalent. As shown in Figure 4 all formulations containing lecithin, such as empty nanocapsules, NE and LD, were associated with a reduction of NO production. Formulations without lecithin produced no reduction on NO levels. These results indicated that lecithin could be the excipient responsible for the inhibitory activity exhibited by empty nanocapsules. As it was previously reported by Aramaki et al. (1996), negatively charged unloaded liposomes containing phosphatidylserine or phosphatidic acid

also elicited an inhibitory effect on NO production by macrophages as a result of suppression of NOS induction. In the present work, soybean lecithin used for nanocapsule preparation contained approximately 40% of phosphatidylcholine and other components, such as phosphatidic acid and phosphatidylserine, which might explain the inhibitory activity exhibited by empty nanocapsules.

The improvement of the inhibitory effect of both xanthenes on NO production through nanoencapsulation could be due to an enhancement of the intracellular delivery of the compounds, as it was previously reported for different drugs (Morin et al. 1994, Wang and Zhang 2001). It is well known that the uptake of nanoparticles by macrophages occurs mainly by phagocytosis, which affords a specific targeting of drugs and immunomodulators to this type of cells (Maaßen et al. 1993, Legrand et al. 1999). Thus, phagocytosis of nanocapsules followed by release of the compounds in the lysosomes, as described by Seyler et al. (1999) for nanocapsules containing a derivative of muramyl dipeptide, might be the main mechanism by which the intracellular delivery of xanthone and 3-methoxyxanthone was promoted. Furthermore, xanthenes might also be transferred by diffusion after adsorption of nanocapsules onto the cells, or be released into the medium in the vicinity of cell membrane, generating a local concentration gradient that would favour the diffusion into the cells, as it was described by Maaßen et al. (1993) and Seyler et al. (1999).

Uptake of nanocapsules by J774 macrophage cell line

In order to confirm the occurrence of a phagocytic process in the internalization of the nanocapsules a fluorescence microscopy technique was adopted and performed at different focus positions that allows identification more clearly if the observed fluorescence is located inside or outside the cells. Microphotographs of cells incubated at 37°C (Figure 5a1 and b1) showed

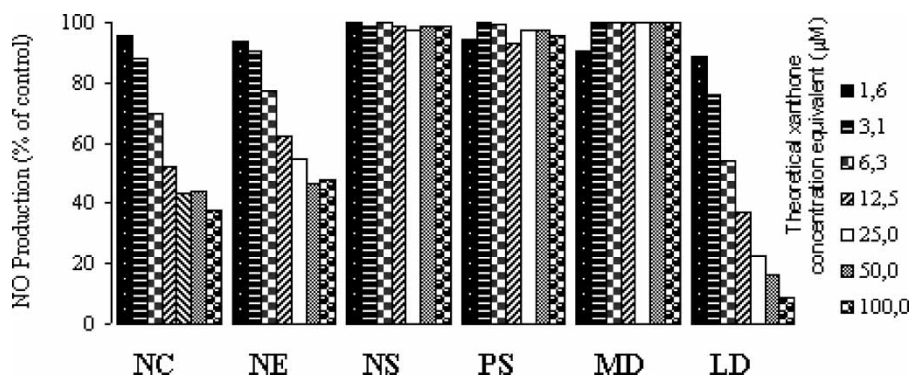


Figure 4. Effect of different formulations without xanthenes on NO production by IFN- γ /LPS stimulated J774 macrophages: Nanocapsules (NC), nanoemulsion (NE), nanospheres (NS), *Pluronic F-68* solution (PS), *Myritol 318* dispersion (MD) and lecithin dispersion (LD). Results show the mean values of three replicates from one representative experiment of two carried out independently.

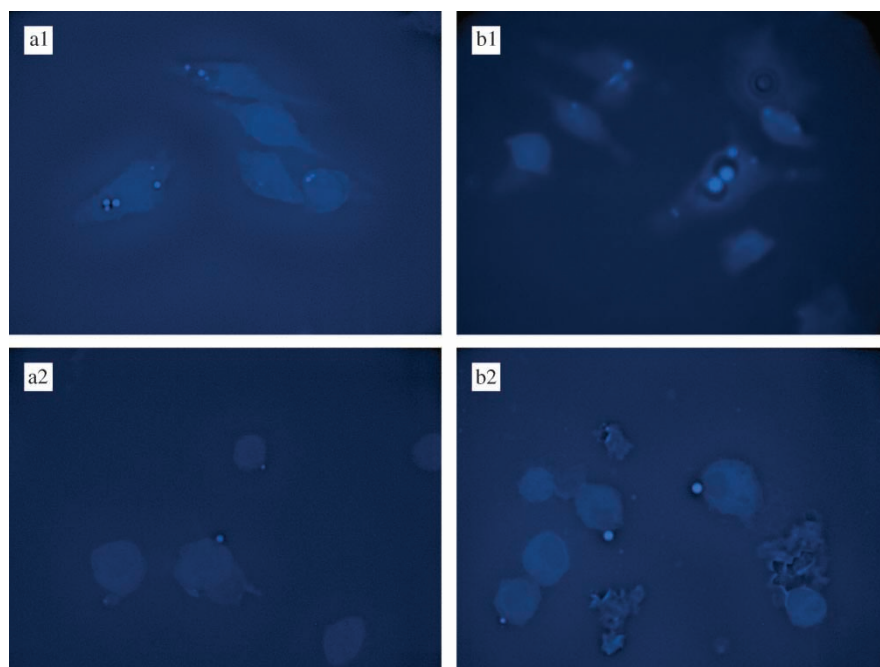


Figure 5. Fluorescent microphotographs of murine macrophage cell line J774 incubated with xanthone nanocapsules (a), and 3-methoxyxanthone nanocapsules (b) at 37°C (a1, b1) and 4°C (a2, b2) for 4 h.

intramacrophage blue fluorescence concentrated in vesicular compartments, corresponding to xanthone- or 3-methoxyxanthone-loaded nanocapsules. Incubation at 4°C resulted in a fluorescence located outside cells near macrophages surface (Figure 5a2 and b2). No fluorescence was observed either inside or outside the macrophages incubated with empty nanocapsules (results not shown). It is well known that the ability of macrophages to phagocytose particles is greatly suppressed at 4°C (Tabata and Ikada 1988). Thus, our results indicate that nanocapsule internalization, observed at 37°C, involves a phagocytic process, whereas at 4°C a passive binding of nanocapsules to macrophage surface may occur. These results are in accordance with those reported by Mosqueira et al. (2001) for PLA nanocapsules containing a fluorescent probe, using the same J774 macrophage cell line.

Conclusions

A weak inhibitory effect on NO production by LPS/INF- γ activated J774 macrophage cell line was observed for xanthone, even at the highest studied concentration (100 μ M). This effect was slightly higher for 3-methoxyxanthone. The encapsulation of these compounds in PLGA nanocapsules afforded a remarkable increase of the inhibitory effect on NO production. For xanthone-loaded nanocapsules a decrease of about 90% of NO production was achieved for 100 μ M concentration, whereas a reduction of

80% was observed for an equal concentration of 3-methoxyxanthone-loaded nanocapsules.

Fluorescence microphotographs confirmed that a phagocytic process was involved in the uptake of xanthone- and 3-methoxyxanthone-loaded PLGA nanocapsules by macrophages. Phagocytosis might be the main responsible mechanism for the enhancement of the intracellular delivery of both xanthenes and consequently for the improvement of their biological effect.

Although the mechanism by which xanthone and 3-methoxyxanthone inhibits NO production by activated macrophages is not yet elucidated, the results presented here suggest that nanocapsule formulations of these xanthenes are promising systems that might be used in disorders associated with production of high amounts of NO by macrophages. Moreover, these results are very encouraging for further studies concerning the improvement of other biological activities of xanthone and its derivatives through their incorporation in nanoparticles.

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