Nanostructured lipid carriers (NLC) for the delivery of natural molecules with antimicrobial activity: production, characterization and in vitro studies

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ABSTRACT

This study describes the preparation, characterization and in vitro activity of nanostructured lipid carriers (NLC) encapsulating natural molecules with antimicrobial activity, namely plumbagin, hydroquinon, eugenol, alpha-asarone and alpha-tocopherol. NLC were prepared by melt and ultrasonication method and characterized by Cryo-TEM for morphology and SdFFF for dimensional distribution and active encapsulation yields. In vitro tests conducted on bacteria, fungi and human cell culture. In vitro tests demonstrated that plumbagin is strongly toxic towards F. oxysporum especially when active molecules are loaded on NLC. Plumbagin was completely non toxic on cyanobacterial model strain up to a threshold over which cell viability was completely lost. NLC loaded with active molecules showed a lower toxicity as compared to their free form on human cultured cells. Notwithstanding further studies have to be performed, these systems can be potentially proposed to control phytopathogenic organisms.

Abbreviations: Nanostructured lipid carriers: NLC; cryogenic transmission electron microscopy: cryo-TEM; photon correlation spectroscopy: PCS; Sedimentation Field Flow Fractionation: SdFFF; Plumbagin: PB, Hydroquinon: HQ, Eugenol: EU, α-Asarone: aA; α-Tocopherol: aT; Methanol: MeOH; dimethyl sulfoxide: DMSO.

Keywords: Nanoparticles, Nanotechnology, Formulation, Nanostructured lipid carriers.
Introduction

The long-term use of synthetic agrochemicals has led European governors and institutions to strongly suggest and support research to find new eco-friendly solutions for agricultural practices reducing environmental pollutants and increasing the use of bio-based products for pest control (Copping and Duke, 2007; Duke et al., 2008; Dayan et al., 2009).

Despite the proved antimicrobial activity against phytopathogens of several natural extracts or pure natural compounds (Balestra et al 2009; Dan et al., 2010; Pradhanang et al., 2003; Slusarenko et al., 2008; Varvaro et al., 2001, Cowan, 1999; Scalbert et al., 1991; Shihabudeen et al., 2010; Tsuchiya et al., 1996), their in-field application points out some toughness, mainly represented by the low solubility in water of the extracts, their instability and also by their handling and storage characteristics. A possible effective solution to these critical points is represented by the use of micro- or nano- encapsulation strategies that may minimize the natural bioactive compound(s) degradation optimizing the efficacy as pest-controller. Micro- or nano- particles can be constituted by non-toxic, chemically inert (inactive), biocompatible polymer or lipid matrix able to alloy both bioactive compound(s) and excipients without altering their chemical and physical properties (Battaglia et al., 2012; Nysrtom and Fadeel, 2012; Cortesi et al., 2002; Esposito et al., 2002; Takei et al., 2008; Glenn et al., 2010; Gonzales et al., 2015).

In the present study, lipid-based nanoparticles, namely nanostructured lipid carriers (NLC), are considered as a versatile tool with a high potential of applications. Indeed, NLC can solubilize a number of molecules with different physico-chemical properties in a biocompatible and biodegradable matrix with well-established safety profiles. Moreover, lipid-based nanoparticles can significantly contribute in the field of green nanosystems due to their derivation from natural source. The production of NLC was carried out using blends of solid (e.g. triglycerides) and liquid (e.g. tricaprylin) lipids at room and body temperatures (Saupe et al., 2005) obtaining an imperfect and
disordered lipid matrix able to accommodate poor water-soluble compounds (Yoon et al., 2013) maintaining their physical stability for long periods of time.

Starting from these premises, the main target of the current study was to produce by standard protocols NLC characterized in terms of morphology, inner structure, dimensional distribution and encapsulation yield (Jores et al., 2004). NLC were then checked as encapsulation and solubilisation tool for natural bioactive pure compounds - namely Plumbagin, Hydroquinone, Eugenol, α-Asarone and α-Tocopherol - chosen for their physical-chemical characteristics and their known in vitro bioactivity (Kumar et al., 2013; Xu et al., 2015; Rajput et al., 2014; Teixeira et al., 2016). Therefore, the aim and novelty of the research is to check the opportunity to use NLC as encapsulating technology for bioactive natural compounds to be used in agriculture, trying to make bio-based products competitive in the market of agricultural treatments products.

Materials and methods

Materials

Plumbagin (PB), Hydroquinone (HQ), Eugenol (EU), α-Asarone (aA) and α-Tocopherol (aT) were purchased from Sigma-Aldrich (Saint Louis, MO, USA) for checking and comparing their biological activities as pure and microencapsulated compounds. The copolymer poly (ethylene oxide) (PEO, a) -poly (propylene oxide) (PPO, b) (a=80, b=27) (poloxamer 188) was from BASF ChemTrade GmbH (Burgbernheim, Germany). Caprylic/capric triglycerides (Miglyol 812 N, miglyol) were from Cremer Oleo Division (Witten, Germany). Tristearin (stearic triglyceride), polysorbate 80 and all other chemicals were from Sigma-Aldrich (Milano, Italy).

Preformulatory study
NLC were prepared by melt and ultrasonication method (Esposito et al., 2008). Briefly, 0.25 g of lipid mixture was melted at 80 °C. The lipid mixture was constituted of tristearin/miglyol 2:1 w/w. 4.75 ml of an aqueous poloxamer 188 solution (2.5 % w/w) at 80 °C were poured into the vial containing the molten lipids. The mixture was then emulsified at 15000 rpm, 80 °C for 1 min, using a high-speed stirrer (Ultra Turrax T25, IKA-Werke GmbH & Co. KG, Staufen, Germany). The emulsion was subjected to ultrasonication (Microson TM, Ultrasonic cell Disruptor) at 6.75 kHz for 15 min and then cooled down to room temperature.

In the case of active containing NLC, 0.2% w/w (with respect to total weight of dispersion) of each active (i.e. PB, HQ, EU, aA and aT) was added to the lipid mixture and dissolved before addition to the aqueous solution. Then the preparation was conducted as above described. The obtained dispersions were stored at room temperature and protected from the light until used.

**Characterization of NLC**

**Cryo-Transmission Electron Microscopy**

Samples vitrified as previously described (Esposito et al., 2008) were transferred to a Zeiss EM922Omega transmission electron microscope for imaging using a cryoholder (CT3500, Gatan). Throughout the examination, the sample temperature was kept below -175 °C. Specimens were examined with doses of about 1000-2000 e/nm^2 at 200 kV. Images were recorded digitally using a CCD camera (Ultrascan 1000, Gatan) by mean of an image processing system (GMS 1.9 software, Gatan).

**Photon Correlation Spectroscopy (PCS)**

Submicron particle size analysis was performed using a Zetasizer Nano Series, Nano SP90 (Malvern Instr., Malvern, England) equipped with a 5 mW helium neon laser with a wavelength output of 633 nm. Glassware was cleaned of dust by washing with detergent and rinsing twice with water for
injections. Measurements were made at 25 °C at an angle of 90°. Data were interpreted using the CONTIN method (Pecora, 2000).

Sedimentation Field Flow Fractionation Analysis

A sedimentation field flow fractionation analysis (SdFFF) system (Model S101, FFFractionation, Inc., Salt Lake City, UT, USA) was employed to determine the size distribution of particles by converting the fractograms, i.e. the graphical results (Contado et al., 1997). Fifty microliter samples were injected through a 50 µl Rheodyne loop valve. As mobile phase demineralized water pumped at 2.0 ml/min was used. After the SdFFF system fractions of 3 ml, being the collecting time 90 sec, were automatically collected by a Model 2110 fraction collector (Bio Rad laboratories, UK).

Active content in NLC

The encapsulation efficiency (EE) and loading capacity (LC) of NLC were determined by centrifugation followed by dissolution of nanoparticles in methanol as previously described (Puglia et al., 2013). 100 µl of each NLC batch was loaded in a centrifugal filter (Microcon centrifugal filter unit YM-10 membrane, NMWCO 10 kDa, Sigma Aldrich, St Louis, MO, USA) and centrifuged (Spectrafuge™ 24D Digital Microcentrifuge, Woodbridge NJ, USA) at 8,000 rpm for 20 min. The amount of active in both the lipid and the hydrophilic phase was determined by high performance liquid chromatography (HPLC), analyses as below reported. The encapsulation parameters were determined as follows.

\[
EE = \frac{L_{active}}{T_{active}} \times 100
\]

\[
LC = \frac{L_{active}}{T_{lipid}} \times 100
\]

where \(L_{active}\) is the amount of active encapsulated in NLC; \(T_{active}\) and \(T_{lipid}\) are the total weight of active and lipid used for the NLC preparation, respectively.
**HPLC Procedure**

The HPLC apparatus consisted of a two-plungers alternative pump (Jasco, Japan), an UV-detector operating at the λ reported in Table 1, and a 7125 Rheodyne injection valve. RP-HPLC analysis was performed using a stainless steel C-18 reverse-phase column (150×4.6 mm) packed with 5 µm particles (Zorbax® Eclipse XDB - Agilent, USA). A pre-column filter Alltima C18 5µm (7.5x4.6 mm) was mounted above the column. Samples of 50 µl were injected through a 50 µl Rheodyne loop valve. For each active the HPLC conditions are summarized in Table I.

**In vitro release studies**

In vitro release studies were performed using the dialysis method. Typically, 1.5 ml of NLC suspension were placed into a dialysis tube (8 cm) (molecular weight cut off 10,000-12,000; Medi Cell International, England), then placed into 30 ml of receiving phase constituted of phosphate buffer (100 mM, pH 7.4) and ethanol (70:30, v/v) and shaken in a horizontal shaker (MS1, Minishaker, IKA) at 175 rpm at room temperature (23-25 °C). Samples of receiving phase were withdrawn at regular time intervals, and analyzed by HPLC method as above described. Fresh receiving mixture was added to maintain constant volume. The encapsulation efficiency of each active was determined four-fold in independent experiments and the mean values ± standard deviations were calculated. The obtained release data were fitted to the following semiempirical equations describing Fickian dissolutive and diffusional release mechanisms, as previously indicated by Esposito et al. (2005).

Precisely,

\[ \frac{M_t}{M_\infty} = K_{diff} t^{0.5} + c' \]  

(3)

\[ 1 - \frac{M_t}{M_\infty} = e^{-K_{diff} t} + c \]  

(4)

where \( M_t \) is the active fraction released at the time \( t \), \( M_\infty \) is the total active content of the analyzed amount of NLC, \( K \), \( c \) and \( c' \) are coefficients calculated by plotting the linear forms of the indicated
equations. The release data up to the plateau of percent were used to produce theoretical release
curves.

In vitro activity against phytopathogens

Clavibacter michiganensis subsp. nebraskense (ATCC 27822), Pseudomonas syringae pv. syringae
(ATCC 19310), Agrobacterium tumefaciens (DMS 30207), Agrobacterium vitis (DMS 6583) were
used as phytopathogen bacterial strains. For testing the antifungal capacity, Alternaria alternata
(SIAPA, Italy) and Fusarium oxysporum f. sp. radicis licopersici (SIAPA, Italy) were employed.
The culture media – all purchased from Oxoid (http://www.oxoid.com/uk/) – were: Tryptone Soya
Broth for C. michiganensis subsp. nebraskense and P. syringae pv. syringae; Nutrient Broth for A.
tumefaciens and A. vitis; Potato Dextrose Broth for A. alternata and F. oxysporum f. sp. radicis
licopersici.
The antimicrobial activity was assessed in triplicate by the microdilution broth method in 96-well
microplates (Microplate reader - Model 680 XR, BIO-RAD) as Minimum Inhibitory Concentration
(MIC) values of the pure and microencapsulated compounds (Furtado et al., 2014). Pure and
microencapsulated compounds PB, HQ, EU, aA and aT were dissolved to reach a final concentration
of 0.1 mg/ml in the specific culture media for each microorganism and dimethyl sulfoxide (2%;
DMSO). This solution was then progressively diluted following a 1:2 ratio for each test dilution to
determine the MIC values considered as the lowest concentration of each compound (pure and
microencapsulated) that inhibits the growth of the microorganisms. Each well contained a final
volume of 200 μL with 2x10⁷ CFU/ml and 1x5x10³ spores/ml, for bacterial and fungal strains
respectively.
The microplates with bacterial strains were incubated under gentle shaking (110 rpm) for 24 h at
28°C. Before (time 0) and after incubation (24h), the microplates were read at 615 nm to check the
bacterial growth. Then, 40 μL of the dye 2,3,5-triphenyl-tetrazolium chloride (20 mg/ml) were added
in each well. Absorbance was recorded at 415 nm (time 0) and after 2 h of further incubation to check
bacterial viability. DMSO (2%) and chloramphenicol (0.1 mg/ml) were used as negative and positive
control respectively.

To check the antifungal activity, the microplates were incubated under gentle shaking (110 rpm) for
5 days at 26°C. After incubation, the fungal growth was visually determined. DMSO (2%) was used
as negative control.

**In vitro activity on algae**

The cyanobacterium Synechococcus elongatus, strain PCC 6301, was grown at 24 ± 1 °C under 14-h
days (150 µmol m⁻² s⁻¹ PAR) and 10-h nights as previously described (Demuner et al., 2013). Late
log-grown cells were settled by centrifugation 5 min at 4,000 x g, and used to inoculate 96-well plates,
0.2 ml per well, to an initial density of about 1.0 mg L⁻¹ chlorophyll. Aliquots (2 µL) of suitable
dilutions of either 50 mg ml⁻¹ active stock solutions in DMSO or active-containing NLC dispersions
encapsulating the same molecules were added to obtain final concentrations ranging from 0.25 to 250
mg l⁻¹. A complete randomized design with eight replications was adopted. Cell growth in each well
was followed for one week by daily determination of absorbance using a Ledetect 96 plate reader
(Labexim, Lengau, Austria) equipped with a LED plugin at 660 nm, subtracting turbidity at 750 nm.
Following logarithmic transformation of data, growth constants were calculated, and expressed as
percent of the mean value for controls treated with the same volume of DMSO or non-active-
containing NLC dispersions. Mean values ± SE over replicates are reported. The concentrations
causing 50% inhibition (IC₅₀) of cyanobacterial growth and their confidence limits were estimated by
nonlinear regression analysis using Prism 6 for Windows, version 6.03 (GraphPad Software).

**In vitro activity on human cells**
Human lung epithelial A549 cells (ATCC; Manassas, VA) were grown in F-12K nutrient mixture (Invitrogen, Carlsbad, CA), supplemented with 10% Foetal Calf Serum (FCS) and 1% penicillin/streptomycin on Vitrogen-coated (Collagen Corporation, Palo Alto, CA) Costar clear Transwells (0.4-mm pore size; Costar Corporation, Cambridge, MA) until a confluent monolayer was established (approximately 4 × 10^6 cells/well).

Human keratinocytes HaCaT cells (a cell line gift from Dr. F. Virgili) were grown in Dulbecco’s modified Eagle’s medium High Glucose (Lonza, Milan, Italy), supplemented with 10% FCS, 100 U/ml penicillin, 100 lg/ml streptomycin and 2 mM L-glutamine as previously described (Sticozzi et al., 2012). HaCaT cells were treated with different NLC doses (1 and 10 mM) at different time points (1 and 24 h).

Free actives were dissolved in ethanol at a concentration of 10 mM as a stock solution then diluted to the required concentrations directly in the medium. The final concentration of ethanol in culture medium did not exceed 0.1% (v/v).

Viability studies were performed 24 h after the treatments and determined by cytofluorimetric assay Muse Count & Viability Kit (Millipore Corporation, Billerica, MA, USA). Briefly, 380 µL with Muse Count & Viability working solution was added to cells (1x10^6 cell/ml), and 20 µL of this cell suspension was incubated for 5 min at room temperature in the dark. L-lactate dehydrogenase (LDH) release was measured by enzymatic assay as previously described (Sticozzi et al., 2012) following the kit instructions (EuroClone, Milan, Italy). All tests were performed in triplicate and repeated at least three times.

**Results**

**Preparation of NLC**

The molecules considered in the present study, namely PB, HQ, EU, A and T were selected on the basis of their physico-chemical characteristics, such as the low solubility in water (see Table 1),
and also of their \textit{in vitro} bioactivity (Hammer et al., 1999; Naz et al., 2007; Prabuseenivasan et al., 2006; Pereira et al., 2007). The production of bio-friendly NLC was performed by a two-step protocol based on the emulsification of the molten lipid phase in an aqueous phase containing poloxamer 188 under high speed stirring at 15000 rpm, followed by treatment with probe ultrasound.

After preparation, the dispersions appeared white milky and free of aggregates and sediment. In the case of PB nanoparticle, due to the color of the molecule, the milky dispersion is yellow colored (Figure 1). Serial dilutions of the dispersions gave rise to a progressive decrease of the scattering intensity and of the milkiness.

The determination of the EE and LC was performed after centrifugation of the dispersions. As reported in Table 2, apart from HQ whose EE was around 31%, all the preparation showed an almost quantitative encapsulation within the lipid matrix of the nanoparticles, as proved by the very high EE. The behavior of the LC results was in agreement with those obtained for EE.

\textit{Characterization of dispersions}

\textit{Cryo-TEM}

In order to shed light on the general morphology and internal structure of the dispersed particles in NLC dispersions, Cryo-TEM analyses were conducted. Figure 2 shows cryo-TEM images of NLCs, including samples of empty NLC (A), PB-NLC (B), EU-NLC (C), HQ-NLC (D), aA-NLC (E) and aT-NLC (F). As reported in literature, the use of blends of lipids in solid (i.e. tristearin) and liquid state (i.e. miglyol) leads to the formation of imperfect lipid matrix structures with the absence of clearly identifiable oil droplets in the NLC samples (Saupe et al., 2005; Esposito et al., 2012). In all panels are visible platelets oriented at 0° and 180° appearing as ellipsoidal platelet-like crystalline particles and dark structures edge-on viewed. No significant differences are appreciable between empty and active loaded nanoparticles indicating that the presence of the active compound does not affect the NLC aspect.
Table 3 summarizes the dimensional size distribution of NLC as determined by PCS measurements. Notwithstanding all the prepared NLC formulations showed a bimodal distribution characterized by a Z-average mean diameter ranging between 200 and 240 nm, they possess the appropriate dimensional characteristics for the environmental distribution by spraying or nebulization. NLC’s size distribution was also determined by SdFFF method that allows to measure the particle size distribution within the sample, by retaining particles of different diameter and eluting them at a calculable time (Merkus et al., 1989). Indeed an observed retention time can be directly related by theory to the mass of the eluting particles and transformed into a size and the UV signal into a mass frequency function (Contado et al., 1997; Esposito et al., 2008). The obtained fractograms are thus converted into PSD plots according to well-proven equations. Since particle in SdFFF are sorted based on their specific mass, knowing the particle density and by supposing a spherical shape, the sizes represent diameter of an equivalent sphere (Bunjes, 2005). The NLC’s density was calculated as previously reported (Esposito et al., 2015). In Figure 3 the PSD plots of three NLC samples taken as an example, namely PB-NLC, EU-NLC and aT-NLC, are reported. In general it can be seen that the three samples subjected to SdFFF are characterized by a size distribution superimposable to that obtained by PCS. Particularly, all the three samples show a quite narrow peak around 100 nm and a secondary broad population spanning between 170 nm up to 600-800 nm. It is interesting to underline that PCS and SdFFF are based on completely different rational principles. Indeed, PCS assesses the size considering the diffusion of the scattered light, thus the obtained results depend on the particles’ position when the laser ray hits them. On the other hand SdFFF "weighs" in some extent the particles being considered as compact spheres of uniform density. In this respect the complementary evaluation of size distribution by PCS and SdFFF are important to obtain realistic information about particle size.
SdFFF was also employed to obtain information about the encapsulation of active compounds. The amount of each molecule encapsulated in the NLC was determined by HPLC after diluting with the mobile phase, by injecting the eluate exiting from the SdFFF channel collected during the fractionation. The injection results are reported in Figure 3 (square dots), where it is clearly evident that each active is entirely associated with NLC.

Concerning stability studies, macroscopic aspect of the formulations and their size stability was evaluated over a period of six months from preparation. The formulations were sealed in glasses vials and maintained at room temperature up to six months. No changes in macroscopic aspect (i.e. precipitation, agglomeration or phase separations) were detectable. In addition, as reported in Table 4, the produced NLC maintain almost unchanged their dimensions.

**In vitro release of active from NLC**

In order to obtain quantitative and qualitative information about the active encapsulated in NLC, namely PB-NLC, EU-NLC, aA-NLC and aT-NLC, an *in vitro* release profile was determined by a dialysis method (Figure 4). Since the tested natural molecules are scarcely soluble in water, their diffusion kinetic in aqueous physiological receptor phases is undetectable. Therefore as suggested by literature, a non-physiological receptor phase composed of phosphate buffer and ethanol (70:30) was used in order to allow the establishment of the sink conditions and to sustain permeant solubilization (Siewert et al., 2003). The theoretical release curves were determined according to the linear form of Eqs. (3) and (4), mimicking a diffusive and a dissolutive model, respectively (Esposito et al., 2005). The comparison between the theoretical curves calculated from equations (3) and (4) and the experimental curves obtained for NLC showed that the experimental curves are superposable to the theoretical curves referring to diffusive kinetics (data not shown). This was confirmed also by R and R² values found by linearization of Eq. (3) reported in Table 5.
Antimicrobial activity against phytopathogens

PB, EU, aA, aT, both as pure compounds and encapsulated in NLC, were tested for antimicrobial activity against phytopathogenic bacteria and fungi (Table 6). The choice of the kind of microorganisms was driven by their particular incidence as etiological agents for important arboreal and herbaceous cultures (Schumann and D'Arcy, 2009). In particular, the bacterial strains belonging to *Clavibacter michiganensis* subsp. *nebraskense* (CM; Leaf freckles disease), *Pseudomonas syringae* pv. *syringae* (PSS; Necrotic leaf spots, stem cankers and other symptoms related to the different plant and part infected), *Agrobacterium tumefaciens* (AT; crown gall disease), *Agrobacterium vitis* (AV; crown gall of grape disease) were used, while *Alternaria alternata* (AL; leaf spot symptoms and other diseases related to the different plant infected) and *Fusarium oxysporum f. sp. radicis licopersici* (FO; severe necrotrophic symptoms) were employed as phytopathogen fungi. HQ, EU, aA, αT did not evidenced interesting bioactivities both as pure and micro-encapsulated showing growth inhibitory properties only at concentrations equal or higher than 200 μg/ml against all employed microorganisms. PB showed instead valuable results against the bacterial strains AT, AV and CM with MIC values ranging from 6.3 to 12.5 μg/ml as pure molecule, and from 12.5 to 25 μg/ml in microencapsulated form. PSS strain was more resistant to both the treatments with PB and PB-NLC showing equal results (MIC=100 μg/ml). Similar sensitivity was evidenced by the fungal phytopathogen AL, but the strongest and most interesting evidences were given by FO, in particular by those cultures treated with PB-NLC (MIC=12.5 μg/ml) showing a sensitivity about 8-folds higher than those treated with the sole pure PB (MIC=100 μg/ml).

Activity on a cyanobacterial model strain

PB, EU, aA, aT, both as pure compounds and encapsulated in NLC, were tested *in vitro* on cyanobacterial strain and human cells. Cyanobacteria, also known as blue-green algae, are ubiquitous photosynthetic prokaryotes that can be found in both terrestrial ecosystems and freshwaters, and are
particularly abundant under eutrophic conditions, as those in most cultivated fields. For these reasons, cyanobacterial strains are well suitable to assess non-target effects of phytochemicals. The addition of increasing levels of PB, EU and aA to the culture medium of a *Synechococcus sp.* model strain caused a marked inhibition of bacterial cell proliferation (Figure 5), with concentrations inhibiting growth by 50% (IC$_{50}$) equal to 60 ± 6, 36 ± 3 and 2 ± 1 µg/ml, respectively. When the same natural compounds were administered as encapsulated in NLC dispersions, in all cases an increased toxicity was evident, with IC$_{50}$ values of 16 ± 2, 15 ± 1 and 0.4 ± 0.2 µg/ml for EU, aA and PB, respectively. Interestingly, while for PB and EU a progressive, dose-dependent toxicity was found, in the case of PB the addition was ineffective up to a concentration threshold, over which cell viability was completely lost. A behavior completely different was observed for aT since, as expected, no toxic effect was detected for aT, either in the free or NLC form.

**Effect on human cell lines of NLC loaded with active compounds**

Experiments on human cells were conducted testing the formulations that showed toxic activity on phytopathogens and cyanobacteria. Concerning human keratinocytes as it is shown in Figure 6, treatment with aA and EU either as free compound or microencapsulated forms did not affect significantly cell viability at the doses ranging from 10.41 to 104.1 and 8.21 to 82.1 µg/ml respectively. Of note is that when EU was loaded in NLC, the higher doses (82.1 µg/ml) significantly reduced cellular viability of about 35%. A completely different response was observed with PB treatment, indeed either alone or loaded in NLC PB resulted extremely toxic to keratinocytes ranging from the doses of 9.41 to 94.1 µg/ml.

In the case of human lung epithelial cells, as it is shown in Figure 7 treatment with aA affected cellular viability in lung cell only at the higher doses (104.1 µg/ml) but this effect was abolished when it was loaded in NLC. Similar pattern was also noted for EU, where at the doses of 82.1 decreases cell viability of about 50%, while once loaded in NLC this effect was eliminate.
Pretreatment with PB, as only active compound, significantly affects cellular viability at all doses of about 55%. This effect was abrogated when PB was loaded in NLC, demonstrating a completely no toxic cellular effect.

**Discussion**

In recent years, the need to use more eco-friendly and natural-derived tools in agricultural treatments for pest control has become increasingly urgent due to dramatic environmental issues and effects on human and animal health caused by agrochemicals. Several researches were aimed to find plant extracts, fractions or pure natural compounds active against plant pathogens. Moreover, the strong need to find a diffusion tool effective in preserving the bioactive molecule and in promoting at the same time the biological activity lowering toxicity was given (Friedman et al., 2002; Balestra et al., 2009; Quattrucci et al., 2013; Pradhanang et al., 2003; Slusarenko et al., 2008; Dan et al., 2010). In light of these premises, nanoparticles enable better penetration into tissues for instance influencing the effect of the carried molecule by inducing faster penetration or direct contact, or increasing the exposure time of the biological active molecule to the phyto-pathogen(s) (Nel et al. 2006, 2009; Margulis-Goshen and Magdassi 2012). Eco-friendly nanoparticles are needed, by employing biocompatible solvents and renewable materials (i.e. lipids or polymers), or organic-solvent-free processes. This study was undertaken in order to provide an eco-friendly and effective encapsulation strategy for natural bioactive molecules to obtain a NLC-Biomolecule tool. The obtained system was studied *in vitro* using different strategies and biological models to check the biological activity against phytopathogens (fungi and bacteria), the potential toxicological impact involving environment (algal model system) and humans (human cell lines) (Kah et al. 2013).

The nanoencapsulation of the selected active molecules for appropriate parameters allowed the improvement of their solubility in aqueous formulation, solving the drawbacks associated with their use up to at least 6 months from preparation. In addition, PCS and SdFFF techniques applied to
investigate the average size of the particles indicated that dimensions slightly increased during 180
days of storage time (data not shown). These data allow us to exclude the occurring of agglomeration
process during the storage of these formulations. Possibly, the surfactant characteristics of poloxamer
present within NLC composition prevent this from happening.

Cryo-TEM images revealed nanoparticles in good dispersion, in the nanometric range (according
with PCS and SdFFF results) with the characteristic ovoidal and ellipsoidal platelet-like crystalline
aspect unaffected by the presence of the active molecule. The analysis of in vitro release of the tested
active molecules from NLC suggested that it has to be ascribed mainly to dissolution rather than
diffusion rate confirming previously reported results concerning molecules encapsulated in lipid
based nanosystems (Esposito et al., 2012).

Results of in vitro assays performed against phytopathogenic bacteria and fungi were not promising
in view of applicative large scale use in integrated or organic agricultural treatments, except for PB.
The results of the pure PB against AT, AV and CM were particularly relevant, but the micro-
encapsulation reduced the efficacy from two- to four-folds, limiting the possibility of an effective use
of PB in agricultural formulations. Not particularly relevant were the results against PSS, with equal
MIC values (100 μg/ml), according to the known evidence of the higher resistance to treatments of
Gram negative bacteria than that of Gram positive ones (Chapman et al., 2016 and references therein).

The most interesting results were obtained against FO (severe necrotrophic symptoms). In fact,
contrary to what obtained with other microorganisms, PB-NLC showed an efficacy 8-folds higher
(MIC=12.5 μg/ml) than that observed in the assays with the pure compound (MIC=100 μg/ml). This
result is also relevant for the selective efficacy of the PB-NLC system suggesting the opportunity to
perform specific treatments without affecting the non-phytopathogenic fraction of the microbiota
pointing out a further eco-friendly aspect of the research approach.

Similar results were obtained in vitro with a cyanobacterial model strain. Also in this case all the
tested molecules increased their efficacy when administered in NLC (demonstrated by lower IC_{50}
values) as compared to the correspondent free form. This suggests that a low uptake may limit their effectiveness, and that lipid encapsulation may help the active principles to reach their targets inside the cells. Consistently, and differently from data obtained for fungi, the addition of PB was completely ineffective up to concentration threshold over which cell viability was completely abolished. Although further experiments are required to shed light on these aspects, this suggests that a higher external concentration of PB is required to allow cell internalization, than to exert cytotoxic effects.

Overall, data indicate that the active molecules considered in this study when loaded on NLC showed a lower activity against phytopathogens as compared to their free form suggesting that NLC is able to prevent their possible toxicity by the slower release of the molecules in the tissues. However, the opposite was true for cyanobacterial cells and PB-NLC, thus suggesting that in particular cases their use may provide better results than the free form. The rationale of testing those molecule in keratinocytes and epithelial lung cells was based on the fact that these tissues (skin and respiratory tract) are the main to be exposed to environmental molecules. From this study it is possible to read that the use of NLC reduced the toxicity of the molecules analyzed most likely because they allow the slow release of the active compound, avoiding the cells to be exposed to high dose (often toxic) of natural extract.

**Conclusions**

Taken together, the results described in the present study allow us to potentially propose NLC loaded with natural molecules as an eco-friendly and biocompatible strategy possibly useful in the control of phytopathogenic organisms in agricultural treatments. To this aim, further intensive studies have yet to be performed in order to investigate the efficiency of these systems in greenhouse and in field.
Acknowledgements

Authors are grateful to Dr. B. Grillini and Dr. G. Pavoni for technical issues. The authors thank the CAMERA DI COMMERCIO, INDUSTRIA, ARTIGIANATO E AGRICOLTURA (CCIAA) of Ferrara BANDO ANNO 2012 project for financial support.
References


Copping LG, Duke SO. Natural products that have been used commercially as crop protection agents. Pest Manag. Sci. 2007;63:524-28.


Table 1 Physico-chemical characteristic and HPLC conditions of the actives considered in the present study

<table>
<thead>
<tr>
<th>chemical structure</th>
<th>PB</th>
<th>EU</th>
<th>HQ</th>
<th>αA</th>
<th>αT</th>
</tr>
</thead>
<tbody>
<tr>
<td>chemical formula</td>
<td>C_{11}H_{8}O_{3}</td>
<td>C_{10}H_{12}O_{2}</td>
<td>C_{6}H_{4}(OH)_{2}</td>
<td>C_{12}H_{16}O_{3}</td>
<td>C_{20}H_{50}O_{2}</td>
</tr>
<tr>
<td>water solubility</td>
<td>low</td>
<td>low</td>
<td>low</td>
<td>not soluble</td>
<td>not soluble</td>
</tr>
<tr>
<td>melting point (°C)</td>
<td>78-79°C</td>
<td>-10°C</td>
<td>172°C</td>
<td>62-63°C</td>
<td>2-4°C</td>
</tr>
<tr>
<td>molar mass (g/mol)</td>
<td>188.18</td>
<td>164.21</td>
<td>110.11</td>
<td>208.25</td>
<td>430.71</td>
</tr>
<tr>
<td>elution phase (v/v)</td>
<td>MeOH/Water (90:10)</td>
<td>MeOH/Water (60:40)</td>
<td>MeOH/Water (40:60)</td>
<td>MeOH/Water (70:30)</td>
<td>MeOH (100)</td>
</tr>
<tr>
<td>flux (ml/min)</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>λ (nm)</td>
<td>254</td>
<td>215</td>
<td>290</td>
<td>258</td>
<td>295</td>
</tr>
<tr>
<td>retention time (min)</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 2 Encapsulation efficiencies and loading capacities of the active in the produced NLC

<table>
<thead>
<tr>
<th>NLC</th>
<th>Encapsulation efficiency (%)</th>
<th>Loading capacity (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB</td>
<td>89.1 ± 1.25</td>
<td>4.21 ± 1.02</td>
</tr>
<tr>
<td>HQ</td>
<td>31.1 ± 1.84</td>
<td>1.67 ± 0.11</td>
</tr>
<tr>
<td>EU</td>
<td>81.4 ± 1.11</td>
<td>3.92 ± 0.71</td>
</tr>
<tr>
<td>αA</td>
<td>92.6 ± 0.77</td>
<td>4.63 ± 0.20</td>
</tr>
<tr>
<td>αT</td>
<td>82.6 ± 1.87</td>
<td>4.13 ± 0.07</td>
</tr>
</tbody>
</table>
Table 3 Dimensional characteristics of the produced NLC as determined by PCS

<table>
<thead>
<tr>
<th></th>
<th>empty-NLC</th>
<th>PB-NLC</th>
<th>HQ-NLC</th>
<th>EU-NLC</th>
<th>aA-NLC</th>
<th>aT-NLC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Z average (nm)</strong></td>
<td>238</td>
<td>206</td>
<td>247</td>
<td>202</td>
<td>203</td>
<td>203</td>
</tr>
<tr>
<td><strong>polydispersity index</strong></td>
<td>0.39</td>
<td>0.24</td>
<td>0.19</td>
<td>0.25</td>
<td>0.25</td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Mean diameter by intensity (nm)</strong></td>
<td>120 (42%) 415 (58%)</td>
<td>103 (76%) 290 (24%)</td>
<td>147 (52%) 408 (48%)</td>
<td>106 (64%) 410 (36%)</td>
<td>124 (59%) 395 (41%)</td>
<td>123 (66%) 397 (34%)</td>
</tr>
</tbody>
</table>

Table 4 Size of the produced NLC over a period of six months, as determined by PCS

<table>
<thead>
<tr>
<th>days</th>
<th>empty-NLC (P.I.)</th>
<th>PB-NLC (P.I.)</th>
<th>HQ-NLC (P.I.)</th>
<th>EU-NLC (P.I.)</th>
<th>aA-NLC (P.I.)</th>
<th>aT-NLC (P.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>238 (0.39)</td>
<td>206 (0.24)</td>
<td>247 (0.19)</td>
<td>202 (0.25)</td>
<td>203 (0.25)</td>
<td>203 (0.26)</td>
</tr>
<tr>
<td>30</td>
<td>233 (0.22)</td>
<td>210 (0.19)</td>
<td>236 (0.17)</td>
<td>222 (0.21)</td>
<td>213 (0.23)</td>
<td>210 (0.24)</td>
</tr>
<tr>
<td>90</td>
<td>245 (0.23)</td>
<td>217 (0.21)</td>
<td>232 (0.18)</td>
<td>219 (0.22)</td>
<td>210 (0.21)</td>
<td>209 (0.20)</td>
</tr>
<tr>
<td>180</td>
<td>248 (0.20)</td>
<td>214 (0.18)</td>
<td>240 (0.19)</td>
<td>215 (0.18)</td>
<td>216 (0.24)</td>
<td>211 (0.23)</td>
</tr>
</tbody>
</table>

P.I.: polydispersity index
Table 5 Release kinetic parameters of active release from the produced NLC

<table>
<thead>
<tr>
<th>Equation\</th>
<th>K</th>
<th>c, c'</th>
<th>R</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\frac{M_t}{M_\infty} = K_{Diff}^{0.5} + c')</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>34.007</td>
<td>-0.06889</td>
<td>0.98294</td>
<td>0.96617</td>
</tr>
<tr>
<td>EU</td>
<td>29.669</td>
<td>23.569</td>
<td>0.87914</td>
<td>0.77289</td>
</tr>
<tr>
<td>aA</td>
<td>52.576</td>
<td>-1.625</td>
<td>0.98964</td>
<td>0.97839</td>
</tr>
<tr>
<td>aT</td>
<td>20.642</td>
<td>-1.2494</td>
<td>0.9913</td>
<td>0.98278</td>
</tr>
<tr>
<td>1−Mt=M_\infty \frac{1}{4} e^{-k_{diss} t} +c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>-0.2481</td>
<td>4.4616</td>
<td>0.97154</td>
<td>0.81449</td>
</tr>
<tr>
<td>EU</td>
<td>-0.26024</td>
<td>4.0062</td>
<td>0.86464</td>
<td>0.74760</td>
</tr>
<tr>
<td>aA</td>
<td>-0.70225</td>
<td>4.5806</td>
<td>0.9869</td>
<td>0.97397</td>
</tr>
<tr>
<td>aT</td>
<td>-0.1229</td>
<td>4.547</td>
<td>0.98279</td>
<td>0.96588</td>
</tr>
</tbody>
</table>

K and c - Mathematical coefficients obtained by plotting the linear forms of the indicated equations,

\(R\) - Regression coefficient

\(R^2\) - Squared regression coefficient
Table 6. MIC (Minimum Inhibitory Concentration; μg/ml) against phytopathogenic bacterial and fungal strains.

<table>
<thead>
<tr>
<th>MIC (μg/ml)</th>
<th>\textit{A. tumefaciens} (AT)</th>
<th>\textit{A. vitis} (AV)</th>
<th>\textit{C. michiganensis} (CM)</th>
<th>\textit{P. syringae} (PSS)</th>
<th>\textit{Alternaria sp} (AL)</th>
<th>\textit{F. oxysporum} (FO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB</td>
<td>6.3</td>
<td>12.5</td>
<td>12.5</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PB-NLC</td>
<td>12.5</td>
<td>12.5</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>12.5</td>
</tr>
<tr>
<td>EU</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>EU-NLC</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>200</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>aA</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>aA-NLC</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>200</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>aT</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>aT-NLC</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
</tr>
</tbody>
</table>
LEGENDS TO FIGURES

Fig. 1 Macroscopic aspect of the produced NLC dispersions: PB-NLC, EU-NLC, HQ-NLC, aA-NLC and aT-NLC.

Fig. 2 Cryo-transmission electron microscopy images (cryo-TEM) of: empty NLC (A), PB-NLC (B), EU-NLC (C), HQ-NLC (D), aA-NLC (E) and aT-NLC (F).

Fig. 3 PSD plots of PB-NLC (A) and EU-NLC (B) and aT-NLC (C).

Fig. 4 In vitro release profile of active molecule from PB-NLC (dot), EU-NLC (diamond), aA-NLC (e), aT-NLC (triangle). Experiments were performed by dialysis method. Data were the mean of 4 experiments ± SD.

Fig. 5 Effect of increasing concentrations of PB (A), EU (B), aA (C) and aT (D) as free form (dot) or encapsulated in NLC (square) on the growth of the cyanobacterium Synechococcus sp., strain PCC 6301.

Fig. 6 Vitality of HaCaT cells after treatment with PB-NLC, EU-NLC and aA-NLC

Fig. 7 Vitality of A549 cells after treatment with PB-NLC, EU-NLC and aA-NLC