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Successful Entrapping of Liposomes in Glucan Particles: an innovative micron-sized carrier to deliver water soluble molecules.

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Mol Pharm. 2014 Oct 6;11(10):3760-5.
doi: 10.1021/mp500374f.

To access the final edited and published work see
<http://pubs.acs.org/doi/abs/10.1021/mp500374f>

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Abstract

Glucan particles (GPs) are monodisperse microspheres derived from baker's yeast, and represent an interesting class of microcarriers for theranostic applications as they show a high affinity towards immune system cells. The typical loading strategy was to harness the ability of the molecule to be loaded to interact with nano- micro-assembled systems through electrostatic or hydrophobic forces. However, small water soluble chemicals could not be steadily retained by the leaky shell of GPs. In this work, we propose an alternative loading approach for small water soluble compounds that is based on their entrapment in the aqueous core of liposomes that are directly formed into the microparticles through the reverse phase evaporation method (REV). The construct obtained may act as biocompatible carrier to deliver and release, even in triggerable way, bioactive compounds.

KEYWORDS: Glucan Particles, Microcarrier, REV liposomes, heat-mediated release, macrophage labeling.

1. Introduction

It is widely demonstrated that the concept of drug delivery has opened up new scenarios in cancer therapy.¹⁻³ Among the various carriers that have been studied for this purpose,⁴⁻⁹ liposomes have played a prominent role owing to the possibility of loading either hydrophilic (confined in the aqueous inner space) or amphiphilic/lipophilic (incorporated in the bilayered surface) drugs.^{10,11} Moreover, phospholipid vesicles have the peculiar ability to allow for a controlled release of their payload, either through exogenous or endogenous stimulations.^{12,13} Recently, Ostroff and co-workers have proposed the use of Glucan Particles (GPs) as micron-sized vehicle to release different therapeutic agents.¹⁴⁻¹⁶ GPs are simply extracted from the common baker's yeast, *Saccharomyces Cerevisiae*. These systems show a porous shell, mainly composed of 1,3-Beta-D-glucan, which encloses a hollow cavity where chemicals can be entrapped.¹⁷ Up to now, the most explored encapsulation method consisted in loading charged macromolecules (*e.g.* DNA, siRNA, proteins) through the formation of tight ionic pairs with polymers of opposite charge.^{15,18} As this method does not allow the entrapment of neutral molecules, an alternative encapsulation strategy, based on the use of hydrogel matrix, has been very recently described.¹⁶ Even though the reported encapsulation efficiency is rather high, the drawback of this approach resides in the impossibility of controlling the release of the payload inside GPs. In fact, a high amount (70-95%) of the entrapped material is released after 36-48 h. As a matter of fact, in some cases, a continuous but slow release might prevent the reaching of the therapeutic dose at the site of interest. Hence, a better control of the release would increase the overall efficacy of the therapy.¹⁹

The aim of this work was twofold: i) to develop a method to entrap small water-soluble molecules (that could not be retained as such) within GPs, and ii) to control the release of this cargo at will.

2. Materials and methods

45 2.1 Chemicals

1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000) were purchased by Avanti® Polar Lipids, Inc. (Alabaster, Alabama, USA). Trypan Blue was obtained by Invitrogen, Life Technologies™ (Carlsbad, California, USA). Zymolyase® 100T was purchased by AMS Biotechnology (Abingdon, UK). All the other chemicals were
50 purchased from Sigma-Aldrich (Milano, Italy).

2.2 Preparation of Glucan Particles

Glucan Particles were prepared as reported elsewhere.¹⁸ Briefly, 100 g of *Saccharomyces Cerevisiae* were suspended in 1 L of sodium hydroxide 1.0 M and heated to 80 °C for 1 h. The insoluble residue containing the yeast cell porous walls
55 was collected by centrifugation at 4000 rpm for 10 min, transferred in 1 L of distilled water, brought to pH 4.5 with hydrochloric acid, and warmed up at 55 °C for 1 h. The insoluble material was recovered by centrifugation and washed once with 1 L of water, four times with 200 mL of isopropanol, and twice with 200 mL of acetone. The resulting slurry was placed in a glass tray and dried at room temperature for a couple of days to obtain 10.9 g of a fine, pale yellow powder.

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2.3 Preparation of liposome-loaded GPs

84.2 mg of DSPC and 15.7 mg of DSPE-PEG2000 were dissolved in 2 mL of chloroform. 100 mg of dry glucan particles were added to the solution and the suspension obtained was left 15 h under magnetic stirring. Then, the suspension was transferred to a round bottom flask, and chloroform evaporated under vacuum. The film
65 obtained was added with 250 µL of methanol and 3 mL of anhydrous diethyl ether, and the resultant suspension was vortexed until the film dissolved. Subsequently, 2 mL of isotonic buffer (HEPES/NaCl) containing carboxyfluorescein (CF) 20 mM were added, and two phases formed. To obtain an emulsion, the sample was sonicated 5 times for 30 s (power 50 W, Sonicator Bandelin Sonopuls HD 2070, Berlin, Germany) keeping the temperature under 10°C. An orange emulsion formed, containing monolayered inverted lipid nanovesicles entrapping a water drop of the
70 carboxyfluorescein solution and exposing the hydrophobic tails to the organic solvent. Next, the organic solvent was slowly evaporated under vacuum. During evaporation, when the ratio between the aqueous and organic phase reached a critical point, a gel composed by bilayered unilamellar liposomes formed both inside and outside GPs. As soon as the organic solvents were totally evaporated, the sample was collected at 60°C by adding 1 mL of carboxyfluorescein 20 mM, and the suspension was vigorously vortexed and sonicated (5 times for 30 s, power 35 W) to pass from
75 multilamellar to unilamellar vesicles. The addition of CF to the film served to avoid the decrease of the dye concentration entrapped in the vesicles consequent to the sonication. After centrifugation (3500 rpm, 10 min), the two phases formed were collected separately. Non GPs-Internalized Liposomes (NILs) were purified by sequential cycles, 4 hours each, of dialysis against 2.5 L of isotonic HEPES buffer at pH 7.4 (4 °C, cut-off 12 kDa) until the buffer was not fluorescent any longer. Liposomes-loaded GPs (Lipo-GPs) were purified by sequential cycles of centrifugation (3500
80 rpm, 10 min/cycle) with isotonic HEPES buffer at pH 7.4. Again, centrifugation was repeated until the supernatant did not display any fluorescence.

To verify the possible loading of CF in GPs, the loading procedure described above was repeated in absence of phospholipids.

Empty GPs were also prepared as control by simply adding 1 mL of isotonic HEPES buffer to 100 mg of dry particles.

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2.4 Characterization of liposome-loaded Glucan Particles

The size of GPs and the number of GPs/mL were determined by means of a Countess® Automated Cell Counter (Invitrogen, Life Technologies™, Carlsbad, California, USA). A suspension of GPs (100 mg/mL) was diluted in HEPES Buffer 1:1000 to enter in the range of detection, and then 1:1 in trypan blue solution to perform the measurements.

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Fluorescence images of Lipo-GPs and empty GPs were acquired with Carl Zeiss Fluorescence Microscope Axio Observer.Z1, equipped with the driving software Axiovision 4.8. Excitation and emission wavelengths were fixed respectively at λ_{exc} 470/440 nm - λ_{em} 525/550 nm, magnification 63x.

Fluorescence was measured by means of a FluoroMax-4 Spectrofluorometer (Horiba Scientific, Edison New Jersey, USA) equipped with the driving software FluorEssence™. The excitation and emission monochromators of the instrument were set to select a band of wavelengths centered on the carboxyfluorescein specific excitation and emission wavelengths (492 and 517 nm, respectively). To check the stability at 4°C of Lipo-GPs, fluorescence emission spectra were continuously acquired for 30 days.

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The fluorescence enhancement (F^{en}) of NILs or Lipo-GPs after addition of Triton was calculated as follows:

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$$F^{en} = \frac{F^{post} - F^{pre}}{F^{pre}} \times 100$$

where *post* and *pre* terms refer to the fluorescence intensity before and after the Triton addition, respectively.

The percentage of release of CF from NILs or Lipo-GPs was calculated according to the following equation:

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$$Rel \% = \frac{F^{post} - F^{pre}}{F^{full} - F^{pre}} \times 100$$

post and *pre* terms have the same meaning as above, whereas F^{full} is the fluorescence of the system in which the dye is fully released.

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2.5 Characterization of non-internalized liposomes

The hydrodynamic diameter of the CF-loaded liposomes was determined by Dynamic Light Scattering (DLS) with Zetasizer Nano 90 ZS (Malvern, UK). Liposomes were diluted 1:100 in filtered HEPES buffer and transferred into a disposable plastic cuvette. Each measurement was performed three times at 20°C, equilibration time 30 s. NILs displayed a diameter of 55 ± 7 nm, with a polydispersity index (PDI) of 0.20. Fluorescence emission spectra of the sample were performed after diluting the sample to have fluorescence intensity within the calibration curve of the dye.

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2.6 Enzymatic degradation of GPs

GPs were digested with the specific enzyme Zymolyase®100T as reported elsewhere¹⁶. The three different samples (Lipo-GPs, NILs, and empty GPs) underwent the same treatment. 60 μ L of each sample were incubated (45°C, 3 hours)

120 in the dark with the enzyme (0.2 mg/mL) in the presence of the reducing agent dithiothreitol (DTT) (10 mg/mL) in 10
mL of Phosphate Buffered Saline (PBS) at pH 6.5. Samples were then cooled down to 20°C and centrifuged at 3000
rpm for 10 min, to pellet undigested GPs. Supernatant was concentrated by means of Vivaspin® centrifugal
concentrators (cut-off 10000 Da) and subjected to fluorescence microscope imaging, Dynamic Light Scattering (DLS),
and Mass Spectrometry (MS). To acquire mass spectra, 50 µl of each specimen (Lipo-GPs, NILs, and empty GPs) were
125 added to 500 µl of methanol. Then, 50 µl of the so-obtained solution were added with 500 µL of formic acid 0.1 % in
methanol:water 9:1, and left 30 minutes at 25°C under gentle agitation. Mass spectrometry was performed by using a
SQ Waters 3100 mass detector in ESI(+) ionization mode, capillary voltage 2.20 KV, cone voltage 20 V, source
temperature 110 °C, desolvation temperature 200°C.

130 2.7 Heat- mediated release

Heat was selected as physical variable to control the release of the material transported by Lipo-GPs. 15 µL of Lipo-
GPs were suspended in 9 mL of isotonic HEPES buffer at room temperature. Afterwards the sample, kept under
magnetic stirring, was heated to 55°C using an oil bath, and the temperature was kept constant for 6 hours. The amount
of CF release from Lipo-GPs was determined spectrofluorimetrically (see Section 2.4). A similar procedure was
135 followed on NILs.

2.8 Determination of lipidic and carboxyfluorescein content in Lipo-GPs

To quantify the amount of phospholipids internalized in Lipo-GPs with respect to NILs, 10 µl of Lipo-GPs and NILs
were diluted in 100 µl of distilled water and digested in 1 ml of concentrated nitric acid (70%) by applying microwave
140 heating (Milestone MicroSYNTH, Microwave labstation equipped with an optical fiber temperature control and
HPR91000/6M six position high pressure reactor, Italy)²⁰. The obtained samples were collected in 2 mL of ultrapure
water and analyzed in terms of phosphorous content by ICP-MS (Thermo Scientific ELEMENT 2 ICP-MS Finnigan,
Rodano, Italy). Values of phosphorous concentration were extrapolated from a calibration curve previously prepared
from 0.02 to 0.2 µg/mL using a phosphorous ICP/DCP standard solution. The proportion of lipids within Lipo-GPs with
145 respect to the total lipids in internalized and non-internalized liposomes was then calculated as follows:

$$\% P_{LipoGPs} = \frac{\text{mol } P_{LipoGPs}}{\text{mol } P_{LipoGPs} + \text{mol } P_{NILs}} \times 100$$

where $\text{mol } P_{LipoGPs}$ and $\text{mol } P_{NILs}$ corresponded to the moles of phosphorous measured in Lipo-GPs and in NILs,
respectively. Similarly, after the measurements of the concentration of carboxyfluorescein in the two loaded samples
(2.4 , 2.5), the percentage of carboxyfluorescein entrapped in liposomes-loaded GPs compared to total encapsulated CF,
150 was calculated as follows:

$$\% CF_{LipoGPs} = \frac{\text{mol } CF_{LipoGPs}}{\text{mol } CF_{LipoGPs} + \text{mol } CF_{NILs}} \times 100$$

where $\text{mol } CF_{LipoGPs}$ and $\text{mol } CF_{NILs}$ indicate the mol of the fluorescent dye found in liposomes-loaded GPs and in non-
entrapped liposomes, respectively. Whereas the encapsulation efficiency of CF with respect to the total amount of
fluorescent dye supplied was as reported hereafter:

$$155 \text{ yield } CF_{LipoGPs} \% = \frac{\text{mol } CF_{LipoGPs}}{\text{mol } CF_{total}} \times 100$$

where $\text{mol } CF_{total}$ corresponded to the total amount of CF supplied.

2.9 Cell culture

160 Mouse macrophages J774A.1 were obtained from the American Type Culture Corporation. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/l glucose, 10% (v/v) FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, and incubated at 37°C in a humidified atmosphere of 5% CO₂. At 80% confluency, macrophages were dislodged from the flask substrate with a cell scraper, aspirated and dispensed into new flasks. DMEM, FBS, glutamine, penicillin and streptomycin were provided by Lonza, Verviers, Belgium.

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2.10 Uptake experiments

0.5x10⁵ J774A.1 cells were seeded in sterile ibidi® open µ-Slide (chambered coverslip) with 8 wells (dimensions of wells w x l x h in mm 9.4 x 10.7 x 6.8), tissue culture treated (ibidi GmbH, Planegg, Germany). After 48 hours the culture medium was discarded and cells were incubated for 60 min with 3.0 µM of CF entrapped either in Lipo-GPs or in NILs. At the end of incubation, cells were washed five times with PBS and fixed with 4% formalin at room temperature for 15 min. Cells were profusely washed again; staining with 8x10⁻⁵ mg/mL Hoechst in PBS was performed for a few minutes, followed by three 5 min washing in PBS at 4°C. Finally cells were imaged through a Leica TCS SP5 confocal microscope (Leica Microsystems s.r.l.), magnification 63x (laser₁ wavelength = 405 nm, laser₂ wavelength = 488 nm, to visualize Hoechst and carboxyfluorescein, respectively).

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3. Results and Discussion

The main goal of this work was the development of a new biocompatible delivery system based on glucan particles and able to release (ideally in controlled way) small water-soluble bioactive molecules. In fact, whereas the ability of GPs to transport nano-constructs have been already reported,^{15,17,18} the loading of hydrophilic small molecules may be challenging due to the size of the pores of the glucan shell. We surmised that a possible solution was to entrap in GPs liposomes encapsulating the small molecule in their aqueous core. However, to achieve this goal, it was necessary to find a method to form the nanovesicles directly inside the particles. This objective was pursued by using the Reverse Phase Evaporation (REV) technique.²¹

This method has been demonstrated to have several advantages with respect to conventional techniques like the hydration of thin lipid film or sonication,^{22,23} because it allows the formation of unilamellar vesicles with a high encapsulation efficiency of water-soluble compounds. We modified the REV method to allow the formation of liposomes, and their successive entrapment, in GPs. First, GPs and the phospholipid components of the liposomes membrane (DSPC:DSPE-PEG 95:5 in moles) were mixed in a 1:1 ratio in chloroform, and left 12 hours under stirring at room temperature. During this time, particles swelled, thus allowing the permeation of the phospholipids through their porous shells. Then, the organic solvent was evaporated under vacuum, and a pale yellow, homogeneous, and thin film was formed. At that stage, phospholipids were homogeneously distributed inside and outside the particles. A mixture of diethyl ether and methanol (12:1 in volume) was added to the film in order to re-dissolve the phospholipids in an organic solvent more suitable for the REV method.

Next, the aqueous solution containing the small water-soluble molecules to be trapped (here the fluorescent dye carboxyfluorescein at a self-quenched concentration of 20 mM) was added until reaching the organic/water volume ratio of 3:1. The resulting biphasic system was sonicated, keeping temperature under 10° C. The procedure led to a W/O emulsion where the hydrophobic tails of the phospholipids pointed towards the organic phase and the polar heads stabilized the aqueous droplets. Importantly, the emulsion formed both inside and outside the GPs. Then, the organic solvent was slowly evaporated under vacuum. During evaporation, when the ratio between the aqueous and organic phase reached a critical point, the W/O emulsion switched to a gel composed by bilayered liposomes. As soon as the

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organic solvents were totally evaporated, the sample was added with 1 mL of CF 20 mM (in isotonic buffer), and the resulting suspension was vigorously vortexed and sonicated (5 times for 30 s, power 35 W) to facilitate the formation of unilamellar vesicles. As sonication induces a destabilization of the bilayers (with exchange of the entrapped material with the bulk), the addition of CF at the same concentration used for the preparation of the liposomes (20 mM) was performed to avoid the loss of the dye from the liposomes with the consequent decrease of concentration below the self-quenching threshold.

At this stage, liposomes formed both inside and outside GPs, and, therefore, separation was necessary. Centrifugation allowed the separation between NILs and Lipo-GPs. Then, NILs were exhaustively dialyzed to remove the untrapped CF, whereas Lipo-GPs were subjected to a repeated washing/centrifugation cycles to remove both untrapped CF and liposomes. Purification cycles were repeated until the fluorescence of the dialysis buffer (or the supernatant) was undetectable.

First, the suspension of Lipo-GPs was observed by fluorescence microscopy. The image reported on the left of Figure 1 clearly highlights the presence of the green fluorescence of CF emitted from the inner core of the particles. GPs prepared according to the procedure reported above, but without using phospholipids, did not display any fluorescence (Figure 1, right), thereby confirming that the dye cannot be entrapped as such in the particles.

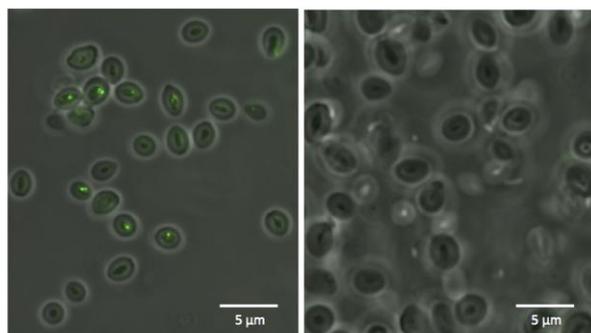


Figure 1: Fluorescence microscopy images of Lipo-GPs (left) and empty GPs (right). λ_{exc} window 470/440 nm- λ_{em} window 525/550 nm.

The diameter of the particles was measured using an automated cell counter and a value of $3.0 \pm 0.7 \mu\text{m}$ was obtained regardless from the entrapment of liposomes. This value is in close agreement with literature data ($2\text{-}4 \mu\text{m}$)^{17,18,24} and confirms that the REV procedure did not affect the morphology of the particles.

Lipo-GPs displayed a fluorescence peak at the fluorescence emission wavelength of CF (520 nm). Fluorescence intensity was monitored over time (at 4 °C) and no changes were observed within one month (Figure 2). Similar results were obtained for NILs, thus confirming that the encapsulation of liposomes in the particles did not affect their (high) stability.

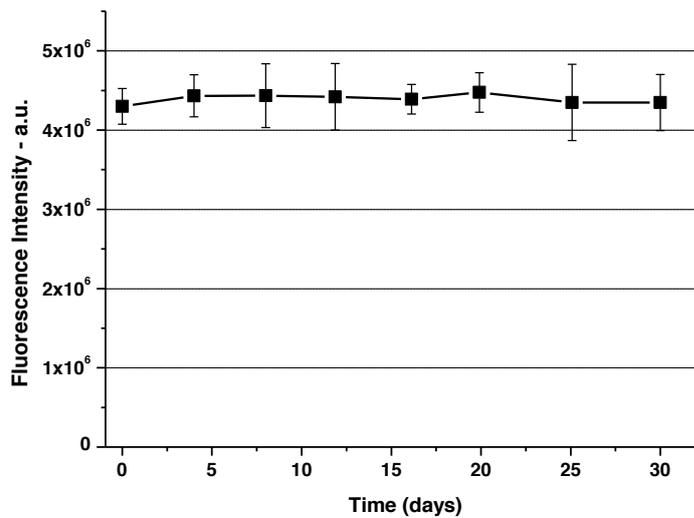


Figure 2: Time evolution of fluorescence intensity for Lipo-GPs at 4 °C.

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To confirm the entrapment of CF-loaded liposomes into GPs, the sample was treated with the non-ionic surfactant Triton X-100, the detergent typically used to disrupt liposomes membranes. Adding Triton X-100 to a suspension of NILs a fluorescence enhancement of $101.7 \pm 11.7 \%$ was observed, due to the well known fluorescence de-quenching caused by the dilution of the dye once released from the vesicles.

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The addition of the same amount of detergent to a Lipo-GPs suspension led to an enhancement of $78.5 \pm 6.3 \%$.

This result is a good indication about the effective entrapment of fluorescent liposomes in the particles. In fact, Triton X-100 experiences a monomer/micelle equilibrium in water (CMC in the range $0.2-0.24 \text{ mM}$)²⁵, and this behavior may reduce the accessibility of the detergent to the core of GPs, thus reducing/slowing down the disruptive effect on the GPs-entrapped liposomes. Actually, the fluorescence enhancement of the Lipo-GPs suspension after treatment with Triton X-100 increased over time and reached a value of $97.2 \pm 4.2 \%$ after one week.

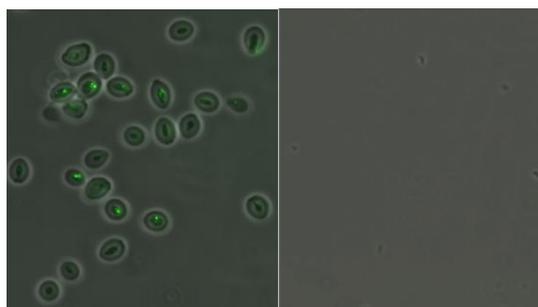
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A further confirmation of the entrapment of intact liposomes in GPs was obtained upon enzymatic degradation of the glucan shell. According to a method published elsewhere,¹⁶ GPs were digested by the enzyme zymolyase that hydrolyses linear glucose polymers at the $\beta-1,3$ position. The incubation was carried out in the dark (PBS pH 6.5, 45°C for 3 hours) in the presence of the reducing agent dithiothreitol that acts as activator of the enzymatic activity. The rationale of this experiment was to induce the release of the liposomes from the GPs core after the enzymatic degradation of the particles shell. Besides Lipo-GPs, also NILs and empty GPs underwent the same treatment. After enzymatic digestion, samples were cooled down to 20°C and centrifuged to remove the residual undigested GPs. Then, supernatants were concentrated and subjected to fluorescence microscopy, DLS, and Mass Spectrometry.

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The hydrolytic action of the enzyme led to clear suspensions that were observed at the fluorescence microscope after centrifugation and concentration. Images reported in Figure 3 confirmed the effective digestion of the GPs shell.



265 Figure 3: Fluorescence images of loaded GPs before (left) and after (right) the treatment with the enzyme Zymolyase®, taken at the
 270 fluorescence microscope, λ_{exc} 470/40 nm- λ_{em} 525/50 nm. Very few particles are still visible.

DLS measurements of NILs incubated with zymolyase revealed nanoparticles with a hydrodynamic diameter of $58.0 \pm$
 270 6.4 nm, *i.e.* almost the same as before the addition of the enzyme (55.0 ± 7.0 nm), thereby showing that liposomes were
 not affected by the enzymatic treatment. DLS of digested Lipo-GPs detected the presence of nanovesicles having a size
 of 64 ± 18 nm, while no nanoparticles were found in DLS measurements of zymolyase-treated empty GPs.

The supernatants were also analysed by Mass Spectrometry (ESI(+) mode). The samples were treated with methanol
 and 0.1% formic acid at 25°C for 30 minutes under gentle agitation. MS spectra of NILs and Lipo-GPs samples showed
 275 three characteristics peaks at $m/Z = 790.6$, 812.5 , and 828.4 Da (Figure 4). These values correspond to the mass of
 DSPC, and its sodium and potassium adducts, respectively. Hence, this result was a further confirmation of the
 entrapment of the main phospholipid in the liposomes membrane inside GPs.

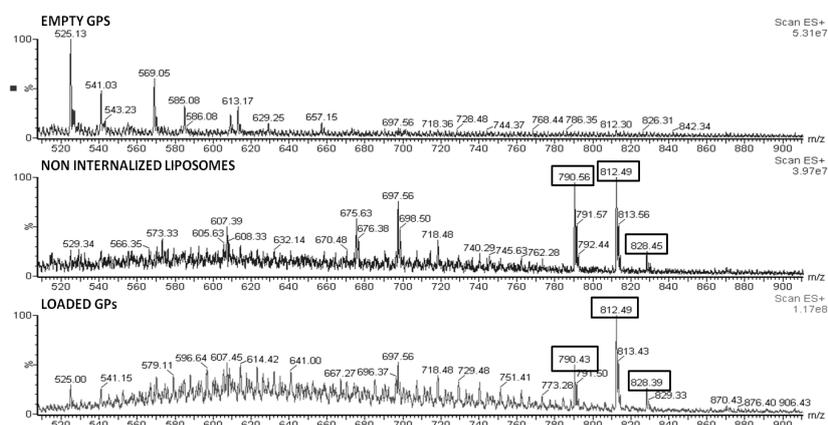


Figure 4: Mass Spectra (ESI+) of empty GPs, NILs and liposomes loaded GPs.

280 Moreover, to conceptually demonstrate the potential of the herein proposed microcarrier to control the release of the
 liposome-entrapped material, Lipo-GPs were heated at a temperature (55 °C) slightly above the gel-to-liquid phase
 transition temperature of DSPC-based liposomes.²⁶ Under such a condition, the liposome bilayer became fluid, thus
 285 allowing the release of the encapsulated hydrophilic molecule. The efficacy of the heat stimulus was checked by
 measuring the fluorescence of CF-loaded NILs before and after heating. Upon the assumption that the addition of Triton

led to a full release of the dye, a release of $88.5 \pm 16.6 \%$ was measured after heating, thus demonstrating the temperature sensitivity of DSPC-based liposomes.

290 The heating of Lipo-GPs led to a release exceeding 100 % (111.5 ± 3.8). However, in this case the full-release value was taken from the fluorescence measured after the addition of Triton to Lipo-GPs, and, likely (as previously discussed) this method is not very accurate to get the full release of CF from Lipo-GPs.

Nevertheless, this observation gave a clear indication about the potential of using heat as trigger to stimulate the release of the material encapsulated in the liposomes entrapped in GPs.

295 In terms of loading capability, the number of liposomes that could be loaded into GPs was estimated to be approximately 300, thus highlighting the possibility of using a single microcarrier particle for delivering high amounts of hydrophilic molecules at the biological target. In particular the portion of phospholipids within Lipo-GPs with respect to the total amount of lipids in internalized and non-internalized liposomes was estimated to be $10.8 \pm 3.1 \%$; similarly, carboxyfluorescein encapsulated in liposomes loaded glucan particles with respect to total entrapped CF was considered to be $8.2 \pm 1.8 \%$. The total efficiency of CF encapsulation was $0.5 \pm 0.2 \%$.

300 As a proof of concept of the possibility to deliver CF to macrophages, J774A.1 cells were incubated for 60 min with either Lipo-GPs or NILs, washed in PBS, fixed and stained with Hoechst. Images obtained are reported below. Figure 5a corresponds to macrophages incubated with Lipo-GPs, whereas figure 5b shows the results obtained with NILs. As it is possible to observe, the delivery efficiency of the newly developed microcarrier is much higher than the delivery ability of liposomes alone. Indeed, no contrast could be observed after incubating the cells with NILs, while a strong contrast could be seen arising from uptaken glucan carriers at the same total concentration of incubated CF. Thus, we
305 have demonstrated that Lipo-GPs retain the function to deliver a payload to phagocytic cells.

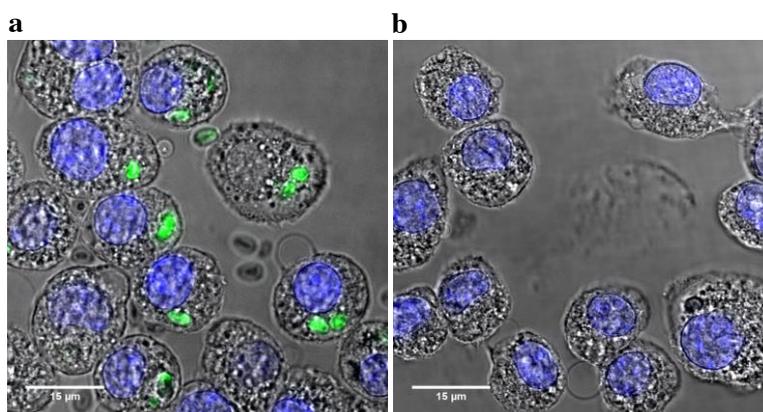


Figure 5: Confocal microscope image of J774A.1 macrophages incubated with a) CF loaded Lipo-GPs (green), b) CF-loaded NILs. Nuclei are stained with Hoechst (blue). Magnification 63X.

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4. Conclusions

To summarize, this work demonstrates that small-sized, and water-soluble molecules can be successfully (and stably) loaded in the inner cavity of Glucan Particles exploiting the *in situ* formation of REV liposomes. Moreover the payload can be efficiently delivered to phagocytic cells. This observation may open new opportunity to encapsulate drugs and other bioactive molecules (*e.g.* imaging agents) using a biocompatible micron-sized carrier that has been demonstrated
315 useful in several biomedical applications. Moreover, depending on the characteristics of the liposomes entrapped in the particles, the release of the liposomal content could be controlled by chemical (*e.g.* pH) or physical (*e.g.* heat) stimuli, thereby further extending the potential of the microcarrier.

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Notes

The authors declare no competing financial interest.

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■ ACKNOWLEDGEMENTS

The financial support of University of Torino/ Compagnia di San Paolo (project “Innovative Nanosized Theranostic Agents”) is gratefully acknowledged. The research leading to these results has received funding from the EU's Seventh Framework Programme (FP7/2007-2013) under grant agreement n°HEALTH-F2-2011-278850(INMiND). This work was carried out within the framework of the COST TD1004 Action.

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▪ LIST OF ABBREVIATIONS

- 395 CF = carboxyfluorescein
CMC = Critical Micelle Concentration
DLS = Dynamic Light Scattering
DMEM = Dulbecco's Modified Eagle's Medium
DSPC = 1,2-distearoyl-*sn*-glycero-3-phosphocholine
400 DSPE-PEG2000 = 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
(ammonium salt)
DTT = dithiothreitol
FBS= Fetal Bovine Serum

405 GPs = Glucan particles
ICP-MS = Inductively Coupled Plasma Mass Spectrometry
Lipo-GPs = Liposomes-loaded GPs
MS = Mass Spectrometry
NILs = Non GPs-Internalized Liposomes
P = Phosphorous

410 PBS= Phosphate Buffered Saline
REV = Reverse phase evaporation