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Preparation, characterization and in vitro antiviral activity evaluation of foscarnet-chitosan nanoparticles

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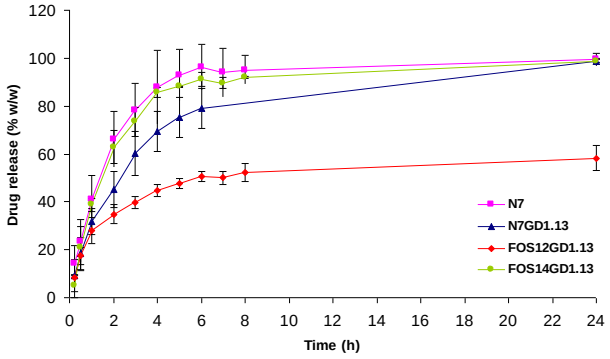
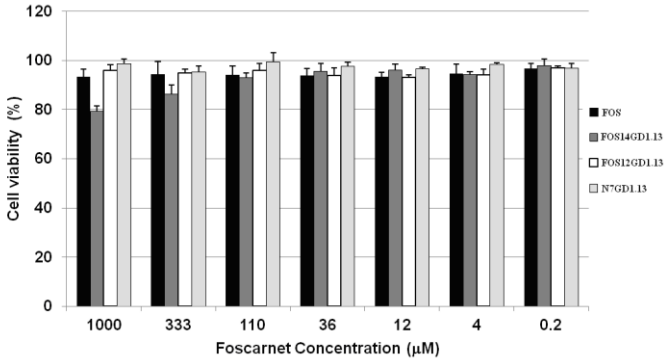
*Graphical Abstract (for review)

Foscarnet release from foscarnet/chitosan nanoparticles



Cytotoxicity of foscarnet/chitosan nanoparticles



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PREPARATION, CHARACTERIZATION AND *IN VITRO* ANTIVIRAL ACTIVITY EVALUATION OF FOSCARNET/CHITOSAN NANOPARTICLES

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1. Introduction

Foscarnet (FOS) is an antiviral agent which inhibits the activity of herpesvirus DNA polymerase (Biron, 2006), including herpes simplex virus types 1 and 2 (Garre et al., 2007; Helgstrand et al., 1978; Oberg, 1989), varicella zoster, and cytomegalovirus (CMV), as well as the human immunodeficiency virus (HIV) and other viruses. Foscarnet is the trisodium salt of phosphonoformic acid, a pyrophosphonate analogue with an extremely simple chemical structure. Due to the instability of its acid form, it is formulated as a hexahydrate trisodium salt, with a molecular weight of 300.1 and a solubility in water of 5% w/w (pH 7) (García Andreu et al., 1997). Foscarnet possesses three acidic hydroxyl groups, with pKa values of 0.49, 3.41 and 7.27, thus the polyanion charge depends on the pH of the solution. Due to its low oral bioavailability, it is usually administered by intravenous infusion (marketed by Clinigen as foscarnet sodium under the trade name Foscavir). A major problem associated with the administration of foscarnet is its marked toxicity. Therefore, a suitable drug delivery system could decrease the incidence of adverse effects and modulate the drug's delivery rate.

In literature, there are a few studies concerning the delivery of foscarnet by controlled release pharmaceutical forms used for the purpose of masking the side and toxic effects of the drug. Claro (Claro et al., 2009) proposed a liposomal formulation which allowed a continuous delivery of foscarnet, with an increasing of its therapeutic index, primarily by confining its action locally to the site of injection. The encapsulation of foscarnet into liposomes was also described by Bergers (Bergers et al., 1997): two types of sized foscarnet-containing liposomes were evaluated with respect to their in vitro efficacy against human CMV in differentiated monocytic (THP-1) cells and embryonic lung fibroblasts. The conclusions of the authors were that even if free foscarnet showed an in vitro antiviral activity equal or greater than liposomes, a therapeutic benefit of administering foscarnet encapsulated in long-circulating liposomes might result from an improved blood residence time and prolonged contact of the drug with the CMV-infected cells. Alternatively, some authors (Hostetler et al., 2000) thought of increasing foscarnet bioavailability by modifying the chemical

structure of the drug to obtain monoalkyl ether lipid analogues. These new compounds, which are bioavailable orally, exhibited an in vitro antiviral activity greater than unmodified foscarnet against human immunodeficiency virus type 1 (HIV-1).

At our best knowledge, no nanoparticulate systems different from liposomes have been described for foscarnet delivery until now. On the other hand, liposomes have a complex structural order due to hydrophobic interactions, are rather unstable in circulation and have a low drug loading capacity due to their small inner volume (Ahsan et al., 2002). The encapsulation of foscarnet into chitosan nanoparticles could be an interesting approach to reduce the toxic effects of this drug and extend its activity (Duceppe and Tabrizian, 2010). Chitosan, a deacetylated derivative of chitin, is an effective vehicle for drug delivery, and is in particular able to enhance the absorption of hydrophobic macromolecular drugs. Chitosan has also been found capable to improve the effectiveness of drug delivery by holding the therapeutic material in closer proximity to its site of action owing to its mucoadhesive cationic nature (Kumar, 2000). Chitosan nanoparticles prepared by ionotropic gelation have been extensively studied in the past decade as delivery media for drugs with low molecular weight and the compound most commonly used for the ionotropic gelation is tripolyphosphate (TPP), which is a non-toxic inorganic polyanion. The molar ratio between chitosan and TPP has been found fundamental for the formation of nanoparticles and especially to achieve good drug release characteristics (Calvo et al., 1997). The mechanism of ionotropic gelation is not well documented in the literature although it seems that all the ionic groups of the TPP can participate in the interaction with the amino groups of chitosan, depending on the pH of the starting solution.

Based on the above considerations, we thought that foscarnet could induce ionotropic gelation of chitosan by the interaction of the amino groups of the polymer with both the phosphonic and the carboxylic groups present in the foscarnet molecule, similarly to what happens between chitosan and TPP. This could allow to obtain nanoparticles in which foscarnet itself could become a structural component of the system.

Obviously, in view of this aim, there was a need to completely investigate such an interaction because it was unlikely that the same molar ratio found suitable for chitosan and TPP could operate for chitosan and foscarnet. This goal has been achieved by studying the experimental conditions for this interaction through statistical experimental design. Moreover, as the greatest problem encountered in a polymeric nanoparticle formulation of this type is nanoparticle stability in physiological environment at high pH (Nayak et al., 2009) we also investigated the effect of glutaraldehyde, a widely used crosslinking agent, on the stability of the formed nanoparticles.
In this paper we describe the properties and the antiviral activity in vitro of chitosan/foscarnet nanoparticles, in which the drug directly participates in the formation of the nanoparticulate system.

2. Materials and methods

2.1 Materials

Chitosan of medium molecular weight (viscosity 200-800 cP, 1 % in 1% acetic acid), with a degree of deacetylation of 85%, sodium phosphonoformate tribasic hexahydrate (foscarnet) and glutaraldehyde solution (25 wt % in H_2O) were all purchased from Sigma (Milan, Italy).
FITC-Chitosan was prepared by Prof. R. Cavalli, University of Torino, Italy.
All the other chemicals were of reagent grade.
Water was purified by Milli-Q Plus system (Millipore, USA).

2.2 Preparation of nanoparticles

2.2.1 Chitosan-foscarnet nanoparticles

Nanoparticles with a positive surface charge were prepared by ionotropic gelation of chitosan induced by foscarnet in acidic solution.
Appropriate amounts of foscarnet aqueous solution (C= 2 mg/mL) were added to a chitosan solution (C= 5 mg/mL in 1% v/v acetic acid). Adding was performed at a controlled flow rate of 0.5 mL/min, by a peristaltic pump with the terminal Teflon tube tip immersed into the liquid, in order to

avoid dropping and consequent local concentration of the reagent, under magnetic stirring (400 rpm). The samples were then diluted, when necessary, with water to the final volume (7.0 mL) and maintained under magnetic stirring at room temperature for 30 min. Afterwards, samples were refrigerated until use.

2.2.2 Fluorescent nanoparticles

Fluorescent nanoparticles were prepared following the same procedure described above using FITC-chitosan instead of chitosan.

2.2.3 Cross-linking of chitosan-foscarnet nanoparticles by glutaraldehyde

Cross-linked nanoparticles were obtained following the procedure described above, by introducing along with the foscarnet solution, a fixed volume (corresponding to 1.13 mg glutaraldehyde) of a glutaraldehyde aqueous solution (25 % w/w).

2.3 Statistical experimental design

Doehlert designs for two variables were used to study, by the response surface methodology, the influences of the different amounts of chitosan and foscarnet in the sample on the size and zeta potential of nanoparticles. Designs of experiments and data analysis were performed using Modde software, Version 6.0 (Umetrics, Sweden) Statistical significance was set at $p < 0.05$.

2.4 Characterization of nanoparticles

2.4.1 Particle size and zeta potential

Particle size (average apparent diameter, D) and polydispersity index (PDI) were determined by dynamic light scattering using a photon correlation spectroscopy (PCS) assembly (Zetasizer 3000 HS, Malvern Instruments, UK). Determinations were carried out at 25°C, at a fixed angle of 90°. Measurements of particle diameter were made, when not otherwise specified, on the prepared

sample without preliminary separation of nanoparticles and subsequent reconstitution of the sample. Results are reported as the mean of three measurements ± SD (standard deviation).

The zeta potential (Z) was measured by laser Doppler anemometry on the same instrument. Measurements were made at 25°C without sample dilution or any salt addition. Results are reported as the mean of ten measurements ± SD.

2.4.2 Drug loading

The amount of foscarnet present in nanoparticles was measured by a direct procedure, based on phosphorus content determination (APHA 1999. Standard Methods for the Examination of Water and Wastewater: 4500-P Phosphorus): nanoparticles were centrifuged at 5000 rpm, 2117∗g, for 3 h at T=10°C, then re-dispersed in water and an accurately measured aliquot (100 µL) of the suspension was evaporated and digested in a mixture of concentrated nitric and sulphuric acid (3 ml, 2:1 ratio) at a temperature of about 80°C for 24 h, in order to eliminate the organic substance. Nitric acid was then evaporated and the remaining sulphuric acid was quantitatively transferred into a volumetric flask of 100 mL. The sample was then diluted with 20 ml of distilled water, and, in the presence of 1 drop of solution of phenolphthalein indicator, 1N NaOH was added up to the appearance of a weak pink.staining After dilution to final volume with water and agitation, 20 mlof the resulting solution were withdrawn and mixed with 4 ml of molybdate reagent (Carlo Erba, Italy) and 0.5 ml of stannous chloride reagent (Sigma Aldrich, Italy).

In the presence of phosphorus the solution showed a blue color, whose intensity was measured photometrically (HP 8452 array spectrophotometer, Hewlett-Packard, USA, managed by HP 89532 software) at λ=690 nm after 10 minutes from the colour onset, on the basis of a calibration curve constructed using a foscarnet standard solution which have undergone the same treatment of the sample (concentration range 50 µg/mL–300 µg/mL, R^2 >0.99). Drug loading was calculated by the following formula:

Drug loading (% w/w) = drug content/ weighed amount of lyophilized nanoparticles *100

Results are reported as the mean of 3 measurements ± SD.

2.4.3. Nanoparticle yield

Each nanoparticle sample was centrifuged as described above and the residue was lyophilised. Nanoparticle yield was calculated by the following formula:

Nanoparticle Yield (% w/w) = weight of the lyophilised nanoparticles / total material weight * 100

Results are reported as the mean of 3 measurements ± SD.

2.5 Drug release study

The release of foscarnet from nanoparticles was evaluated by the dialysis method. nanoparticles were recovered from the initial suspension (V= 110 mL) by centrifugation and re-dispersed in water (3.0 mL). Salts were added to the obtained suspension in order to obtain the composition of the phosphate buffer saline at pH 7.4 (PBS) used as the receptor phase. Dialysis bags (Spectra/Por-7; MWCO: 1000, Spectrum Laboratories, Inc., Laguna Hills, CA) containing 3.0 mL of the suspension in PBS were then immersed in a thermostatted beaker containing 60.0 mL PBS at 37.0 °C±0.1 °C and maintained under agitation with a stirring bar rotating at 300 rpm. At predefined intervals, aliquots (V=2.0 mL) of receiving buffer were withdrawn and spectrophotometric determination of foscarnet was then carried out by measuring the absorbance at λ= 233 nm on the basis of a calibration curve obtained from standard solutions of foscarnet in PBS (concentration range 20 μg/mL–200 μg/mL, R^2 >0.99). The study was carried out at least in duplicate.

Following the same method, the diffusion of free foscarnet from the dialysis bag into PBS was also investigated. At this aim, a foscarnet amount equivalent to that contained in a nanoparticle sample was dissolved in PBS and transferred to a dialysis bag.

Sink conditions were respected throughout the experiment.

2.6 Stability study

The average mean diameter, D, of nanoparticles was used as the indicator to evaluate their stability. After centrifugation, a nanoparticle sample was re-dispersed in PBS and maintained at 37 °C in the cuvette of the photon correlation spectrometer registering D variation for at least 4 h. The study was carried out at least in duplicate.

2.7 Biological studies

2.7.1 Cells and Viruses

Low-passage-number human embryonic lung fibroblasts (HELFs) were grown as monolayers in minimum essential medium (MEM) (SIGMA) supplemented with 10% fetal calf serum (FCS) (SIGMA), 1 mM sodium pyruvate and antibiotics.

A (BAC)-derived HCMV strain Towne incorporating the GFP sequence (Marchini, 2001) and HCMV strain AD-169 (ATCC VR-538) were propagated on HELFs. The GFP marker facilitates the identification of infected cells. HCMV titers were determined on HELF cells by using the median tissue culture infective dose (TCID50) method (Mahy et al., 1996).

2.7.2 Antiviral assays

To assay the antiviral activities of foscarnet and foscarnet-loaded nanoparticles, we performed an infectivity inhibition assay where cells were infected with GFP-expressing HCMV at a multiplicity of infection (MOI) of 0.1 for 2 hours at 37°C and then exposed to the drug. Three days postinfection, cells infected were examined by an inverted Zeiss LSM510 fluorescence microscopy. AD-169 HCMV-infected monolayers (MOI 0.004 pfu/cell) were washed and overlaid with 1.2% methylcellulose medium in presence of serial dilutions of the test formulations. After five days incubation, cells were fixed with cold methanol and acetone for 1 min and subjected to HCMV-specific immunostaining using an anti-HCMV IEA monoclonal antibody (11-003; Argene, Verniolle, France) and an UltraTech HRP streptavidin-biotin detection system (Beckman Coulter,

Marseille, France). Immunostained plaques were counted, and the percent inhibition of virus infectivity was determined as described previously (Donalisio et al., 2012). $IC50_s$ and 95% CI_s were determined using Prism software (GraphPad Software, San Diego, California, U.S.A.) to fit a variable slope-sigmoidal dose-response curve. All data were generated from duplicate wells in at least three independent experiments.

2.7.3 Cell viability assay

HELF cell viability was measured by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium;] assay. Cells were seeded at a density of 5.5 x 10^3/well in 96-well plates. After 24 h, they were either incubated with different concentrations of the compounds in triplicate or left untreated. After 96 h treatment, cell viability was determined by the CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions (Cavalli et al., 2012). The effect on cell viability of compound at different concentrations was expressed as a percentage, by comparing absorbances of treated cells with the ones of cells incubated with culture medium alone.

2.7.4 Cytotoxicity assay

The cytotoxic effect of nanoparticles on HELF cells was measured by analyzing the release of lactate dehydrogenase in culture medium of treated cultures according to the manufacturer's protocol of CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA). 50% cytotoxic concentration (CC50) values and 95% CI_s were determined using Prism software.

2.7.5 Evaluation of cellular internalization by confocal laser scanning microscopy

HELF cells were seeded at a density of 2 x 10^4/well in 24-well plates on glass coverslips. Subsequently, the cultures were incubated with 1 or 5 μg/mL of the labelled fluorescent nanoparticle suspension for different time points (1, 3 and 24 hours) and analysed on an inverted

Zeiss LSM510 fluorescence microscope as described previously (Bencini et al., 2008; Cavalli et al., 2009).

3. Results and discussion

Positively charged nanoparticles were prepared as indicated above, according to a starting Doehlert design for two variables, without replications, whose plan is represented in Table 1.

The starting amounts of foscarnet have been identified by referring to the study of the TTP/chitosan nanoparticle system (data not shown) and adjusting for the foscarnet/chitosan system.

The Doehlert design for two factors is a uniform shell design represented by a regular hexagon with a centre point. It allows extending or displacing design in any of the six directions, partially overlapping with the original design and requiring only three extra experiments to be performed.

The Doehlert design was used in order to find a suitable combination of variables that yielded nanoparticle samples characterized by low average diameter and narrow size distribution.

As can be seen from Figure 1, this design indicated that an increase in the amount of foscarnet could cause a decrease in nanoparticle size in the presence of lower amounts of chitosan, with a slight decrease of zeta potential. Among the samples obtained by this design, N7 was chosen for a preliminary investigation, as it appeared to show promising characteristics not only in regard to size and zeta potential but also in terms of yield and drug loading. The characteristics of a re-prepared N7 sample are summarised in the Table 3. Size and zeta potential of this re-prepared sample are slightly different from the previous ones, likely due to the use of a different lot of chitosan.

The design was then extended in the direction which potentially allowed to obtain nanoparticles of reduced particle size (Table 2).

The progressive reduction of particle size with increasing foscarnet amount while maintaining low values of chitosan amounts, was in line with the hypothesis of a greater packing of the polymer chains due to the higher number of amino groups of chitosan involved in the electrostatic interaction with the drug.

The results of the second design confirmed the above hypothesis, showing that an additional displacement of the experimental domain towards higher values of foscarnet amount might be useful to obtain nanoparticles with further reduced size. For this purpose, two other Doehlert experimental designs were set and explorative runs were carried out. These runs are highlighted in green and red respectively. Their foscarnet content was 12 and 14 mg (green) , and 17 and 19 mg (red);as shown in Figure 2 the chitosan content remained fixed at 4.5 mg.

Among the samples of nanoparticles obtained by these runs, those containing 17 and 19 mg of foscarnet were discarded because, despite having an appropriate particle size, the nanoparticle yield was too low.

Table 3 shows the data obtained from the new formulations, respectively named FOS12 and FOS14: it can be seen that the particle size decreased when the drug content increased, at a fixed chitosan amount.

A preliminary stability study carried out on the N7 sample showed that it was relatively unstable in PBS at 37°C, but the addition of glutaraldehyde improved its stability very significantly as shown in figure 3.

The drug release curves obtained from the N7 and N7GD1.13 samples are depicted in figure 4. Free foscarnet diffused almost immediately across the membrane of dialysis bag to receiving buffer (data not shown), thus indicating that also non-crosslinked nanoparticles gave a sustained release. As can be seen from the figure, the drug release was tendentially lowered when nanoparticles were crosslinked with glutaraldehyde.

On the basis of these results, glutaraldehyde was also used in the preparation of FOS12 and FOS14, so obtaining the corresponding crosslinked samples, named FOS12GD1.13 and FOS14GD1.13, respectively. In Table 4 the composition and characteristics of these new samples are reported along with those of N7GD1.13.

It is evident that nanoparticle size significantly decreased with the increasing of foscarnet content whereas drug loading and zeta potential remained substantially unchanged. Nanoparticle yield

presented a significant decreasing in the case of FOS14GD1.13. On the whole, these data indicated that a greater foscarnet content was able to reduce particle size, without effect on zeta potential up to a certain limit, beyond which nanoparticle formation was impeded, likely due to the strong excess of negative ions present into the system.

The corresponding stability curves of different nanoparticles prepared along with the drug release curves are depicted in Figure 5.

The stability curves clearly showed that when glutaraldehyde was added during nanoparticle formation, their size remained at around 500 nm up to 5 h in PBS at 37°C. The drug release curves showed that no significant differences existed among the various samples, with the exception of FOS12GD1.13, which presented a very sustained release of foscarnet.

It is noteworthy that the release curve of FOS14GD1.13 was almost superimposable with that of N7, in spite of the greater content of foscarnet in the formulation, crosslinking and smaller nanoparticle size. This result might be explained considering that the foscarnet excess, when greater than that used in the FOS12GD1.13 formulation, could yield a chitosan chain configuration that allowed a easier drug release from nanoparticles.

3.2 Results of biological studies

The formulations selected for biological studies in vitro were N7GD1.13, FOS12GD1.13 e FOS14GD1.13.

To compare the antiviral activity of plain foscarnet and N7GD1.13 nanoparticles on HCMV infection, cell monolayers were infected with GFP-expressing virus and exposed to the drugs. Figure 6 shows images of untreated and treated cell cultures after 72 hours. As is evident from the figure, untreated cells showed a high number of infected cells expressing GFP protein, while both foscarnet and foscarnet-loaded N7 nanoparticles exerted a dose-dependent antiviral effect to a similar extent. To quantify the inhibition, we performed a plaque-reduction assay in the presence of serial dilutions of the test formulations. The dose-response curve shown in Figure 7 indicates that

the antiviral activity of N7GD1.13 nanoparticles was similar to that of free foscarnet. The IC_{50} values for free foscarnet and N7GD1.13 nanoparticles determined at 5 days post infection were found to be 41.53 μM (95%CI: 31.45 to 54.85 μM) and 58.27 μM (95% CI: 41.10 to 82.60 μM), respectively. The IC_{50} value for plain foscarnet corresponded to previously published values (Kini et al., 1997).

The same assay was repeated to test the antiviral activity of FOS12GD1.13 and FOS14GD1.13 nanoparticles.

Similarly to plain foscarnet, the foscarnet-loaded nanoparticles, FOS12GD1.13 and FOS14GD1.13, exerted a strong inhibitory activity against HCMV, as reported by the dose-response curves in Figure 8 and by EC_{50} values of 59 μM (95%CI: 41 to 84 μM) and 40 μM (95% CI: 31 to 52 μM), respectively.

To exclude that the inhibitory activities observed were due to cytotoxicity, cell viability assay (MTS assay) and cell cytotoxicity assay were performed on treated HELFs. Nanoparticles showed no antiproliferative activity at the concentrations and time points chosen, as depicted in Figure 9. Furthermore, no significant difference in the release of the cytoplasmic enzyme LDH was observed between nanoparticle-treated and untreated tissues (Figure 10). These experiments revealed that N7GD1.13, FOS12GD1.13 and FOS14GD1.13 were not cytotoxic and that CC_{50} values (50% cytotoxic concentration) were higher than 1 mM.

We examined HELF cells treated with fluorescent nanoparticles by confocal laser scanning microscopy in order to investigate whether nanoparticles could penetrate inside cells. For this purpose the assay was carried out on living unfixed cells to avoid misleading due to the cell fixation protocols. As represented in Figure 6, no nanoparticle was internalised by cells different time points at the concentration of at 1 μg/ml Similar results were observed using a concentration of 5 μg/ml nanoparticles (data not shown).

These data suggest that the release of foscarnet from formulations occurred in the extracellular environment followed by its cellular penetration as free drug. On the other hand, the image g of

Figure 6 highlighted that after 1 h of exposure, FOS14GD1.13 was able to bind cell surface although in a completely reversible way. Fluorescence was not detected in control cells that had not been exposed to the labelled compounds (data not shown).

4. Conclusion

Foscarnet, acting in a manner similar to sodium tripolyphosphate, induced chitosan gelation and allowed to obtain positively charged nanoparticles, with small size and high zeta potential. This result was achieved through a proper selection of the drug/polymer ratio. These nanoparticles, although not internalized by infected cells, maintain the antiviral activity of the free drug and show no cytotoxicity. They represent a delivery system that could improve the therapeutic effect of foscarnet. In fact, foscarnet encapsulation into potentially long-circulating nanoparticles might lead to a prolonged blood residence and in vivo exposure of infected cells to the drug, accompanied by reduced toxic effects. On the other hand, an absorption increase by mucosal epithelium, due to the mucoadhesive properties of chitosan, could also make both the oral and topical route more effective for the administration of foscarnet.

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We would like to thank Professor Roberta Cavalli (University of Turin) for kindly gift the FITC chitosan.

Figure Captions

Figure 1 :Contour plots for D (nm) (A) and Z (mV) (B), derived from the first Doehlert design represented in Table 1.

Figure 2 : The Doehlert designs considered to explore the experimental domain for chitosan/foscarnet interaction

Figure 3: Stability curves in PBS at 37°C, registered for N7 sample and a sample of the same composition, prepared in the presence of 1.13 mg of glutaraldehyde (N7GD1.13). Measurements were carried out in duplicate. Data are presented as mean ± S:D.

Figure 4: Drug release curves obtained from samples N7 and N7GD1.13. Measurements were carried out in duplicate.. Data are presented as mean ± S:D.

Figure 5: Stability and drug release curves of non-crosslinked and crosslinked nanoparticles. The data are presented as the mean ± S.D. of at least two replicates.

Figure 6: Inhibition of GFP-expressing HCMV by free foscarnet and foscarnet loaded into N7GD1.13 nanoparticles. Helf cells were infected at a MOI of 0.1 and then exposed for 72 h to different drug concentrations. Cells were analyzed by confocal laser scanning microscopy without fixation.

Figure 7: Antiviral activity of free foscarnet, and foscarnet loaded into nanoparticle N7GD1.13 against HCMV strain AD-169. Helf cells were infected at a MOI of 0.004, washed to remove unbound virus and overlaid with 1.2% methylcellulose medium in presence of different drug concentrations. Five days postinfection, the immunostained plaques were counted, and the percent infection was calculated by comparing treated to untreated cultures. Values are the means of three separate determinations ± SD.

Figure 8. Antiviral activity of free foscarnet, and foscarnet loaded into nanoparticles FOS12GD1.13 and FOS14GD1.13 against HCMV strain AD-169. Helf cells were infected at a MOI of 0.004, washed to remove unbound virus and overlaid with 1.2% methylcellulose medium in presence of different drug concentrations. Five days postinfection the immunostained plaques were

counted, and the percent infection was calculated by comparing treated to untreated cells. Values are the means of three separate determinations ± SD.

Figure 9. Effect of foscarnet, N7GD1.13, FOS12GD1.13 and FOS14GD1.13 on the viability of non-infected Helf cells as a function of the drug concentration at 96 h. X axis: foscarnet concentration, Y axis: cell viability (% of untreated control). Each point represents the mean ±S.D. (n=3).

Figure 10. Effect of N7GD1.13, FOS12GD1.13 and FOS14GD1.13 on Lactate deydrogenase (LDH) release of non-infected Helf cells as a function of the drug concentration at 96 h. X axis: foscarnet concentration, Y axis: LDH release (% of untreated control). Each point represents the mean ±S.D. (n=3).

Figure 11. Evaluation of cellular uptake of fluorescent N7GD1.13, FOS12GD1.13 and FOS14GD1.13. Helf cells were incubated with the formulation for the times indicated and then analyzed by confocal laser scanning microscopy without fixation. The upper panels show the fluorescence images while the lower panels show fluorescence images merged with phase-contrast images.

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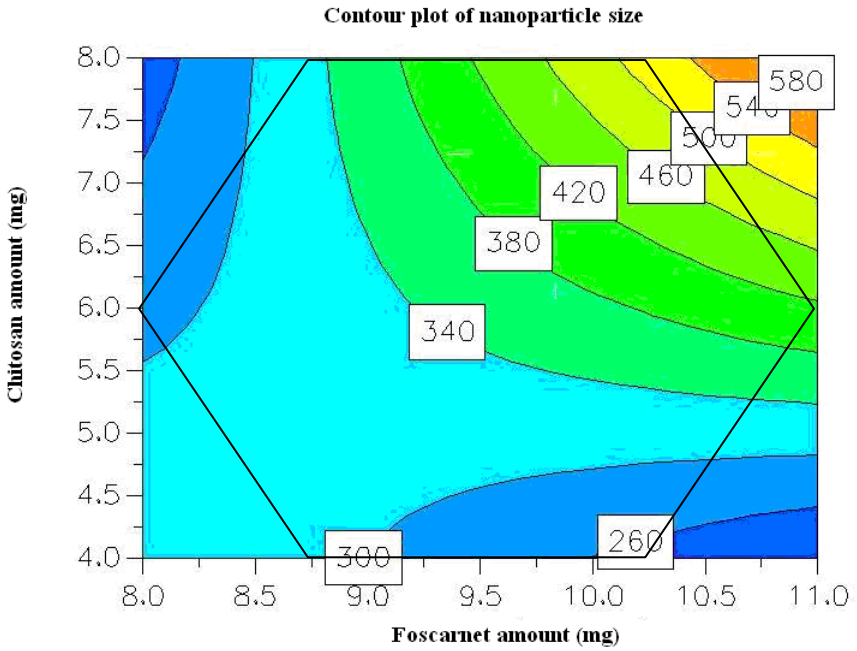
Figure 1



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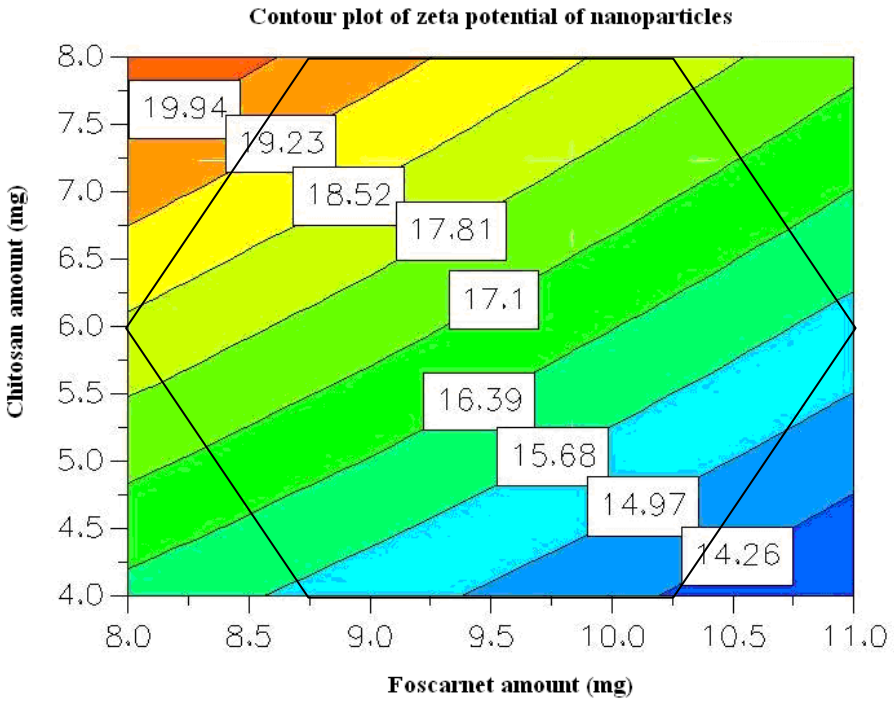

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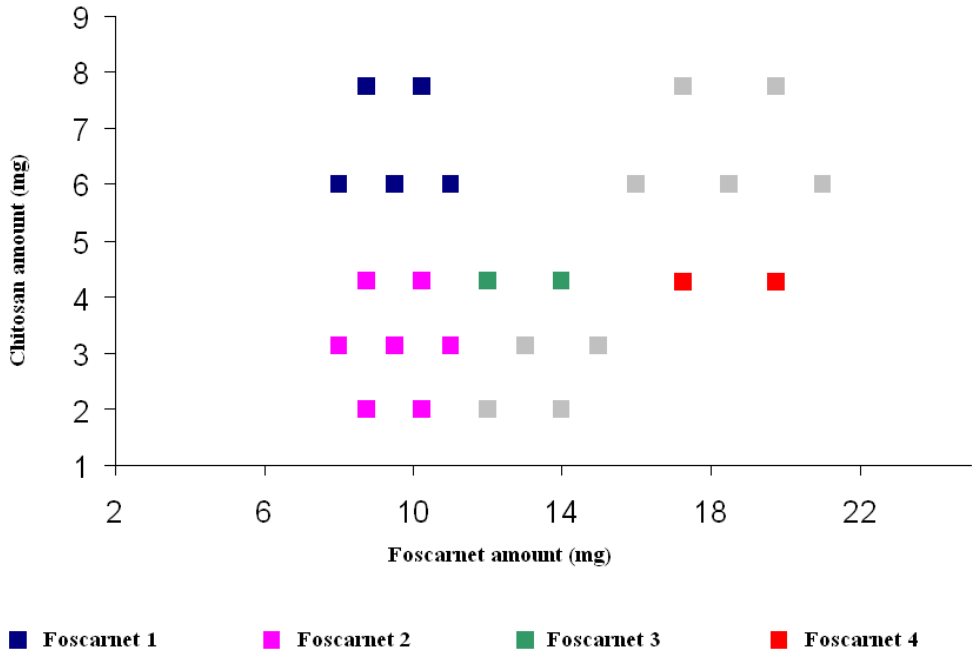

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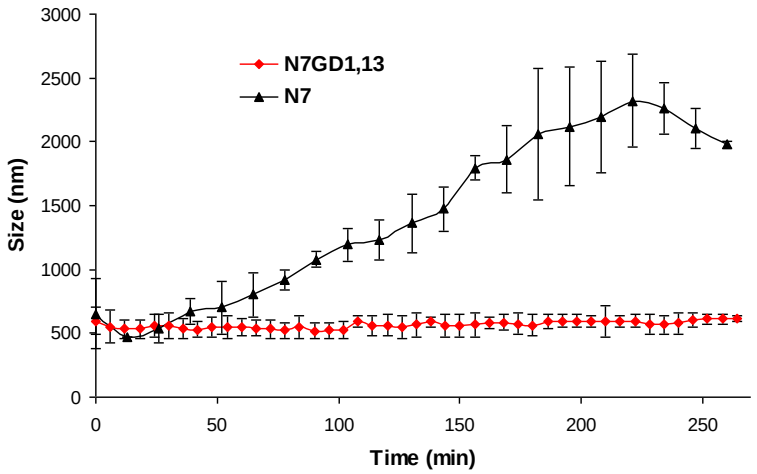

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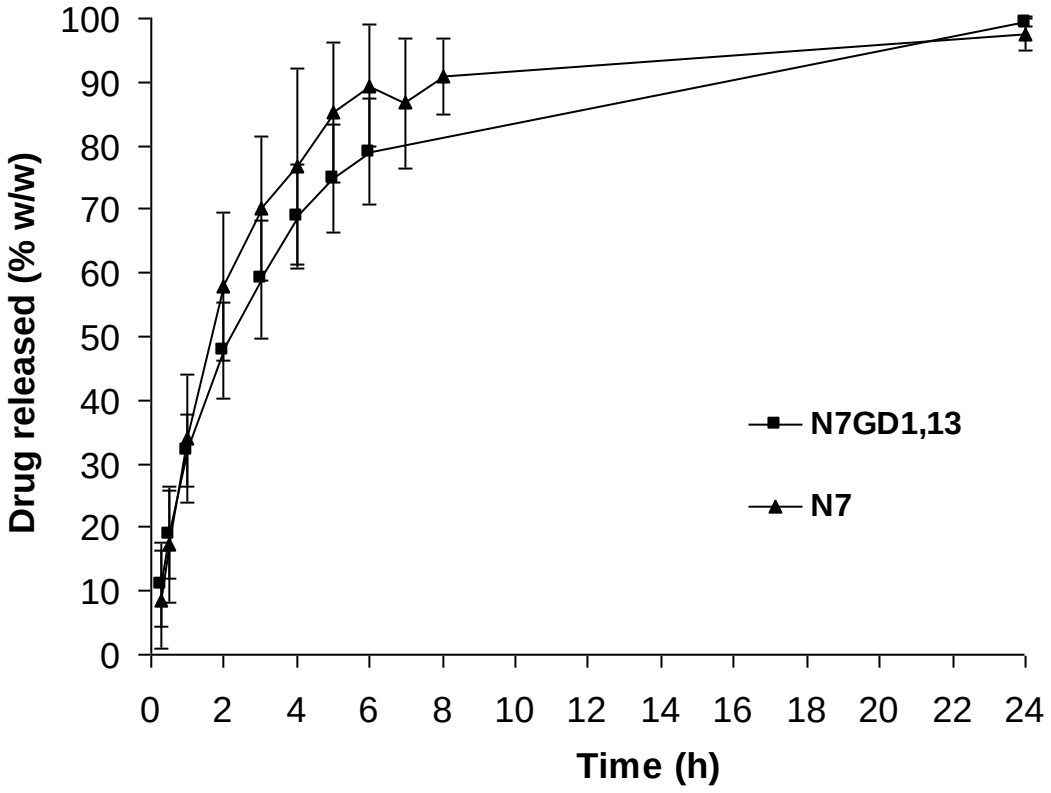

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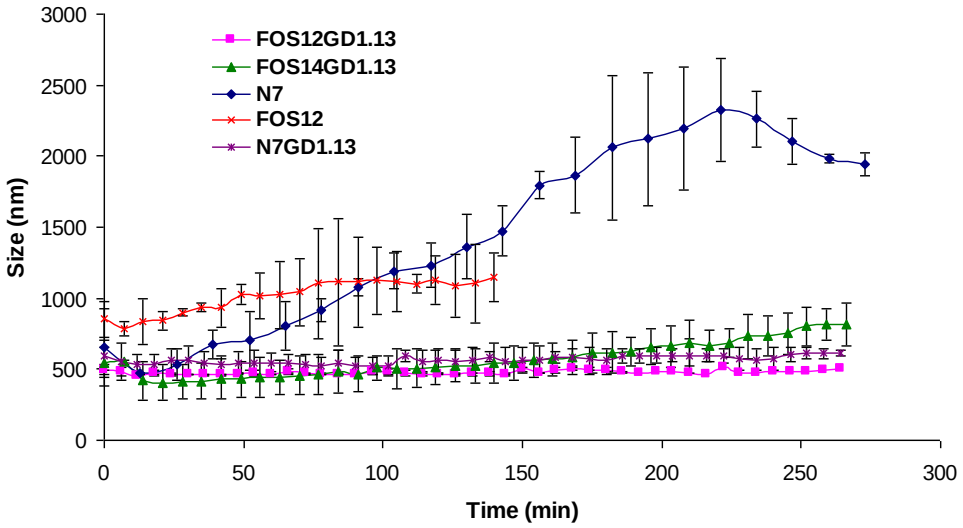

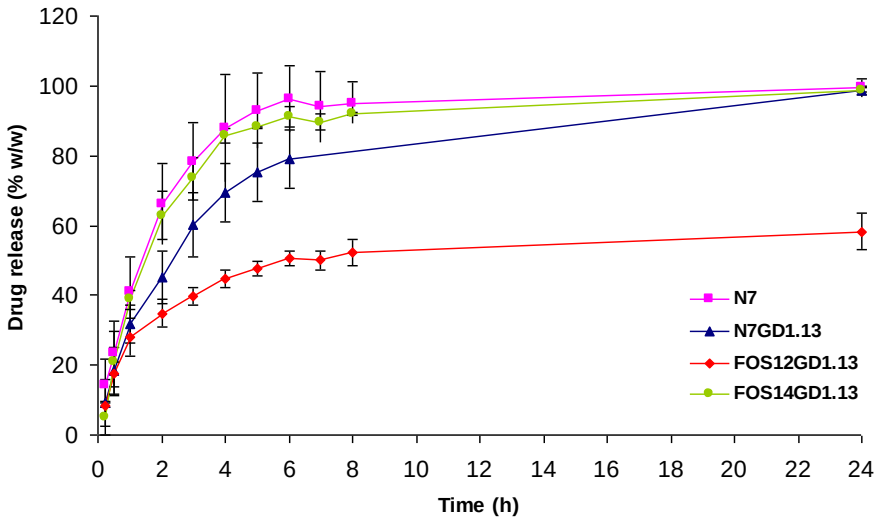

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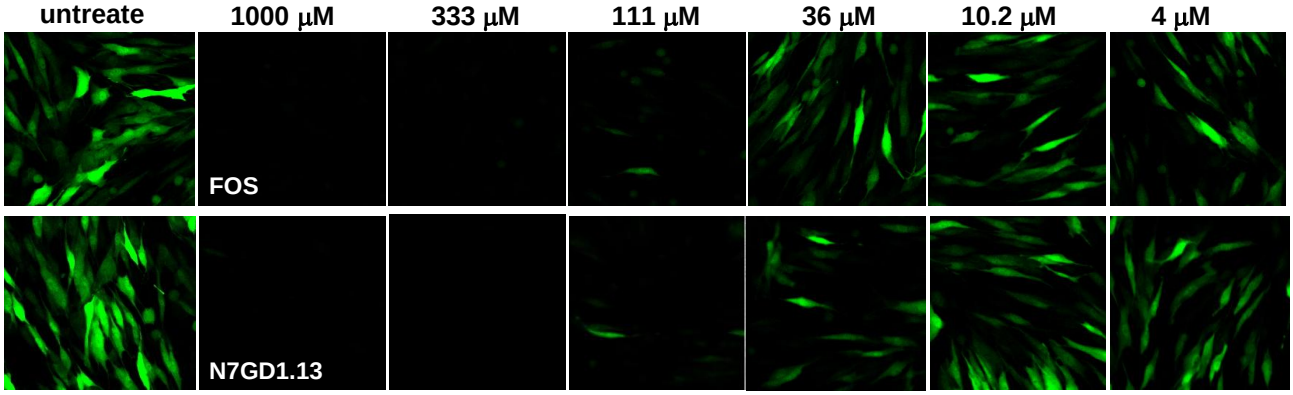

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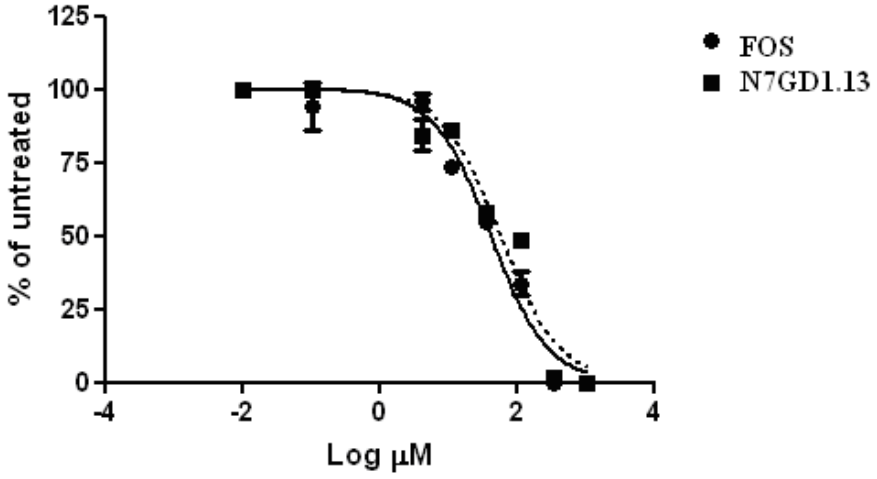

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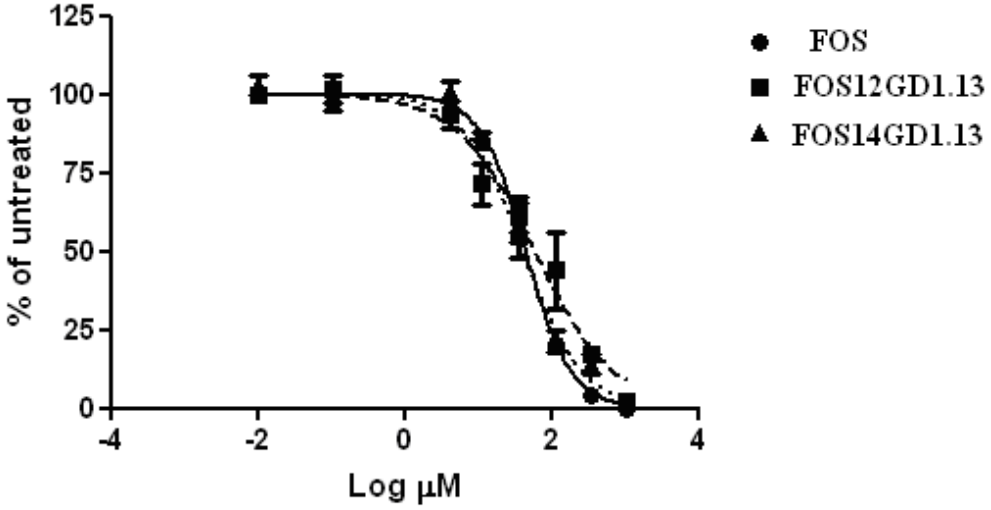

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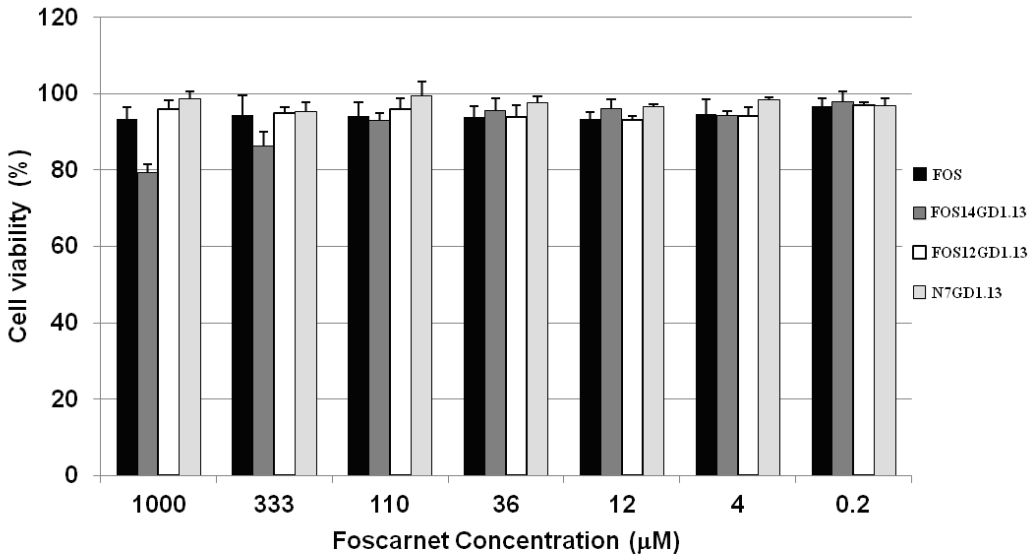

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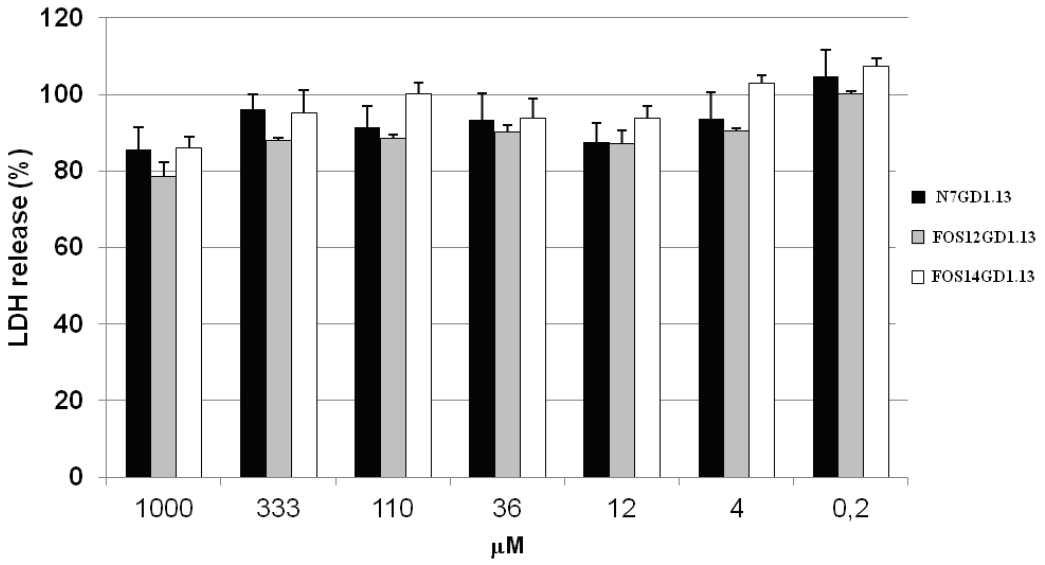

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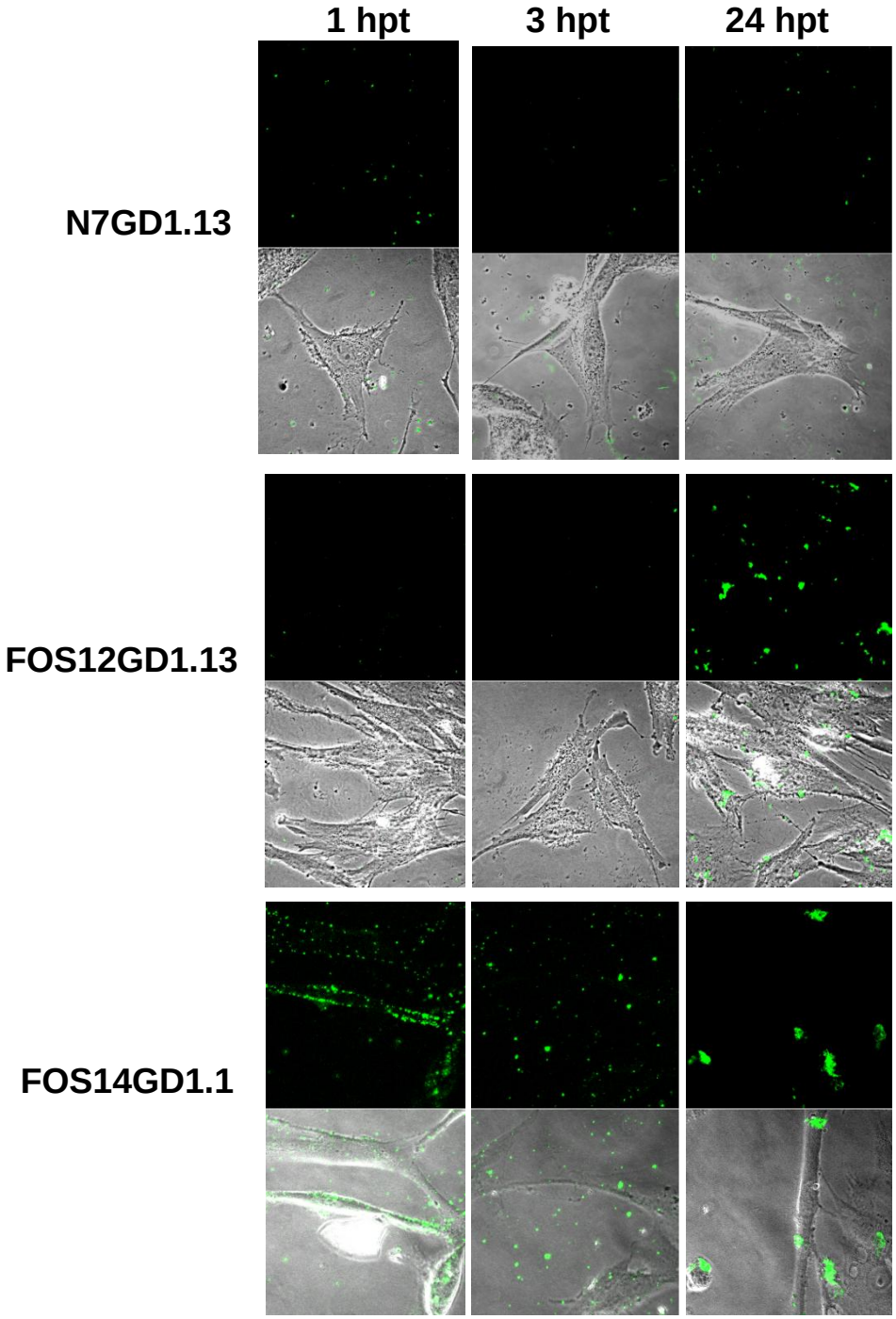

Table(s)

Table 1

The experimental plan of the first Doehlert experimental design (Foscarnet1). Experimental data are reported as mean values ± S.D.

Sample name	**Chitosan amount (mg)**		**Foscarnet amount (mg)**		**D (nm)**	**Z (mV)**
	Real value	Coded value	Real value	Coded value		
N1	6.00	0	9.50	0	352 ± 4	15.6 ± 0.5
N2	6.00	0	8.00	-1	292 ± 5	18.4 ± 0.3
N3	7.73	0.866	8.75	-0.5	338 ± 13	19.9 ± 0.3
N4	7.73	0.866	10.25	0.5	497 ± 13	17.7 ± 0.4
N5	6.00	0	11.00	1	427 ± 4	16.0 ± 0.3
N6	4.27	-0.866	10.25	0.5	265 ± 7	14.3 ± 0.4
N7	4.27	-0.866	8.75	-0.5	317 ± 7	16.2 ± 0.4

Table 2

The matrix of a Doehlert design (Foscarnet2) whose domain was shifted to higher values of foscarnet amount with respect to (Foscarnet 1) design. Experimental data are reported as mean values ± S.D.

Sample name	**Chitosan amount (mg)**		**Foscarnet amount (mg)**		**D (nm)**	**Z (mV)**
	Real value	Coded value	Real value	Coded value		
N1	3.14	0	9.50	0	178 ± 2	12.1 ± 0.2
N2	3.14	0	11.00	-1	149 ± 2	10.4 ± 0.4
N3	4.27	0.866	10.25	-0.5	206 ± 1	14.3 ± 0.4
N4	4.27	0.866	8.75	0.5	237 ± 2	16.2 ± 0.4
N5	3.14	0	8.00	1	204 ± 4	14.6 ± 0.3
N6	2.00	-0.866	8.75	0.5	139 ± 3	8.5 ± 0.5
N7	2.00	-0.866	10.25	-0.5	105 ± 1	7.5 ± 0.3

Table 3

Summary of the characteristics of samples N7, FOS12 and FOS14. Experimental data are reported as mean values ± S.D.

Sample name	D (nm)	Z (mV)	Yield (%w/w)	Drug loading (%w/w)
N7	349 ± 6	21.4 ± 0.5	37 ± 2	45 ± 2
FOS12	196 ± 2	22.4 ± 0.7	18 ± 1	32 ± 1
FOS14	208 ± 3	21.7 ± 0.7	14 ± 1	38 ± 1

Table 4

Composition and characteristics of nanoparticle samples obtained by crosslinking with glutaraldehyde. Experimental data are reported as mean values ± S.D.

Sample name	CHI (mg)	FOS (mg)	D (nm)	Z (mV)	Yield (%w/w)	Drug loading (%w/w)
N7GD1.13	4.5	8.8	302 ± 3	22 ± 1	26 ± 1	56 ± 2
FOS12GD1.13	4.5	12	279 ± 3	20.5 ± 0.7	24 ±1	56 ± 1
FOS14GD1.13	4.5	14	199 ± 3	24.5 ± 0.3	15 ± 2	53 ± 3