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New chitosan nanospheres for the delivery of 5-fluorouracil: preparation, characterization and *in vitro* studies

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Abstract

The aim of this work is to develop new chitosan nanospheres for the delivery of 5-fluorouracil (5-FU). Drug loaded nanospheres were prepared using a technique derived from a combination of coacervation and emulsion droplet coalescence methods. The size and morphology of nanospheres were characterized by laser light scattering and transmission electron microscopy. The 5-FU interaction with chitosan nanospheres was investigated by DSC analysis and FT-IR spectroscopy. The *in vitro* release was studied by dialysis bag technique. Cytotoxicity of 5-FU loaded chitosan nanospheres was evaluated *in vitro* on HT29 and PC-3 cell lines. The effects of 5-FU loaded chitosan nanospheres on adhesion of tumor cells to human umbilical vein endothelial cells (HUVEC) were also investigated. 5-FU loaded chitosan nanospheres appeared with a spherical shape, with a mean diameter of about 200 nm and a negative zeta potential of about - 6.0 mV. The successful interaction between drug and chitosan nanosphere matrix was demonstrated by both DSC and FT-IR analyses. The quantitative determination of 5-FU was assayed by UV-Vis analysis. The encapsulation efficiency of 5-FU content was about 70%. A kinetic study of *in vitro* release demonstrated that the percentages of 5-FU delivered from nanospheres was approx. 10% after 3 hours. The *in vitro* studies showed that 5-FU loaded nanospheres were effective in reducing tumor cell proliferation in a time- and concentration-dependent manner. 5-FU nanospheres were also able to inhibit both HT29 and PC-3 adhesion to HUVEC after 48 hours of treatment.

Keywords: Chitosan, drug delivery systems, 5-fluorouracil, nanospheres, sustained release

Introduction

The delivery of insufficient drug concentrations to the tumor site, after both intravenous and oral administration, and the high systemic toxicity of many anticancer drugs are the major reason for limited efficacy of chemotherapy. Nanomedicine, the medical application of nanotechnology, was proposed as a strategy to improve many current cancer treatments by allowing various advantages, such as the increase of water solubility, the site-specific accumulation and the sustained delivery of drugs [1]. In recent years, nanoparticles have been developed as drug carriers and they seem to be effective, providing low side effects and targeted action on cancer cells [2-5].

5-Fluorouracil (5-FU), or 5-fluoro-2,4-pyrimidindione, is one of the oldest chemotherapeutic agents and it has played a dominant role for decades in the treatment of breast cancer and of a variety of other solid tumors. 5-FU is an antimetabolite of the pyrimidine analog class which is widely used alone or in combination with chemotherapy regimens. It interferes with the synthesis of nucleic acid, inhibits DNA synthesis, and, eventually, inhibits cell growth [6]. As a consequence of a very rapid *in vivo* metabolism, 5-FU shows a short biological half-life [7]. Intravenous administration of 5-FU results in a large systemic distribution, with only a small fraction of the dose reaching the site of action. In addition, oral delivery is not a realistic option for delivering, because the drug displays a not uniform oral absorption. Moreover, tumor cells often undergo significant development of drug resistance. These problems lead to the need of high doses of 5-FU, so its use has been restricted by its systemic toxicities, as severe gastrointestinal toxicities, hematologic side effects and severe bone marrow disturbances [8].

Various polymeric nanoparticles were proposed to improve the 5-FU administration [9-12]. Different polymers have been studied for 5-FU delivery, such as poly(glycolide-*co*-lactide-*co*-caprolactone) nanoparticles [13], self-assembled thermo-sensitive polyelectrolyte complex nanoparticles [14] and bovin serum albumin (BSA) nanoparticles [15, 16]. Various liposomal formulations of 5-FU have been described [17]. Among polymeric nanoparticulate systems, chitosan is a polymer largely used for the 5-FU delivery, because of it can easily form nanoparticles by crosslinking with different agents, such as glutaraldehyde, sodium citrate, sodium hydroxide, formaldehyde [18, 22].

The aim of this work is to prepare small-sized polymeric nanospheres for the delivery of 5-FU. Chitosan was selected for nanospheres matrix, because of its biocompatibility, biodegradability and low toxicity [23, 24]. It is a linear cationic polysaccharide, composed of randomly distributed β -(1-4) linked D-glucosamine and N-acetyl-D-glucosamine, derived from deacetylated chitin [25-27]. The positive charge of the chitosan macromolecule promotes the interaction with polyanions. It is worth noting that this process has been widely used to obtain drug carrier systems through complexation, forming nano-/microparticles specifically suitable for hydrophilic molecules [28-31]. The present work is focused on the improvement of the preparation process to obtain small chitosan nanospheres by the combination of coacervation and emulsion coalescence methods.

Material and Methods

Materials

5-Fluorouracil (5-FU), chitosan (medium molecular weight), Arlacel®83, Tween® 80 and sodium citrate monobasic were supplied from Sigma Aldrich. Fetal calf serum (FCS) (endotoxin tested) was obtained from Hyclone Laboratories (Logan, UT). Trypsin was provided by Difco Laboratories (Detroit, MI). M199 and RPMI-1640 (endotoxin tested), thiazolyl blue tetrazolium bromide were purchased from Sigma-Aldrich (St. Louis, MO). A chitosan aqueous solution (0.7% w/w) was obtained dissolving the polymer in a 0.1 M acetic acid solution. Milli Q water was used for all the experiments. All the other reagents were of analytical grade. The experiments were performed in triplicate.

Preparation of 5-FU loaded chitosan nanospheres

The nanosphere preparation method consists of a combination of coacervation and emulsion droplet coalescence methods [32, 33]. This method exploits an emulsion as template, in order to obtain nanosized particles, starting from nanosized emulsion droplets. For this purpose, an W/O emulsion, containing a chitosan aqueous solution at pH=5.0 with 5-FU (10 mg/ml) as internal phase, was prepared. Arlacel 83 and Tween 80 were selected as surfactants and mineral oil as external phase. The qualitative-quantitative composition of the W/O emulsion is reported in the Table 1. The emulsion was obtained after homogenization for 3 minutes using an high shear homogenizer (Ultraturrax, IKA, Germany) and sonication for 10 minutes in order to reduce drop sizes. Then the W/O system was dropped by a syringe, using a roller pump into a sodium citrate aqueous solution (0,1 M) under magnetic stirring (Fig.1). This semi-automatic technique was tailored to guarantee a high reproducibility to the preparation process, avoiding manual mistakes. Completed the drift phase, the nanosphere dispersion was left under stirring for 30 minutes; then, after further 60 minutes, it was centrifuged to remove mineral oil derived from the W/O emulsion and then it was washed. Finally, the aqueous suspension of nanospheres was filtered through a filter paper. The nanosphere aqueous dispersion was stored at 4°C. Blank nanospheres were obtained using the same preparation method in the absence of 5-FU.

Samples of the two nanosphere aqueous dispersions were freeze-dried using a Modulyo freeze-drier (Edwards, UK) to obtain a dry product.

Table 1 W/O Emulsion qualitative-quantitative composition.

	Component	Amount (%)
Oily phase	Mineral oil	46.15
	Arlacel 83	3.50
	Tween® 80	0.35
Aqueous phase	Chitosan aqueous solution (pH 5.0)	50.00

Figure 1 Schematic representation of the experimental set-up for chitosan nanosphere preparation

Characterization of 5-FU loaded chitosan nanospheres

- Determination of particle size and Zeta potential

Mean particle diameter, polydispersity index and Zeta potential of the 5-FU loaded nanospheres and blank nanospheres were determined by Photon Correlation Spectroscopy using a *90 Plus Instrument Brookhaven*, at a fixed scattering angle of 90°, at room temperature. The nanosphere dispersion was diluted with filter water and analyzed in triplicate.

- Evaluation of nanosphere morphology

The morphology and size of 5-FU loaded nanospheres were observed by Transmission Electron Microscopy (TEM) (CM10, Philips). A drop of the 5-FU loaded nanosphere aqueous dispersion was diluted with filtered water and placed onto a copper micro-grid and evaporated in air at room temperature before observation.

- Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was carry out using a DSC7 Perkin Elmer instrument, connected to a computer with Pyris Software Version 3.7.1. The instrument was calibrated with indium for melting point and heat of fusion. DSC thermograms of blank nanoparticles, free 5-FU, 5-FU-loaded nanoparticles were evaluated.

A weighed amount of 3-4 mg of freeze-dried chitosan nanospheres containing 5-FU and of 1.5-2 mg free 5-FU were put in suitable aluminum pans. The analysis was performed in the temperature range between 25°C and 300°C, at a speed of 10 °C per minute under a nitrogen flow.

- Fourier transformed infra-red (FT-IR) spectroscopy

Fourier transform infra-red spectroscopy (FTIR) was applied to 5-FU loaded chitosan nanospheres, blank chitosan nanospheres and free 5-FU using a Perkin Elmer system 2000 spectrophotometer. The spectra were recorded between 4000–400 cm⁻¹ using KBr pellets.

Determination of 5-FU encapsulation efficiency in the nanospheres

The quantative determination of 5-FU content in the nanospheres was assayed by UV-Vis analysis with a *Beckman-Coulter DU 730* spectrophotometer. 5-FU concentrations were obtained by reference to a calibration curve. The calibration curve is linear in the range 2–15 µg/ml with a r² of 0.9996. The wavelength selected for determination of 5-FU concentration was 266 nm. The

encapsulation efficiency and loading capacity of 5-FU-loaded chitosan nanospheres were evaluated by separation of nanospheres from aqueous medium containing non-associated 5-FU by ultracentrifugation at 8000 rpm for 30 minutes at 25°C. The amount of free 5-FU in the supernatant were diluted with water and analyzed spectrophotometrically.

In vitro drug release studies

The in vitro release of 5-FU from the chitosan nanospheres was investigated in phosphate buffer solution at pH 7.4.

The *in vitro* release studies were carried out using multicompartiment rotating cells with a hydrophilic dialysis membrane (*Spectra/Por, Spectrum®*, cut-off 12000-14000 Da). For this purpose, a phosphate buffer suspension of 5-FU-loaded nanospheres (drug concentration: 1 mg/ml) was compared to free 5-FU (1 mg/ml) also dissolved in phosphate buffer (pH = 7.4). The experiment was conducted for 5 hours and the receiving phase, which consisted of phosphate buffer pH 7.4, was completely withdrawn and replaced with fresh medium after fixed time intervals. The amount of drug released was measured spectrophotometrically at 266 nm. The experiment was carried out in triplicate.

Cell and culture conditions

HT29 cells were derived from human colon adenocarcinoma and PC-3 cells were derived from human prostate carcinoma. Both of them were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultured at 37 °C in a 5% CO₂ humidified atmosphere in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin.

Cell proliferation

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide) analysis was performed in 96-well plates. 1000 cells/well were seeded in 100 µl of complete medium. After 24 hours, they were treated for 48-72 hours with increasing concentration of 5-FU or nanospheres of 5-FU (10⁻⁶-10⁻⁵M). Subsequently, cells were supplemented with 11 µl of 5 mg/ml thiazolyl blue tetrazolium bromide for 2 hours. Thereafter, the medium was removed and cells were lysed with 100 µl of DMSO. Absorbance was recorded at 570 nm by a 96-well-plate ELISA reader.

Fluorescent labeling of HT29 and PC-3 cells

Commercial fluorescent cell linker kit PKH67 was used for membrane labeling of HT29 and PC-3 cells, following the manufacturer's directions as described in the kit. The staining efficiency was monitored by fluorescent microscopy.

Cell adhesion assay

HUVECs (Human Umbilical Vein Endothelial Cells) were isolated from human umbilical veins by trypsin treatment (1%). They were cultured in M199 medium, with the addition of 20% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 5 UI/ml heparin, 12 mg/ml bovine brain extract, and 200 mM glutamine. HUVECs were grown up to confluence in flasks and they were used at passages two through five. The use of HUVEC was approved by the Ethics Committee of the “Presidio Ospedaliero Martini” of Turin (Italy) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all donors.

HUVECs were grown up to confluence in 24-well plates, washed, and rested for one day in M199 plus 10% FCS. For titration experiments, HUVECs were treated or not for 24-48 hours with increasing concentrations of 5-FU or with 5-FU loaded chitosan nanospheres (10^{-7} - 10^{-5} M). After the treatments, HUVECs were incubated for one hour with prelabeled tumor cell line (1×10^5 cell/well). One hour incubation time was chosen in order to allow a full sedimentation of the adhering cells [34]. After incubation, non-adherent cells were removed by washing three times with M199. The center of each cell was analyzed by fluorescence image analysis. Adherent cells were counted using Image Pro Plus Software for microimaging (version 5.0; Media Cybernetics, Bethesda, MD). Single experimental points were assayed in triplicate, and the SEM of the three replicates was always close to 10%. Data are shown as percentage of inhibition versus the control adhesion measured on HUVECs not treated with the drug. This control adhesion was 65 ± 4 cells/microscope field ($n = 5$) for HT29 cells and in a similar range for PC-3.

Statistical analysis

Data are expressed as means $\pm$ SEM of $n=5$ experiments. Statistical analysis was performed with GraphPad Prism 4.0 software. Significance was assessed with Student's t-test for paired varieties or one-way ANOVA and the Dunnett test with $p \leq 0.05$ as the cut-off.

Results and discussion

In this work, the developed preparation technique consists of a combination of coacervation and emulsion droplet coalescence methods. The coacervation exploits the physico-chemical property of chitosan, insoluble at alkaline pH, to precipitate when it comes in contact with basic solutions. Nevertheless, this method is not suitable to obtain systems in the nanometric range and with a high drug loading. Generally, chitosan nanoparticles were obtained using the ionic gelation of the polymer with sodium tripolyphosphate (TPP) solutions [35]; nanoparticles formed through the intermolecular cross-linking of amino groups of chitosan with the negative phosphate groups of TPP. Tuning the molecular weight, degree of deacetylation and the concentration of chitosan, it is possible to prepare small nanoparticles, with uniform nanoparticles [36]. Alternatively, a complex coacervation can be used to prepare chitosan nanoparticles, consisting of the addition to a chitosan solution an oppositely charged polyelectrolyte solution.

The emulsion-droplet coalescence method uses the principles of both emulsion cross-linking, based on the linking between chitosan and different cross-linking agents, and precipitation in a base solution. In this protocol, a stable water-in-oil emulsion of chitosan solution and a similar one containing a NaOH solution were prepared. Mixing both the emulsions, a precipitation is induced due to the coalescence of chitosan droplets with NaOH droplets [37].

The preparation method developed in this work exploits an emulsion as template, in order to obtain nanosized particles, starting from nanosized emulsion droplets. Recently, template methods have been proposed to obtain nanoparticles with a monodisperse size distribution [38]. We developed a semi-automatic technique, based on the use of a roller pump to drop the emulsion at constant rate

through a syringe needle, to ensure a high reproducibility of the preparation process avoiding manual mistakes. Moreover, this experimental set-up is ease for scaling-up. It is worth noting that no toxic solvents were used to obtain the emulsion and all the components are admitted for pharmaceutical use.

Average diameter, polydispersity, and zeta potential of prepared blank chitosan nanospheres and 5-FU loaded chitosan nanospheres were about 210 nm, 0.17, and -8.2 mV and about 199 nm, 0.10 and - 6.5 mV, respectively. (Table 2).

Table 2 Characteristic of blank chitosan nanospheres and 5-FU loaded chitosan nanospheres.

	<i>Average diameter (nm)</i>	<i>Polydispersity index</i>	<i>Zeta Potential (mV)</i>
Blank Chitosan Nanospheres	209.5 ± 24.0	0.17 ± 0.02	-8.2 ± 0.3
5-FU loaded Chitosan Nanospheres	198.6 ± 14.3	0.10 ± 0.04	-6.5 ± 0.8

For cancer therapy, the average size and size distribution of nanoparticles are critical determinants for their bioavailability [39]. In fact, particles with an average size lower than 500 nm can mainly extravasate and accumulate in tumor parenchyma, though a passive mechanism, exploiting the enhanced permeation and retention effect (EPR).

A representative TEM micrograph of the 5-FU loaded nanospheres showed that nanoparticles have a spherical shape and smooth surface. TEM analysis also reveals that 5-FU-loaded chitosan nanospheres are discrete and non-aggregated (Fig.2a), as confirmed by the particle size analysis (Fig. 2 b)

Figure 2 a) TEM micrograph b) Size distribution graph of 5-FU loaded nanospheres

The 5-FU interaction with chitosan nanospheres was confirmed by DSC analysis. The DSC thermograms of 5-FU-loaded nanospheres, blank nanospheres and free 5-FU are showed in Fig.3. The drug shows an endothermic peak at about 280°C, in correspondence to the 5-FU fusion. This peak is not present in the DSC thermograms of both 5-FU-loaded nanospheres. This disappearance of the drug melting peak indicates that 5-FU is molecularly dispersed in the chitosan matrix, which composes nanospheres, and it is not able to crystallize, confirming the 5-FU interaction with the nanosphere structure.

Figure 3 DSC thermograms of free 5-FU, 5-FU-loaded nanospheres and blank nanospheres.

FTIR spectra of 5-FU loaded chitosan nanospheres, blank chitosan nanospheres and free 5-FU are shown in Fig.4. In the free 5-FU spectrum the specific peaks of the drug molecule are visible, such as the peak of the carbon-fluorine bond between $1400\text{--}1200\text{ cm}^{-1}$. The spectrum of chitosan shows an enlarged band between $3500\text{--}3000\text{ cm}^{-1}$, where hydroxyl groups and amino groups stretching overlap. Bands relative to CO and COC groups, both very frequent in the molecule of chitosan, are also clearly visible between $1200\text{--}1000\text{ cm}^{-1}$. The successful interaction between drug and nanospheres is demonstrated in the spectrum of the 5-FU loaded nanospheres from the displacement and the peak change of the typical bands of the drug.

Figure 4 FTIR spectra of free 5-FU, 5-FU loaded chitosan nanospheres and blank chitosan nanospheres

In order to determine the 5-FU content in the nanospheres UV-visible spectrophotometric analysis was carried out. The mean of encapsulation efficiency percentages with the standard deviation of 5-FU content was 69.9 ± 3.9 and the loaded capacity was about 42%. It is supposed that the drug loss may be caused by the purification processes, aimed to eliminate the oily phase. The drug loading did not change over time.

The advantages of nanoparticles for the delivery of anticancer agents can include: prolonged drug release, enhanced drug accumulation in cancer tissues, prolonged half-life in blood circulation and

increased cellular trafficking of the drug incorporated in their structure [40]. In this work the release profile of 5FU from nanospheres showed much slowly compared to the free drug. The results of the *in vitro* release kinetics study of 5-FU from the chitosan nanospheres in comparison with free 5-FU are shown in Fig. 5.

5-FU-loaded nanospheres did not show an initial burst effect, proving that the active molecule was incorporated into the chitosan matrix of nanospheres and not adsorbed on their surface. After 3 hours the amount of 5-FU released from nanospheres was approx 10%. The constant and slow release profile of the drug might be related to the slow diffusion of 5-FU within the cross-linked polymer matrix. A sustained and low release of 5-FU can prevent the non-specific toxicity of 5-FU decreasing the amount of free drug and related side effects, as previously reported [41].

Figure 5 Percentage of 5-FU released from chitosan nanospheres in comparison with free 5-FU over time. Each point represents the mean (n = 3).

The anti-tumor efficacy of 5-FU loaded chitosan nanospheres, blank chitosan nanospheres and free 5-FU was carried out on HT29 cells, derived from human colon adenocarcinoma, are reported in Figs. 6a and 6b, respectively. HT29 cells were treated for 48-72 hours with increasing concentration of the drugs (10^{-6} - 10^{-5} M). The results show that both formulations were effective in reducing cell proliferation in a time- and concentration-dependent manner. After 48 and 72 hours of treatment, 5-FU decreased HT29 growth and 5-FU loaded chitosan nanospheres showed a statistically greater efficacy. In order to confirm the higher anti-tumor efficacy of the 5-FU loaded chitosan nanospheres, experiments were repeated with PC-3, a different cancer cell lines, derived from human prostate carcinoma. The inhibition effect of 5-FU was lower on these cells, being active only after 72 hours of incubation (Fig.6c and 6d). Nevertheless, the 5-FU nanospheres inhibited PC-3 proliferation to a similar extent, and with a similar kinetics to that displayed on HT29. By contrast, blank chitosan nanospheres were completely ineffective in all the experiments (data not shown), as previously reported for different chitosan formulation[42].The nanosphere formulation may increase the amount of 5-FU entering the two cell lines. Indeed fluorescent chitosan nanospheres can be internalized by cancer cells (data not shown).

Figure 6 Inhibition of proliferation following 5-FU and 5-FU loaded chitosan nanospheres treatment. HT29 (a,b) and PC-3 (c,d) (1000 cells per well) were treated with increasing concentrations (10^{-6} - 10^{-5} M) of 5-FU and 5-FU loaded chitosan nanospheres for 48 (a,c) – 72 (b,d) hours. Results are expressed as % inhibition of control and shown as mean $\pm$ SEM ($n = 5$). * $P < 0.05$; ** $P < 0.01$, significantly different from control; # $P < 0.05$ significantly different from 5-FU; one-way ANOVA and Dunnett's test.

To assess the effect of 5-FU and 5-FU loaded chitosan nanospheres on tumor cells adhesion to ECs, HUVECs were treated or not with different concentrations of the two formulation (10^{-7} - 10^{-5} M) for 24-48 hours. After that, they were used in the adhesion assay with each tumor cell line. Fig. 7 (a= HT29; b= PC-3) shows that both formulations were effective in reducing tumor cell adhesion. However, a significant difference was revealed after 24 hours of treatment, being the 5-FU loaded chitosan nanospheres more effective at the highest concentration tested. Maximal inhibitions were $58 \pm 5\%$ for 10^{-5} M 5-FU loaded chitosan nanospheres and only $33 \pm 2\%$ for 10^{-5} M 5-FU. It is worth noting that only the 5-FU loaded chitosan nanospheres were able to inhibit HT29 adhesion to HUVEC after 48 hours of treatment.

Figure 7 Effect of HUVEC treatment with 5-FU and 5-FU loaded chitosan nanospheres on HT29 cell line adhesion. HUVECs were pretreated or not with increasing concentrations (10^{-7} - 10^{-5} M) of 5-FU and 5-FU loaded chitosan nanospheres for 24 (a) - 48 (b) hours and then incubated with HT29 cell line for 1 hour. Results are expressed as % inhibition of control and shown as mean $\pm$ SEM ($n = 5$). * $P < 0.05$; ** $P < 0.01$, significantly different from control; $\# P < 0.05$; $\#\# P < 0.01$ significantly different from 5-FU; one-way ANOVA and Dunnett's test.

Fig. 8 shows micrographs of the HT29 adhesion assays on untreated HUVECs (panel a) or those that were treated with 10^{-5} M of 5-FU (panel b) or 5-FU loaded chitosan nanospheres (panel c). It is worth noting that only the 5-FU loaded chitosan nanospheres were able to inhibit HT29 adhesion to HUVEC after 48 hours of treatment.

Figure 8 Fluorescent microscopy of HT29 cells adherent to HUVECs. HUVECs were not treated (panel a), or treated with 5-FU (panel b), and 5-FU loaded chitosan nanospheres (panel c) (x100 magnification).

The adhesion experimental test was repeated using the PC-3 cell line. The results were similar to those obtained with HT29 cell line (Fig.9).

Figure 9 Effect of HUVEC treatment with 5-FU and 5-FU loaded chitosan nanospheres on PC-3 cell line adhesion. HUVECs were pretreated or not with increasing concentrations (10^{-7} - 10^{-5} M) of 5-FU and 5-FU loaded chitosan nanospheres for 24 (a) - 48 (b) hours and then incubated with PC-3 cell line for 1 hour. Results are expressed as %

inhibition of control and shown as mean $\pm$ SEM ($n = 5$). * $P < 0.05$; ** $P < 0.01$, significantly different from control; $\# P < 0.05$; $\#\# P < 0.01$ significantly different from 5-FU; one-way ANOVA and Dunnett's test.

Conclusion

5-FU loaded chitosan nanospheres of about 200 nm sized were obtained using a purposely tuned set up, ease to be scaled. They showed a good encapsulation efficiency and prolonged release profile of the drug. The incorporation of the 5-FU in the nanocarrier may be exploited to improve the therapeutic effect and to overcome the drug resistance.

Development of resistance by cancer cells to chemotherapeutic agents has currently become a major clinical problem, limiting the effectiveness of the treatment of hematological malignancies as well as solid tumors. One mechanism of drug resistance is due to the prevention of a drug from entering cells; this can depend from deficiencies in membrane nucleoside transporters or the overexpression of ATP-dependent drug efflux transporters like P-glycoprotein. Therefore, the drug accumulation is substantially reduced when the expression of such nucleoside transporters is deficient or the activity of drug efflux transporter proteins elevated. We suggest that 5-FU loaded chitosan nanospheres may overcome these transport defects by entering into the cells without a specific carrier-mediated transport. This mechanism is under investigation and it will be described in a future work.

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