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Couple delivery of Imatinib Mesylate and Doxorubicin with nanoscaled polymeric vectors for a sustained downregulation of BCR-ABL in Chronic Myeloid Leukemia

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In this work, we have investigated the potential benefits of combining biodegradable pH sensitive core-shell PCL NPs loaded with IM and enzyme sensitive polyelectrolyte complexes (PECs) loaded with Doxorubicin (DOX). Our *in vitro* studies confirmed the excellent antileukemic activity of dual drug loaded nanoparticles on CML cells. As compared with drug alone, co-treatment with IM and DOX loaded in nanoparticles allowed a sustained downregulation of BCR-ABL and significant CML stem cell death. This further more showed that couple formulation of nanoparticles enhanced drug's kinetics and efficacy, combined with the pH sensibility of core-shell PCL NPs loaded with IM and enzyme sensitive polyelectrolyte complexes (PECs) loaded with DOX. Our study demonstrates that dual drug loaded nanoparticles work in a synergistic manner lowering the dose and confirming that both drugs reach the target cell specifically, maximizing the cytotoxicity while minimizing the chances of cell resistance to any one drug.

Introduction

Chronic Myeloid Leukemia (CML) is a pluripotent hematopoietic stem cell disorder, characterized by the Philadelphia chromosome (Ph). The Ph chromosome is the product of a chromosomal translocation involving the c-abl oncogene on chromosome 9 and the bcr oncogene on chromosome 22.[1] The chimeric oncogene, p210 BCR-ABL is present in almost all cases of CML. BCR-ABL tyrosine kinase activates a number of cell signalling pathways, leading to increased cell proliferation, blocked differentiation, and resistance to apoptosis induced by drugs. Imatinib Mesylate (IM, STI571, Gleevec®) inhibits BCR-ABL activity by occupying its ATP-binding pocket.[2] Targeted therapy with IM induces complete cytogenetic responses in more than 80% of newly diagnosed patients in the chronic phase.[3,4,5,6] Although active in initial stages of CML, IM is incapable to eliminate malignant progenitors and numerous patients develop drug resistance after long-time use. It is therefore of great importance to develop an alternative strategy to overcome both IM resistance and the reduced sensitivity to DNA-damaging drugs in CML cells. Synergistic combinations of two or more drugs can overcome toxicity and other side effects linked with high doses of single drugs by countering biological compensation, allowing reduced dose of each drug or accessing context-specific multitarget mechanisms. Most of all, the attention is concentrated on the development of combination treatments to avoid relapse of CML. A supplementary drug seems to be indispensable, since BCR-ABL may cause epigenetic/genetic modifications in leukemia cells which renders them insensitive to BCR-ABL inactivation.[7] One of the candidates for the combined therapy could be doxorubicin (DOX). The anticancer activity of DOX is largely used and the mechanisms of its action are defined.[8] The idea of drug targeting and controlled drug delivery is used as an effort to increase the therapeutic index of drugs by increasing their localization to specific cells and by reducing their possible toxic side effects at normal sensitive sites. Controlled drug delivery implicates the association of a drug with a carrier system, thereby allowing modulation of the drug pharmacokinetic properties and biodistribution. Thus, many polymeric nanoparticles have been introduced as drug delivery systems to improve the anticancer drug delivery efficacy based on the capability to target specific sites in the body. Our recent reports have confirmed that anticancer IM loaded nanoparticles could readily afford the sustained drug delivery for the target leukemia cells and reduce the relevant toxicity towards normal cells thereby increasing the intracellular concentration of the targeted drug by the synergistic effect of nanoparticles.[9,10] In the present study, we explore a strategy to encourage the sustained and synergistic anti CML activity combining two type of polymeric nanoparticles (NPs) for quick and slow release of

IM and DOX. The approach taken in this work is to prepare and characterize biodegradable pH sensitive core-shell PCL NPs loaded with IM and enzyme sensitive polyelectrolyte complexes (PECs) loaded with DOX for delivery of an anticancer cocktail to pursue the goal of multi drug delivery. Different *in vitro* studies conducted on BCR-ABL positive cells confirmed the excellent antileukemic activity of dual drug loaded nanoparticles on the KU812 model cell line. To better estimate synergistic effects of the combinations at molecular level western blot was done to detect the downregulation of BCR-ABL and significant CML stem cell death demonstrating that couple formulation of nanoparticles enhanced drug's kinetics and efficacy, combining the pH sensibility of core-shell PCL NPs loaded with IM and enzyme sensitive polyelectrolyte complexes (PECs) loaded with DOX.

Materials and methods

Materials

All tissue culture media and serum were purchased from Sigma-Aldrich, cell lines were purchased from American Tissue Type Collection (ATTC). The suppliers of the chemicals were as follows and were supplied by Sigma-Aldrich: thiazolyl blue tetrazolium bromide (MTT), dextran sulphate (DXS), polyallylamine hydrochloride (PAH), phosphate buffered saline (PBS), anti-clathrin light chain monoclonal antibody, Fluoroshield with DAPI, Chitosan (CH) low molecular range with degree of deacetylation 75-85%, Poly-(-caprolactone) (PCL) with an average molecular weight (MW) of 14.800 Da, Polyvinyl alcohol (PVA, MW 13-23 kDa, 87–80% hydrolyzed). LysoTracker from Life Technology.

DOX-PECs and IM-PCL NPs preparation

DOX-PECs were obtained by premixing dextran sulphate (DXS; 2 mg/ml in 0.1 M NaCl, pH 6.5) with DOX at a final concentration ranging from 10 to 100 μM under agitation overnight at room temperature. The mixture was then dialyzed against pure water for 8 hours and the product (DOX-DXS) was harvested by freeze drying. DOX-DXS solution was mixed in equal volume with poly(allylamine hydrochloride) (PAH; 1 mg/ml in 0.5 M NaCl, pH 6.5) under agitation at room temperature for 2 hours. DOX-PEC suspensions were then isolated by centrifugation at 13.400 rpm for 30 min.
PCL nanoparticles were prepared by an emulsion-diffusion-evaporation method. Briefly, a solution of IM (10 to 100 μM) was complexes with a 1% (w/v) chitosan in acetic acid aqueous solution by incubating at room temperature for 24 hours on a rotary shaker. A 100 mg amount of PCL was dissolved in 10 mL organic phase consisting of 9 mL ethyl acetate and 1 mL acetone for 1 hour under mild heating at 30°C. As for the aqueous phase, 100 mg PVA were stirred in 5 mL water for 2 hours at room temperature until a clear solution was obtained, after, IM-CH complex solution was mixed. The organic phase was passed through a 0.22 μm syringe filter to remove any undissolved solids and subsequently added drop wise to the aqueous phase under constant stirring. The resulting microemulsion was kept under constant agitation on magnetic stirrer at 1.000 rpm for 1 hour and was subsequently sonicated for 30 minutes. This colloidal preparation was diluted to a volume of 50 mL by adding water drop wise under stirring conditions (1.000 rpm, magnetic stirrer), which resulted in nanoprecipitation. In order to remove the organic solvent and to harden the PCL nanoparticles, the suspension was treated with a rotary evaporator at 50 mbar at 40°C for 20 minutes. Next, the nanoparticle suspension was washed three time with water by centrifugation at 12.000 rpm for 10 minutes and then resuspended in water. The finished nanoparticle suspension was stored at 4°C for further use. To determine the concentration of nanoparticles (weight per volume), this suspension was centrifuged to 12.000 rpm for 10 minutes; the supernatant was removed and the pellet was allowed to dry under a nitrogen stream before weighing. For preparing fluorescent CH-FITC or CH-TRITC PCL nanoparticles, a 1 mg/mL CH-FITC or CH-TRITC was added to PCL solution and the formulation was carried out as described earlier. The labeled nanoparticles were stored in the dark at 4°C until use.

Nanoparticles size, surface charge studies and stability

The average particle size and zeta potential of the prepared nanoparticles were determined by photon correlation spectroscopy using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., USA) equipped with a 4.0 mW He-Ne laser operating at 633 nm and an avalanche photodiode detector. Measurements were made at 25°C in aqueous solutions (pH 7). The NPs solution (1 mg/mL) was passed through a 0.45 μm pore size filter before measurements and appropriately diluted if necessary according to the instrument's requirements. The stability of IM-CH PCL NPs and DOX-PECs was tested under physiological conditions. Briefly, nanoparticles were incubated in complete RPMI medium at 37°C, and the size variation was measured over a period of 8 days by Dynamic Light Scattering (DLS) analysis. Representative measurements of three distinct sets of data have been reported (*Student t- test, $P < 0.05$*).

Atomic Force Microscopy (AFM)

The morphological characterization has been performed by tapping mode AFM using a Solver PRO Scanning Probe Microscope (NT-MDT) in air at room temperature, we used TESPA (Veeco, USA) silicon cantilevers of 20-80 N/m spring constant and resonance frequency of around 300 kHz. A drop of sample suspension was applied to a silicon support and then drying overnight.

Determination of drug loading and *in vitro* drug release

The drug loading efficacy of the nanoparticles was determined by analysing the supernatant of the final emulsion, once the nanoparticles was removed from it, by centrifugation. For the estimation of drug content present in the supernatant (100 μL of supernatant diluted in 1 mL with PBS 1x) were evaluated using an UV–visible spectrophotometer (Varian Cary® 300 Scan; Varian Instruments, CA, USA) at a wavelength of 260 nm (for IM) and 485 nm (for DOX). The dose of drug loaded into NPs was calculated using Equation 1:

$$C_c = \frac{(V_i\ C_i - V_s\ C_s)}{V_c} \quad (1)$$

Where C_c, C_i and C_s represent drug concentration in NPs, drug feeding concentration and drug concentration in supernatant after incubation, respectively. V_i, V_s and V_c refer to the volume of drug feeding solutions, the volume of supernatant and the volume of NPs, respectively. The concentration in supernatant is derived from UV–visible adsorption referring to a standard curve.

Release behaviour of drugs from NPs was investigated at pH 4.0 (approximate pH in endosome or lysosomes), pH 6.0 (pH of the environment around the tumour), and pH 7.4 (pH of physiological blood). A known amount of lyophilized nanoparticles (50 mg) were dispersed in 5 mL of phosphate buffer (PBS 1x) pH 7.4 and 500 µl of sample was dialyzed in a large volume of PBS 1x pH 4.0, pH 6.0 and pH 7.4, and maintained at 37°C ± 0.5°C under stirring at 50 rpm. At specified time intervals, the samples were centrifuged and supernatants were collected. Samples were taken and analysed in triplicates. The concentration of IM or DOX release was determined from the corresponding absorbance measured in spectrophotometer at 260 nm or 485 nm, respectively. Representative measurements of three distinct sets of data have been reported (*Student t- test, $P < 0.05$*).

Cell culture

Human BCR-ABL positive cells (KU812) were established from a CML patient and maintained as previously reported,[9] and human normal B lymphoblast (C13589) were cultured in Roswell Park Memorial Institute 1640 (RPMI; Sigma-Aldrich, MO, USA) containing 10% fetal bovin serum (Sigma-Aldrich), 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM l-glutamine (Sigma-Aldrich) at 37°C, 5% CO_2.

Cell uptake efficiency of DOX-PECs and IM loaded PCL NPs

KU812 leukemic cells were seeded in 24-well plates at a density of 10^5 cells/well in complete culture media and were incubated with drug loaded nanoparticles for 1, 2, 3 and 4 hours, respectively at 37 °C. For each sample, we have seeded six wells for positive control and six wells for samples. At the designated interval, the sample wells were washed three times with cold PBS 1x. After that, all the wells were lysed by 0.5% Triton X-100 in 0.2 N NaOH solution. The amount of IM (excitation wavelength of 254 nm and an emission wavelength of 380 nm) and DOX (excitation wavelength of 480 nm and an emission wavelength of 590 nm) in the cells was fluorometrically determined for the lysate with an using a fluorescence spectrometer. The cellular uptake efficiency was expressed as the percentage of the fluorescence associated with the cells *vs* that present in the positive control solution. Representative measurements of three distinct sets of data have been reported (*Student t- test, $P < 0.05$*).

Uptake modality and intracellular localization

To determine the cellular uptake of the NPs, KU812 CML cells and healthy C13895 cells (10^5 cells/mL) were incubated with the fluorescent NPs dispersions at a concentration of 0.05 mg/mL. After 3 hours of incubation at 37°C, the culture medium was removed, and the cells were washed three times with phosphate buffered saline. For fluorescent microscopic observation, cells were fixed in situ for 5 minutes in 3.7% formaldehyde and mounting with fluoroshield with DAPI.
The uptake modality of the PCL NPs was evaluated incubating KU812 cells (10^5 cells/mL) with 0.05 mg/mL PCL-FITC NPs for 1-3 hours, then washed three times with 0.27% glucose/PBS 1x and fixed in ice-cold methanol. After several washes with PBS, cells were incubated at room temperature for 50 min. with blocking buffer composed of PBS 1x with 4% horse serum, 0.3% Triton X-100 and 1% bovine serum albumin to block non-specific binding. They were then incubated with anti-clathrin light chain monoclonal antibody (10 µg/mL; Sigma-Aldrich) at 37°C for 1 hour. The primary antibody was revealed using TRITC conjugated anti-mouse antibody (4 µg/ml; Millipore, MA, USA) as secondary antibody and mounting with fluoroshield with DAPI.
To study the intracellular localization of FITC-PCL NPs, DOX-PECs and combination of DOX-PECs + IM-CH PCL NPs immunostaining with LysoTracker Yellow (Life technology), was performed, in according with manufactur's instructions, to label lysosomes. Confocal micrographs were taken with Leica confocal scanning system mounted into a Leica TCS SP5 (Leica Microsystem GmbH, Mannheim, Germany), equipped with a 63 X oil immersion objective and spatial resolution of approximately 200 nm in x-y and 100 nm in z.

***In vitro* cytotoxicity assay**

To test the effect of with free IM, IM-CH loaded in PCL NPs, free DOX, DOX-PECs, combination of free drugs and combination of DOX-PECs + IM-CH PCL NPs suspension (10-100 nM) on cell growth, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) survival tests were performed in accordance to manufacturer's instructions (Sigma-Aldrich, USA). The IC_{50} of IM was calculated. The absorbance was spectrophotometrically measured at wavelength 570 nm and the background absorbance measured at 690 nm subtracted. The percentage viability is expressed as the relative growth rate (RGR) by Equation 2:

$$RGR\ (\%) = \frac{D_{sample}}{D_{control}} * 100 \qquad (2)$$

where D_{sample} and $D_{control}$ are the absorbances of the sample and the negative control. Representative measurements of three distinct sets of data have been reported (*Student t- test, $P < 0.05$*).

Apoptosis evaluation

KU812 leukemia cells (10^5 cells/mL) were incubated with free IM, IM-CH loaded in PCL NPs, free DOX, DOX-PECs, combination of free drugs and combination of DOX-PECs + IM-CH PCL NPs suspension to final concentration of 10 nM and after 72 hours of incubation, the cells were washed with PBS 1x and resuspended in the same buffer. They were centrifuged on a slide and then stained with May-Grünwald and Giemsa (Sigma-Aldrich, USA) and were analyzed for the morphometric analysis using a light microscope BX61 (Olympus).
The apoptotic cells were evaluated mixing 9 mL of cell suspension (10^5 cells/mL) with 5 µL of dye mixture composed by 100 mg/mL Acridine orange (AO) and 100 mg/mL ethidium bromide (EtBr). After incubation for 2-3 min., cells were visualized under fluorescence microscope BX61 (Olympus) with excitation filter at 510-590 nm. The percentage of total apoptotic cells was determined by the Equation 3:

$$\%\ \text{of apoptotic cells} = \frac{\text{Total number of apoptotic cells}}{\text{Total number of normal and apoptotic cells}} * 100 \qquad (3)$$

Ten different fields were randomly selected for counting 300 cells. Representative measurements of three distinct sets of data have been reported (*Student t- test, $P < 0.05$*).
In addition, apoptosis was investigated by staining the cells with HOECHST 33342 in accordance to manufacturer's instructions (Sigma-Aldrich, USA). The Hoechst-stained nuclei excitation and emission wavelengths were set at 358-461 nm, respectively, and visualized using a fluorescence microscope BX61 (Olympus).

Detection of oligosomal DNA fragmentation

We detect apoptotic cell death via DNA fragmentation and formation of oligosomal ladders. KU812 leukemia cells and C13895 healthy cells (10^6 cells/ml) were incubated at 37°C in 5% CO_2, 95% relative humidity for 12 hours with free IM, IM-CH loaded in PCL NPs, free DOX, DOX-PECs, combination of free drugs and combination of DOX-PECs + IM-CH PCL NPs suspension to final concentration of 10 nM. The control (NT) was complete culture medium only. Phenol extraction by cell lysate was carried out, and DNA electrophoresis was performed in a 1% agarose gel containing 1 µg/mL ethidium bromide at 70 V, and the DNA fragments were visualized by exposing the gel to ultraviolet light, followed by imaging.

Validation of BCR-ABL inhibition by Western Blotting

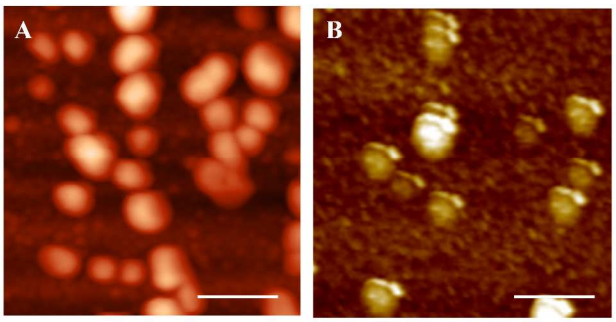
CML cells incubated for 8 days with free IM, IM-CH loaded in PCL NPs, free DOX, DOX-PECs, combination of free drugs and combination of DOX-PECs + IM-CH PCL NPs suspension to final concentration of 10 nM were washed once in PBS 1x at 4°C and resuspended in lysis buffer (50mM tris HCL, pH 7.4; 1% Triton X-100; 5 mM EDTA; 150 mM NaCL; 1 mM Na_3VO_4; 1 mM NaF; 1 mM phenylmethylsulfonylfluoride), and protease inhibitor cocktail (10 µM benzamidine-HCl and 10 µg of aprotinin,leupeptin and pepstatin A per mL) followed by incubation on ice for 30 min. Lysates were clarified by centrifugation at 13000 rpm for 15 min. at 4°C and protein concentration was determined using the BCA protein assay (Pierce, IL, USA). Protein bands were separated on SDS-polyacrylamide gels, immunoblotting was performed using Immobilon-P nitrocellulose membrane (Millipore Corp., MA, USA). Primary incubations were 1-3 hours. Antibody against phosphotyrosine (clone 4G10) was form Upstate Biotechnology, whereas the anti-c-ABL (clone K-12) was from Santa Cruz Biotechnology Inc. (CA, USA). Secondary incubations were for 1 hour with HRP-conjugated anti-mouse or anti-rabbit antibodies (Amersham, IL, USA). Proteins were visualized by chemiluminescence (Super Signal, IL, USA).

Results and discussion

Although many chemotherapeutic drugs are used clinically, the overall survival of leukemia patients is far from satisfactory. Recent advances in nanotechnology have encouraged different applications in biomedicine where nanoparticles are used as drug delivery carriers permitting rational manipulation of the pharmacological profiles of drugs encapsulated in them and hence their concomitant therapeutic indices. In the present study, we examined the relative susceptibilities of CML cells to the induction of apoptosis by DOX and IM used singly or in combination. The rationale behind a combination therapy was to achieve a synergistic action of drugs loaded in NPs so that the overall total dose of the drugs required is reduced, which is anticipated to result in a better therapeutic outcome with fewer side effects than single drug therapy. Our study reports an approach to enhance the efficient accumulation and utilization of chemotherapic drugs for CML therapy by using a controlled drug delivery system. In this regard two type of polymeric nanoparticles for quick and slow release of IM and DOX were synthesized carrying either single or dual drugs.

Synthesis and characterization of DOX-PECs and PCL nanoparticles loaded with IM

The physical and chemical stabilities of PEC formulations have been previously described,[10,11] and appeared as monodispersed spheres with an average diameter of 252.1 nm ± 0.26 nm (**Fig. 1A**). The spontaneous self-assembly of preloaded DOX-Dextran and poly(allylamine hydrochloride) polyelectrolytes did not affect the regular morphology of PECs assessed by AFM analysis.
The IM-CH loaded PCL nanoparticles were prepared by an adapted emulsion-diffusion-solvent evaporation method. IM-CH complexes were formed in aqueous solution and then were mixed with aqueous solution of PVA and this aqueous core (water phase) was coated with the oil phase containing the PCL molecules. The synthesis protocol was optimized to obtain non-aggregating, spherical in shape with smooth surface NPs as shown the AFM images (**Fig. 1B**), with an average diameter of 247.43 nm ± 0.577 nm. The small sized nanoparticles allowed efficient intracapillary and transcapillary passage along with escape from macrophage uptake.
The resulting NPs were characterized for their physicochemical properties, as size and surface zeta potential. As shown in the table of **Fig. 1C**, DOX-PECs showed a zeta potential of 10.4 mV ± 0.72 mV, while IM-CH loaded PCL nanoparticles had a zeta potential of -11.3 mV ± 0.542 mV.
The stability of the DOX-PECs and IM-CH loaded PCL NPs was tested under physiological conditions. NPs were incubated in complete RPMI medium at 37°C, and the size variation was measured over a period of 8 days by DLS analysis. As shown in **Fig. 1D,E**, over a period of 8 days both NPs maintain their hydrodynamic size of about 252 and 247 nm, demonstrating no significant aggregation. In addition, no significant alteration in PdI was detected, supporting evidence of particle stability in physiological settings.



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	ζ potential (mV)	Size (nm)
DOX-PECs	10.4 ± 0.72	252.1 ± 0.26
IM-PCL NPs	-11.3 ± 0.54	247.43 ± 0.57

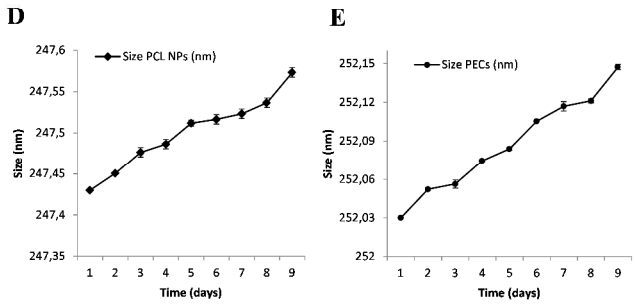

Fig. 1. AFM images of dried PECs (A) and PCL Nps (B) SEM image showing the size and morphology of dried sample. *Scale bars*: 500 nm (C) Surface zeta potential distribution and size of DOX-PECs and IM-PCL NPs. (D,E) Size distribution from DLS analysis showing the effect of the incubation of PCL NPs (D) and PECs (E) in complete RPMI medium at 37°C on the stability of the NPs. Size distribution of the NPs are determined by DLS analysis performed each time point. Representative measurements of three distinct sets of data have been reported (*t-Student test, P < 0.05*).

Drug loading in NPs and drug release kinetics under different physiological conditions

The optimization of the formulation and purification process led to a high encapsulation efficiency (approximately 98% DOX loaded in PECs and 88% for IM loaded in PCL NPs) without altering nanoparticles spherical morphology and size. Encapsulation of therapeutic agents in polymeric nanoparticles provides enzymatic protection and possible sustained release over prolonged time periods.[12] A successful nanoparticular system should have a high loading capacity to reduce the quantity of the drug required for administration.[13] In our study the drugs were efficiently loaded in the nanoparticles with high encapsulation efficiency. Controlled release of drug from a delivery can appreciably enhance its therapeutic effect. A sustained release phenomenon is used to achieve a constant concentration of drug over an extended period of time thereby maintaining an optimum concentration of drug being delivered to target site for maximum therapeutic effect. The advantage of a sustained formulation includes very accurate dosing, ability to have a certain release patterns, potential for local delivery, and possible biological drug stability enhancement.[14] As shown in **Fig. 2**, release of drug molecules (DOX and IM) depended on pH of the medium and release time. Drug release at pH 7.4 was slow and sustained. However, at lower pH, drugs release rate was much faster. The single drug formulations were capable of showing a biphasic release pattern which consisted of an initial burst release (due to drugs present at or just beneath the surface of the nanoparticles) followed by a slower sustain release of drugs from the core of nanoparticles. It may be hypothesized that most drug will remain in the carrier for a considerable time period at normal physiological conditions (pH 7.4), indicating the potential for prolonged drug retention time in blood circulation and thereby greatly reducing the side effects to normal tissues.

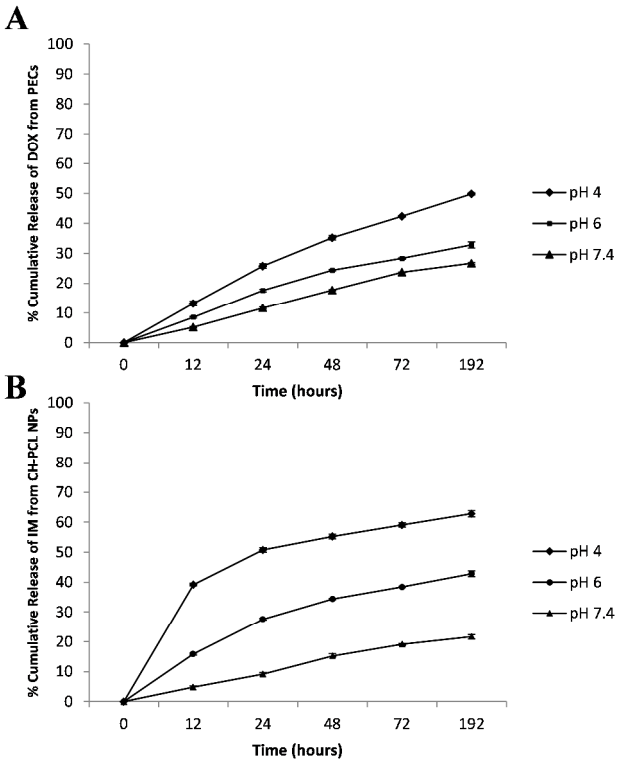

Fig. 2. *In vitro* DOX release profiles from PECs (A) and IM release profiles from CH PCL NPs (B) at neutral condition (pH 7.4) and acidic conditions (pH 6.0 and 4.0) at 37 °C. Representative measurements of three distinct sets of data have been reported (*t-Student* test, *P < 0.05*).

We have chosen to use two different type of NPs, in particular a pH sensitive core-shell PCL NPs loaded with IM and enzyme sensitive polyelectrolyte complexes (PECs) loaded with DOX for CML delivery of an anticancer cocktail to pursue the goal of multi drug delivery. The incubation of CML cells with both drug formulation loaded in different stimuli sensitive NPs to allow to improve the sustained targeting of BCR-ABL with low therapeutic dose, thanks the synergistic action of both the drugs.

Cellular uptake and intracellular studies of drug loaded NPs

Uptake of DOX-PECs and CH-PCL NPs in KU812 leukemia cells and in healthy C13895 cells was studied by confocal laser scanning microscopy (CLSM). After 3 hours of incubation, DOX-PECs and CH-PCL NPs appeared as fluorescent spots with a uniform distribution inside the cell, both in the cytoplasm and the nucleus (**Fig. 3**). Similar cellular distribution, we have observed in our previous study on the uptake of nanocomplexes of polyelectrolytes (PECs) on cells of chronic myeloid leukemia[10] and we previously reported that the size of particles could impact their uptake modality and,

consequently, the accessibility of their therapeutic cargo to specific intracellular targets (e.g., cytosolic and/or nuclear pools of BCR-ABL).[9,10]

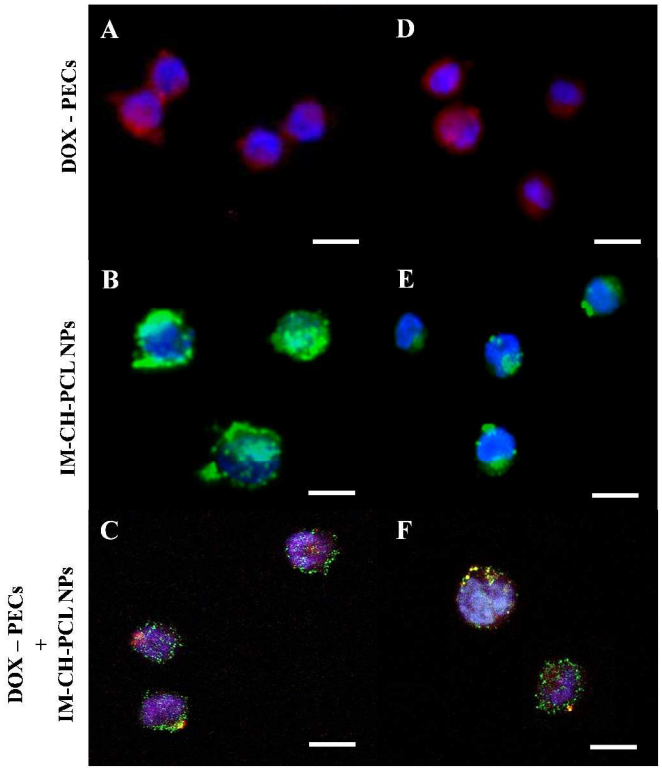

Fig. 3. Confocal laser scanning microscopy images of KU812 leukemic cells (A,B,C) and healthy C13895 cells (D,E,F) after 3 hours of incubation with DOX-PECs (A,D-red), IM-CH-FITC PCL NPs (B,E-green) and combination of DOX-PECs + IM-CH PCL NPs (C,F). Cell nuclei were counterstained with DAPI (blue). *Scale bars: 10 μm*

To improve the therapeutic prospective of nanoparticle based carriers for intracellular drug delivery, it is imperative to understand the cellular uptake mechanism and the intracellular trafficking of nanoparticles.[15] In this regard, a time dependent uptake study was conducted. The cellular uptake efficiency of DOX-PECs and IM-CH loaded PCL NPs was analyses after 1,2,3 and 4 hours in culture. It can be seen in **Fig. 4** that the DOX-PECs and IM-CH loaded PCL NPs exhibited enhanced cellular uptake for KU812 leukemia cells after 1,2,3 and 4 hours. The fluorescence intensity of drug loaded nanoparticles was increase in the mean fluorescence intensity after 2 hours suggesting that maximum uptake of nanoparticles occurs during the initial period of incubation (**Fig. 4**).

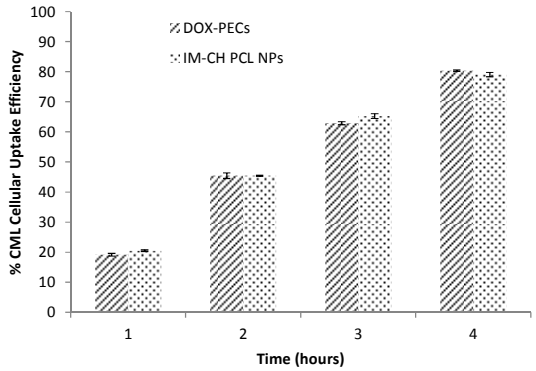

Fig. 4. Time-dependent cellular uptake efficiency of DOX-PECs and IM-CH loaded PCL NPs by KU812 cells after 1, 2, 3 and 4 hours of incubation, respectively. Representative measurements of three distinct sets of data have been reported (*t-Student test*, $P< 0.05$).

Next, we monitored the internalization process of drug loaded nanoparticles and their intracellular dispersion, as the intracellular fate of the macromolecular carriers is strongly affected by the route of entry. To date, numerous reports have discussed the internalization of polymeric nanoparticles into the cells by endocytic pathways.[16,17,18] In this regard, we previously reported that the initial uptake of polyelectrolyte capsules with a diameter of 3 μm (PMCs) was principally nuclear in KU812 cells.[9] On the contrary, nanosized PECs were internalized with a clathrin-mediated endocytosis mechanism.[10] This disagreement could be also considered taking into account the distinctive morphological features of CML cells, which have a large nucleus (**Fig. 3**). It seems possible that the uptake of big particles could be mediated by fusion between the outer plasma membrane and the perinuclear envelope of BCR-ABL+ leukemic blasts through micropinocytosis/phagocytosis;[19] while nanoparticles can be internalized via caveolin- or clathrin-mediated endocytosis pathways.[20,21,22,23] Whereas the size of caveolin-coated vesicles limits the internalization of NPs larger than 20-40 nm, clathrin-coated invaginations on the cell membrane recruit cargo molecules of variable shape and size.[24,25] Based on our CH-PCL NPs with nanoscaled size (247 nm in diameter), we supposed clathrin-mediated endocytosis as a accepted mechanism of internalization. To confirm this hypothesis, we determined if the staining pattern of the clathrin light chain could be spatially associated with CH-PCL NPs fluorescence in treated CML cells. A polarized colocalization of red clathrin (**Fig. 5A**) and green CH-PCL NPs (**Fig. 5B**) was already observed in the cytoplasm within 1 hours of CH-PCL NPs incubation (**Fig. 5D**), and after 3 hours were evident a redistribution of clatrin inside the cells (**Fig. 5E-H**). These findings suggested the rapid induction of clathrin vesicles at the outer plasma membrane in response to CH-PCL NPs incubation, and their quick redistribution in specific subcellular compartments.

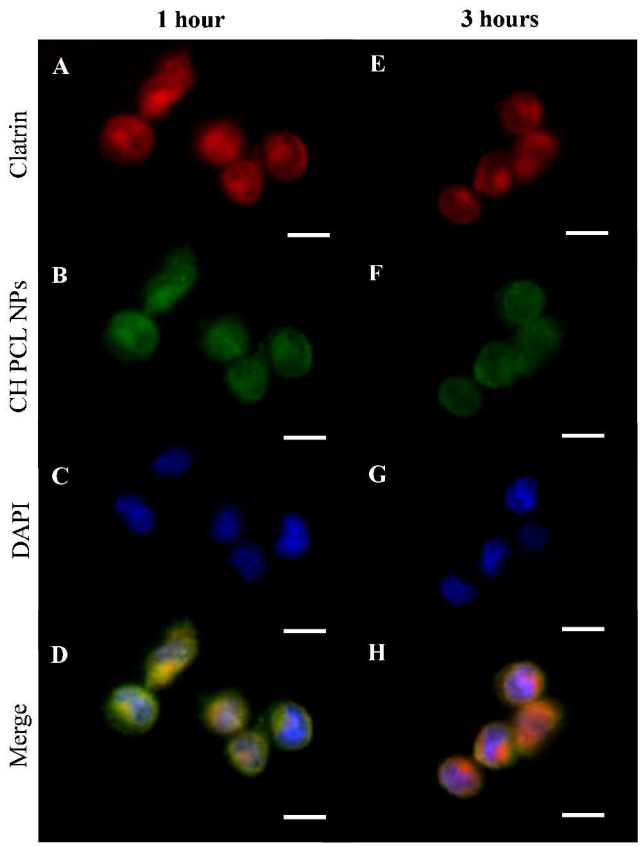

Fig. 5. Clatrin (A,E) immunofluorescence (red) in KU812 leukemia cells after 1 (A) and 3 (E) hours of incubation with (B,F) CH-FITC PCL NPs (green). Cell nuclei (C,G) were counterstained with DAPI (blue). Merge images (D,H) shows colocalization of NPs with clatrin. *Scale bars: 10 μm.*

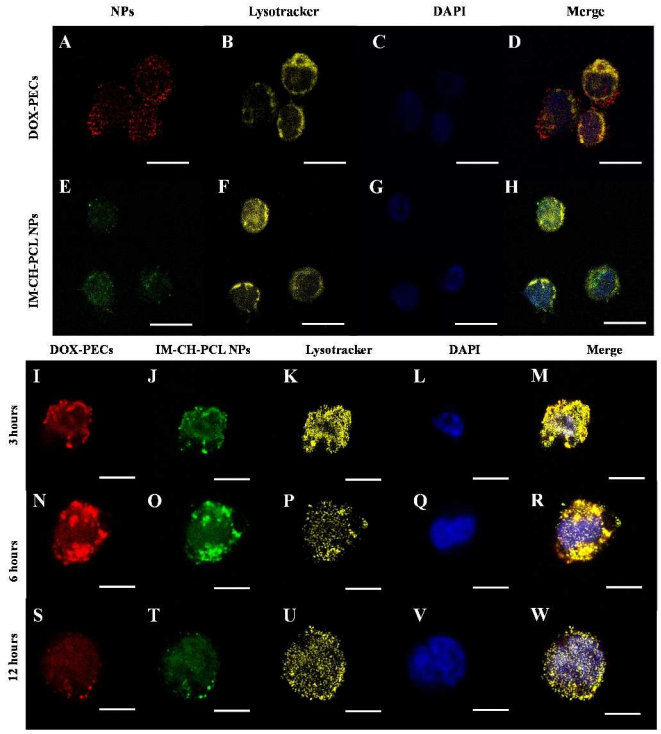

Fig. 6. Intracellular localization of DOX loaded PECs (A,I - red) and IM-CH-FITC loaded PCL NPs (E,J - green) in KU812 leukemia cells. CLSM images of cell staining with Lysotracker (B,F,K - yellow). In merge images are shown colocalization of DOX-PECs (D), IM-CH-FITC loaded NPs (H) with Lysotracker after 3 hours treatment and combination of DOX-PECs + IM-CH PCL NPs (M,R,W) with Lysotracker after 3, 6 and 12 hours treatment. Cell nuclei were counterstained with DAPI (blue) as shown in the CLSM images C,G,L,Q,V. *Scale bars: 10 μm*

In this study, the intracellular localization of DOX-PECs, IM-CH-FITC loaded NPs and combination of DOX-PECs + IM-CH PCL NPs in KU812 leukemic cells was investigated by using specific probes, Lysotracker Yellow and DAPI, for fluorescence imaging of lysosomes and cell nucleus, respectively. As shown in **Fig. 6M**, the combination of the blue, yellow, green (associated with IM-CH loaded in FITC PCL NPs) and red (associated with DOX-PECs in dual drug NPs cell incubation) clearly indicates the colocalization of both drug loaded NPs in lysosomes. Conversely, high colocalization (yellow present in the merge channel of CLSM images of **Fig. 6H**) of green fluorescence CH-FITC PCL NPs and yellow fluorescence of Lysotracker marker was observed and CH-FITC PCL NPs were primarily transported to lysosomes, on the contrary low colocalization signal of red DOX-PECs with Lysotracker was observed and this indicates that the DOX-PECs is not typically localized within lysosomes (**Fig. 6D**).
Combination of DOX-PECs and IM-CH PCL NPs escape form lysosomes after 6 hours and released into cytosol and distributed in cells. During the 6-12 hours process, the fluorescence from DOX-PECs and IM-CH PCL NPs changed sharply (**Fig. 6N-W**). These results suggest that the DOX and IM was release with a slow process and the combination of NPs could be an prolonged drug release system.

Synergistic effect of DOX and IM on cytotoxicity of CML cells

To explore the relationship between treatment duration, drug and drug concentration, the pharmacodynamics of different single and dual formulation of free or loaded drugs at lower therapeutic drug dose (nanomolar range, 10-100 nM) were studied on CML cells and healthy C13895 cells over 8 days. No adverse effects on healthy C13895 cells was observed by using freely soluble or encapsulated drugs at 100 nM (**Fig. 7B**). Our study revealed that all the drugs were capable of exhibiting some antileukemic effect on KU812 cells, with the nanoformulations showing better activity than their native equivalents (**Fig. 7A**). To corroborate the hypothesis that dual drug therapy is more effective than single drug therapy, combination of DOX-PECs and IM-CH PCL NPs was used against KU812 typical Philadelphia positive CML cell line. Results observed show that a combined nanoparticulate drug therapy was relatively more effective than single drug formulations (**Fig. 7A**) to low concentrations and in KU812 when a combinational regime was followed using the nanoparticles, the cells were more prone to cell death.

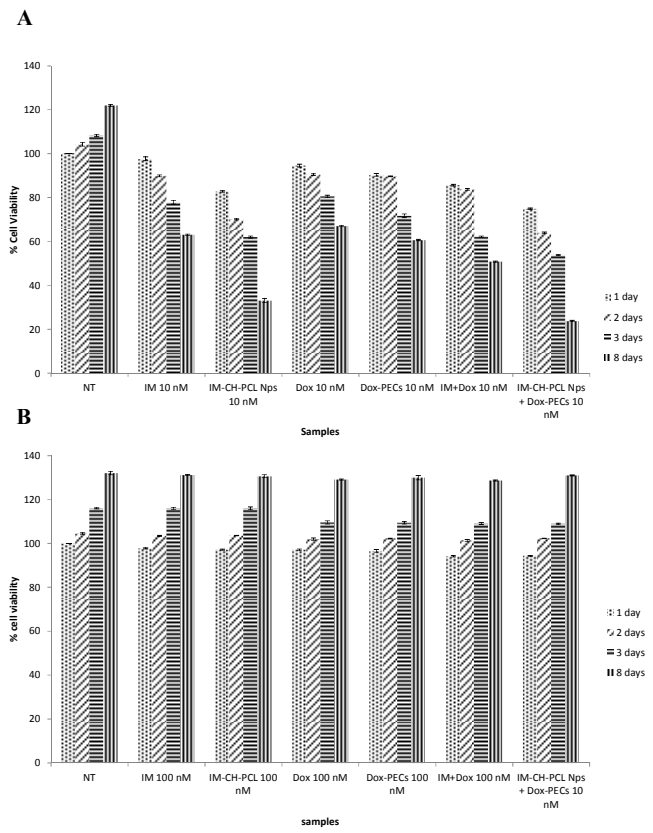

Fig. 7. MTT test for cellular viability of KU812 leukemia cells (A) and C123895 control cells (B) cultured for 1, 2, 3, 8 days in the absence (NT) or in the presence of free IM (10-100 nM), IM-CH loaded in PCL NPs (10-100 nM)free DOX (10-100 nM), DOX-PECs (10-100 nM), combination of free drugs (10-100 nM), and combination of DOX-PECs + IM-CH PCL NPs (10-100 nM). Representative measurements of three distinct sets of data have been reported (*t-Student test, $P < 0.05$*).

Induction of CML apoptosis and sustained downregulation in CML

The inhibition on cancer cell proliferation by anticancer drugs could be the results of induction of apoptosis or cell cycle arrest, or a combination of these two modes. Apoptotic cell death was further confirmed by morphological changes, membrane integrity, DNA fragmentation and nuclear condensation as examined by Hoechst nuclear staining.

The May-Grunwals-Giemsa staining of leukemic KU812 cells cultured for 72 hours after treatment with drugs free or loaded in NPs (**Fig. 8B-G**) indicate a significant morphological evidence of apoptosis in the cells treated with the single and combinatorial drug nanoformulations. Similar evidence were observed performed AO/EtBr assay (**Fig. 8I-N**) to determine the loos of membrane integrity. In particular, in AO/EtBr staining cells observed as orange coloured cells are apoptotic and necrotic cells were observed as red colour fluorescence due to their loss of membrane integrity. The Hoechst nuclear staining of control cells (no drugs) had intact round nucleus and had normal morphology emitted a weak blue florescence (**Fig. 8O**), furthermore the cells treated with single and combinatorial drug nanoformulations (**Fig. 8P-V**) for 72 hours exhibited bright blue colour emission concluding nuclear fragmentation with increased chromatin condensation leading to induction of apoptosis.

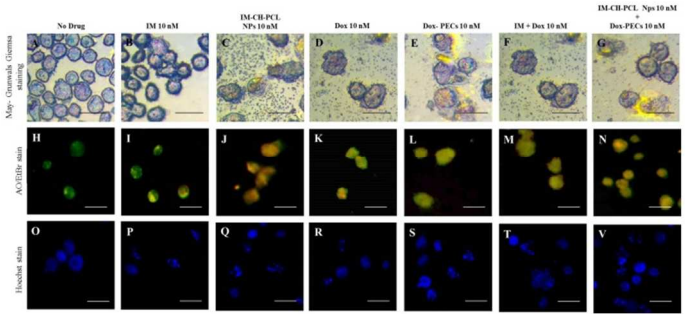

Fig. 8. May-Grunwals-Giemsa (A-G), AO/EtBr (H-N) and Hoechst (O-V) staining of KU812 leukemia cells after 72 hours of treatments, untreated cells as control (A,H,O), KU812 treated with free IM (10 nM, B,I,F), IM-CH loaded in PCL NPs (10 nM, C,J,Q), free DOX (10 nM, D,K,R), DOX-PECs (10 nM, E,L,S), combination of free drugs (10 nM, F,M,T) and combination of DOX-PECs + IM-CH PCL NPs (10 nM, G,N,V). *Scale bars: 25 μm*.

The doses of single drugs tested in NPs or as single agent are substantially smaller than the IC_{50} value and this explained why apoptosis of KU812 cells was only slightly increased (10-20% increase) by using freely soluble DOX or IM at 10 nM (**Fig. 9A**). On the contrary, drugs loaded in the NPs improved the drug's kinetics and efficacy, causing high level of apoptosis (60-80% apoptotic cells) as shown in **Fig. 9A**.

To examine whether the single and combinatorial drug nanoformulations induced apoptosis in human leukemia cells, we tested internucleosomal DNA fragmentation, which is characteristic for cells in apoptosis, by use of neutral agarose electrophoresis after 12 hours of drug treatment. Indeed, the characteristic DNA ladder-like pattern of internucleosomal DNA cleavage was detected at 12 hours for KU812 CML cells but not for healthy C13895 cells (**Fig. 9B**).

Chronic myeloid leukemia is typified by constitutive activation of the BCR-ABL kinase. Because of the importance of this protein, extensive efforts have been made by several research groups to understand its mechanisms of action in malignant cells. As we have already identified the enhanced efficacy of combination formulation over single drugs (evident from *in vitro* cytotoxicity and apoptosis studies) further *in vitro* studies (i.e. western blot) was carried out using single and combination formulations of drugs. Western blot analysis of dual drug treated KU812 cells showed that in control cells BCR-ABL tyrosine kinase was in highly activated state and on drug treatment the tyrosine kinase activity of the protein decreased resulted in a more significant decrease in the tyrosine kinase property of the protein with the nanoparticles showing a better result than their native counterparts (**Fig. 9C**) causing a long-lasting inactivation of BCR-ABL activity that is mandatory to significantly promote CML cell death and growth inhibition. Our combination formulation was capable of modulating the activity of bcr-abl gene and its product BCR-ABL fusion protein. The approach taken in this work is to combine biodegradable pH sensitive core-shell PCL NPs loaded with IM and enzyme sensitive polyelectrolyte complexes (PECs) loaded with DOX. Our couple delivery strategy allowed the downregulation of BCR-ABL for long times combining two type of polymeric nanoparticles for quick and slow release of IM and DOX.

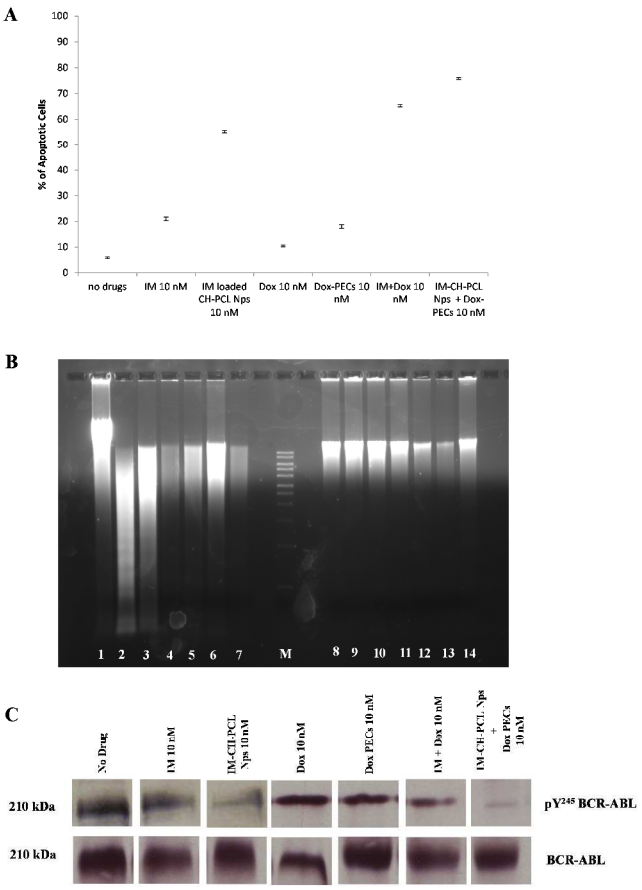

Fig. 9. (A) Percentage of CML apoptotic cells after 72 hours of incubation with KU812 treated with free IM, IM-CH loaded in PCL NPs, free DOX, DOX-PECs, combination of free drugs and combination of DOX-PECs + IM-CH PCL NPs suspension to final concentration of 10 nM. Representative measurements of three distinct sets of data have been reported (*t-Student* test, $P < 0.05$). (B) DNA fragmentation assay of KU812 leukemic cells (lanes 1-7) and C13895 healthy cells (lanes 8-14) treated for 12 hours with combination of DOX-PECs + IM-CH PCL NPs (10 nM, lanes 2-9), combination of free drugs (10 nM, lanes 3,10), free IM (10 nM, lanes 4,11), IM-CH loaded in PCL NPs (10 nM, lanes 5,12), DOX-PECs (10 nM, lanes 6,13) and free DOX (10 nM, lanes 7-14). Lanes 1,8 represent the control untreated cells and lane M represent DNA marker. (C) Western Blotting analysis for the expression (anti BCR-ABL) and phosphotyrosine activation levels (anti pY^{245} BCR-ABL) of the oncoprotein BCR-ABL in KU812 cells cultured for 8 days in the absence (No drug), in the presence of free IM, IM-CH loaded in PCL NPs, free DOX, DOX-PECs, combination of free drugs and combination of DOX-PECs + IM-CH PCL NPs suspension to final concentration of 10 nM.

Conclusions

The approach undertaken in this work is to prepare and characterize biodegradable pH sensitive core-shell PCL NPs loaded with IM and enzyme sensitive polyelectrolyte complexes (PECs) loaded with DOX for delivery of an anticancer cocktail to pursue the goal of multi drug delivery. Our *in vitro* data suggests that the nanoformulations maintain the biological activity of drugs for longer periods and lead to a continuous release of active drug. Combination of DOX-PECs and IM-CH PCL NPs was used against CML cells and our results showed that the combined drug loaded nanoformulations was relatively more efficient than single drug formulation to low concentrations. The effective inhibition of the BCR-ABL tyrosine kinase activity was assessed by Western Blot analysis to 3 days of incubation with low concentration of drug loaded nanoparticles cocktail and the same concentrations of free combination drugs. Using free combination drugs at 10 nM we observed a partial inhibition of BCR-ABL protein kinase activity. On the contrary, by using drug loaded nanoparticles cocktail, CML cell viability was permanently blocked showing that combining the pH sensibility of core-shell PCL NPs loaded with IM and enzyme sensitive polyelectrolyte complexes (PECs) loaded with DOX can improve drug's kinetics and efficacy at single cell level consistent with a inactivation of BCR-ABL autokinase activity that is required to endorse apoptosis.

Further studies will be focused to apply the proposed technology to improve the delivery of pharmacological and nucleic agents, using the coupled delivery such as IM and DNA oligonucleotides antisense or small-interfering RNAs and combining the functional protein inactivation and gene silencing of BCR-ABL.

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