

INVESTIGATION OF CYCLODEXTRIN BASED NANOSPONGES COMPLEXES WITH ANGIOTENSIN I CONVERTING ENZYME INHIBITORS (ENALAPRIL, CAPTOPRIL, CILAZAPRIL)

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Abstract

Cyclodextrin based nanosponges are cross-linked polymeric networks formed by cyclodextrins and cyclodextrin derivatives; they are obtained by reacting cyclodextrin with a cross-linker agent such as carbonyldiimidazole, organic carbonates or ($\pm$) epichlorohydrin and have many important applications as drug delivery systems or for pollutants removal in environmental issues. Several β -CD, sulfobutylether- β -CD and hydroxypropyl- β -CD nanosponges were prepared using epichlorohydrin as cross-linker. After lyophilization, the polymers were purified and characterized by FT-IR and ¹H NMR, respectively. The complexing properties of the CD polymers were investigated against enalapril, captopril and cilazapril (effective drugs for controlling blood pressure, congestive heart failure, and prevention of stroke and hypertension acting as angiotensin converting enzyme -ACE- inhibitors), in order to improve their stability and bioavailability and to overcome the possible drug-excipient interactions. The drug-nanosponge complexes prepared through lyophilization were characterized by FT-IR and ¹H NMR; the phase-solubility diagrams, the solubility profile and the loading capacity of the cyclodextrin polymers were also determined.

Rezumat

Nanospongii derivați de ciclodextrină sunt polimeri reticulați formați de ciclodextrine sau derivați de ciclodextrine, prin reacția cu un agent de reticulare, cum sunt carbonil diimidazol, carbonați organici sau ($\pm$) epichlorhidrina. Nanospongii au aplicații importante ca sisteme de transport și eliberare a substanțelor medicamentoase sau pentru îndepărtarea poluanților din mediu. Au fost sintetizați mai mulți nanospongi, pornind de la β -ciclodextrină (β CD), sulfobutyleter- β CD și hidroxipropil- β CD, folosind ca agent de reticulare epichlorhidrina. După liofilizare, polimerii au fost purificați și caracterizați prin FT-IR și ¹H-RMN. Capacitatea de complexare a polimerilor sintetizați a fost studiată în raport cu enalaprilul, captoprilul și cilazaprilul (substanțe medicamentoase eficiente, care acționează ca inhibitori ai enzimei de conversie a angiotensinei I, utilizate pentru controlul presiunii sanguine și tratamentul insuficienței cardiace congestive, ca și pentru prevenirea accidentelor vasculare sau a hipertensiunii arteriale), cu scopul ameliorării stabilității și biodisponibilității acestora și pentru a preveni posibile interacțiuni substanță medicamentoasă-excipient. Complecșii substanță medicamentoasă-nanospongi au fost preparați prin liofilizare și au fost caracterizați prin FT-IR și ¹H-RMN; au fost determinate, de asemenea, diagramele de fază solubilitate și capacitatea de încărcare a ciclodextrinelor polimerizate.

Keywords: nanosponge, captopril, enalapril, cilazapril, β -cyclodextrin, sulfobutylether- β -cyclodextrin, hidroxipropil- β -cyclodextrin, solubility

Introduction

Cyclodextrins are macrocyclic molecules synthesized by enzymatic action on hydrolysed starch. The most common cyclodextrins are α , β and γ and they consist of six, seven and eight glucopyranose related units in a toroidal shape. The interior of the toroids is not hydrophobic, but considerably less hydrophilic than the aqueous environment and thus able to host other

hydrophobic relatively small molecules, as many lipophilic drugs [1]. As it is already well known, cyclodextrins are especially useful in the pharmaceutical industry as they can increase the apparent solubility of drugs and can modify the dissolution rate and sometimes even the dissolution profile. Moreover, cyclodextrins improve the stability of several labile drugs or mask some of the organoleptic characteristics leading to an enhanced compliance [2]. In order to overcome some of their

disadvantages (i.e., renal toxicity) and to extend the inclusion capacity of native cyclodextrins, two major ways are prominent, the synthesis of chemically modified cyclodextrins and the synthesis of cyclodextrin based polymeric materials, usually exhibiting enhanced encapsulating ability [1, 3-8].

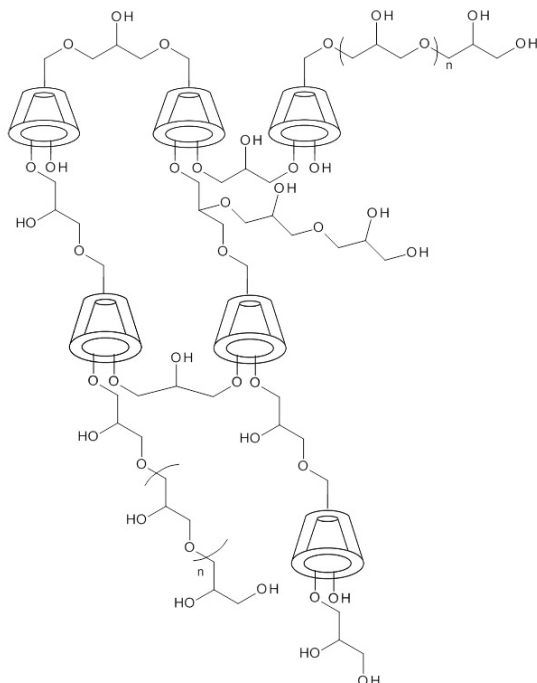


Figure 1.

Reticulate structure of the β CD nanosponge

These polymers are obtained by reacting cyclodextrins with a cross-linking agent such as

epichlorohydrin (when more hydrophilic nanosponges are obtained), or carbonyl compounds (e.g. diphenyl carbonate, dimethyl carbonate, carbonyldiimidazole), organic dianhydrides (e.g., pyromellitic anhydride) and diisocyanates, leading to more hydrophobic nanosponges. Cyclodextrin based nanosponges are able to form complexes with drug molecules due to both their nanoporous structure and the existing native cyclodextrins cavities (Figure 1) [9-12].

As drug delivery systems, cyclodextrin based nanosponges act to improve the release profile of drugs or drug-cyclodextrin inclusion complexes and, consequently, the bioavailability of lipophilic pharmaceutical molecules (they enhance the solubility and the chemical stability of the drug substances), and also to modulate the drug release; they are also capable of targeting the drugs to specific organ or tissues [12].

For our study we have selected three widely used antihypertensive agents belonging to the angiotensin I converting enzyme (ACE) inhibitors class, i.e. captopril (CP), enalapril (ENP) and cilazapril (CZP) (Figure 2). As the renine-angiotensin system is a hormone system that regulates blood pressure and fluid balance and plays an important role in the pathophysiology of cardio-vascular diseases (CVDs) such as congestive heart failure and hypertension and the hormone angiotensin II (resulted in the proteolytic cleavage of angiotensin I) is a vasoconstrictor and its formation increases blood pressure, ACE inhibitors are mostly used to lower hypertension and prevent CVDs.

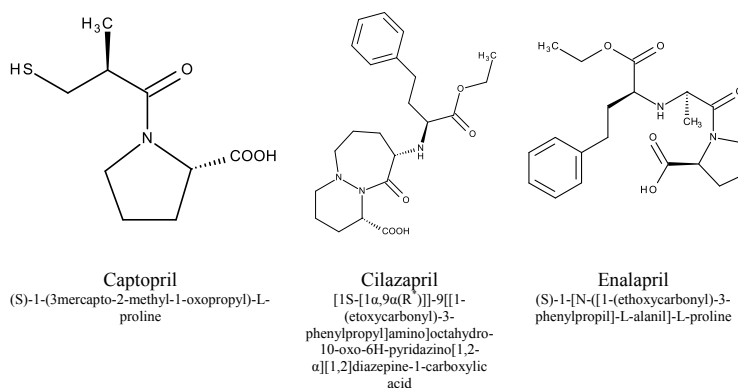


Figure 2.

Chemical structures of captopril, cilazapril and enalapril

It is important to say that, although ACE inhibitors show a remarkable effect in treating hypertension, they cause several unpleasant side effects (cough, allergic reactions, taste disturbances and skin rashes) mostly due to angiotensin I converting enzyme blocking in various organs and tissues. CP, ENP and CZP are rather soluble, but they have low

bioavailability (generally only 50 to 75% of the administered dose is absorbed) and are relatively unstable in the presence of specific excipients. For example, ENP maleate suffers degradation in the solid state in the presence of different excipients used in tablet formulation (microcrystalline cellulose, magnesium stearate, Eudragit E etc.).

These interactions cause major degradation of ENP maleate resulting in the formation of enalaprilat and enalapril diketopiperazine by hydrolysis of the ethyl ester moiety and by intramolecular cyclization of the drug. Many researchers have studied the possibilities to improve the stability and the bioavailability of ACE inhibitors, mostly captopril and enalapril, using β -cyclodextrin and some of its derivatives [1, 14-15].

Aiming to achieve CP, ENP and CZP stability and bioavailability improvement and to overcome possible undesired drug-excipient interaction, we have synthesized nanosponges (NS) from β -cyclodextrin (β CDNS), sulfobutylether- β -CD (SBE β CDNS) and hydroxypropyl- β -CD (HP β CDNS) in solution, using epichlorohydrin as a cross-linker and we have characterized them by IR and ^1H -RMN spectroscopy, SEM. Then mutual complexes of each ACE inhibitor and NS were prepared by lyophilization and the solubilization efficiency and loading capacity of each NS against ENP, CP and CZP were determined and the cellular toxicity of the synthesized NSs and ACE inhibitor-NS complexes was tested using eukaryotic cell culture (HeLa, ATTC CCL-2).

Materials and Methods

Captopril, enalapril and cilazapril detection: CZP concentration was determined by UV spectrometry at 210 and 240 nm, according to its UV absorption spectrum. The calculated specific absorbance of CZP was $A_{1\text{cm}}^{1\%} = 11250$. CP concentration was determined by measuring the amplitude of the first derivative of the UV absorption spectrum at 215 nm and ENP concentration was determined by measuring the amplitude of the first derivative of the UV absorption spectrum at 218 nm. The presence of CDs and CD based nanosponges did not affect the absorbance of CZP, ENP or CP in water. The spectrometric methods were validated. All measurements were made with a UV-Vis Perkin Elmer Lambda2 spectrometer.

Nanosponges preparation: β CD, SBE β CD, HP β CD were dissolved in NaOH 33% under stirring and the mixtures were stirred overnight at 30°C. Epichlorohydrin (EP) was rapidly added (CD:EP 1:10) to the CD solution under vigorously stirring. The reaction was stopped by acetone addition (20 mL); the acetone was removed by decantation. The mixture was stirred overnight at 50°C. After cooling, the colloid was neutralized with 6 M HCl and lyophilized (Alpha 1 -2 /LD2-2, Martin Christ lyophiliser).

Fourier Transform Infrared Spectroscopy – Attenuated Total Reflectance (FTIR-ATR): IR absorption spectra were recorded on a FT-IR 4200 JASCO equipped with an ATR device.

Nuclear Magnetic Resonance (NMR): The ^1H -NMR spectra were recorded with a digital resolution of 0.56 Hz/point, 16 K data points, a sweep width of 4,500 Hz and an acquisition time of 1.998 s. All chemical shifts were measured relative to trimethylsilane (TMS).

Complexes preparation: ENP, CP and CZP, respectively, and β CDNS, SBE β CDNS, respectively HP β CDNS (1:4, 1:5, and 1:5) were suspended in 25 mL of water and stirred for 24 h, then the colloidal solution was filtered and lyophilized and the resulting powder was characterized.

Solubilization efficiency of β CD-NS, SBE β CD-NS and HP β CD-NS: A mixture consisting of an excess amount of ENP, CP and CZP (50 mg, 50 mg and 25 mg, respectively) and 10 mg CD-NS was suspended in 20 mL of water and shaken overnight at room temperature. After proper dilution, the filtrate absorbance was measured for CZP estimation. For ENP and CP we measured the first derivative of the UV absorption spectrum using an UV-Vis Perkin Elmer Lambda2 spectrometer.

Phase-solubility studies: Phase-solubility studies were carried out according to the method of Higuchi and Connors [12]. An excess amount of CP, CZP and ENP (approx. 10 mg) was added to rising amounts of nanosponges and water and shaken for 24 h at $25^\circ\text{C} \pm 0.5^\circ\text{C}$. After equilibration, samples were filtered through a 0.45 μm Nylon filter membrane (Whatman® Puradisc™) and the filtrate absorbance was measured for CZP. For ENP and CP we measured the first derivative of the UV absorption spectrum using an UV-Vis Perkin Elmer Lambda2 spectrometer.

Loading capacity: 5 mg lyophilized complex were suspended in 5 mL CH_3OH and after 10 min sonication the solution was filtered and the absorbance and the first derivative of the UV absorption spectrum, respectively, were measured.

Cell cycle analysis: The eukaryotic cell culture used in our assay was represented by HeLa (ATTC CCL-2) that was maintained as an adherent culture in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, USA) supplemented with 10% heat-inactivated foetal bovine serum (Sigma, USA) at 37°C , 5% CO_2 , in a humid atmosphere.

In order to evaluate the effects of our compounds on HeLa's cell cycle, 1×10^5 cells were seeded in each well of 24 wells plate and after cell recovery, 100 $\mu\text{g/mL}$ of nanosponge compounds were added into medium. They were harvested after 24 h and washed in a cold solution of phosphate buffered saline (PBS) (pH 7.5), then fixed in cold ethanol (70%) and stored at -20°C overnight. The samples were washed two times with PBS and re-suspended in 100 μL PBS, treated with RNase A (1 mg/mL) for 15 minutes at 37°C , and labelled with propidium

iodide (100 µg/mL) at 37°C. The samples were incubated at 4°C for 30 minutes prior to the measurement. The cell DNA content was quantified with a Beckman Coulter EPICS XL flow cytometer and analysed using the FlowJo 8.8.6 software (Ashland, Oregon, USA). *Cytotoxicity evaluation.* The viability test was performed using trypan blue method and propidium iodide (PI) /fluoresceindiacetate (FdA) assay. HeLa cells (1×10^5) were seeded in each well of a plate with 24 wells and after 72 h were treated with 100 µg/mL CD-complexes. The effects on cell viability were evaluated after 72 h by adding 10 µL PI (0.1 mg/mL) and 10 µL FdA (0.1 mg/mL). FdA is taken up by viable cells which convert the non-fluorescent FdA into the green fluorescent metabolite fluorescein depending on esterase, while PI pass through disordered areas of dead cell

membranes, and intercalates with the DNA double helix of the affected cell. Fluorescence was quantified using an Observer D1 Carl Zeiss microscope.

Results and Discussion

A preliminary characterization of the bulk NS was performed using FTIR-ATR and ^1H -RMN spectroscopy and scanning electronic microscopy.

IR and RMN studies

βCDNS , $\text{HP}\beta\text{CDNS}$ and $\text{SBE}\beta\text{CDNS}$ were synthesized using 1:10 CD/cross-linker ratio. After purification, these NS and their mutual (1/5 drug/NS) complexes with CP, ENP and CZP were prepared and characterized through ^1H -NMR and ATR-IR spectra.

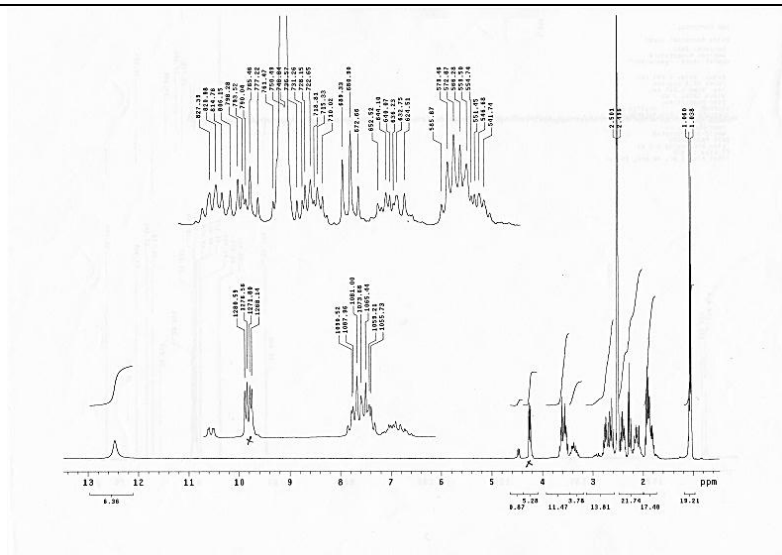
Table I

^1H NMR assignments, chemical shifts, δ (ppm) and chemical shifts variations for CP, CZP and ENP protons

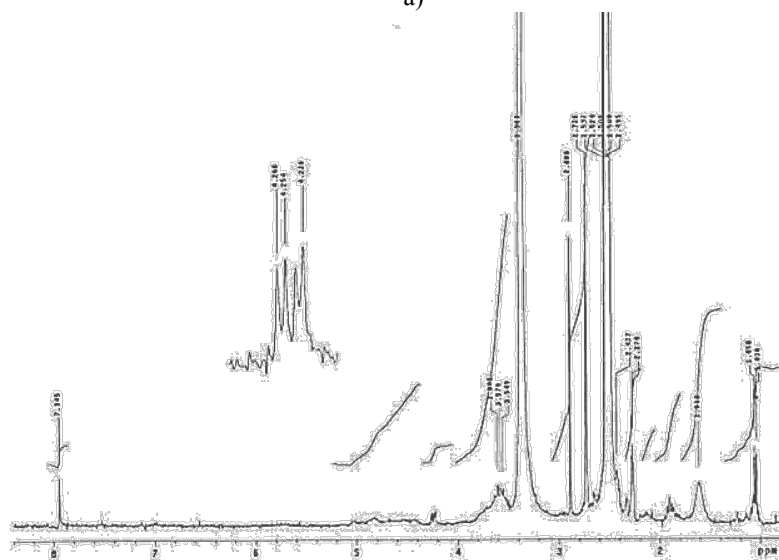
CP	CP- βCDNS	$\Delta \delta$ ppm	CP-SBE βCDNS	$\Delta \delta$ ppm	CP-HP βCDNS	$\Delta \delta$ ppm
δ ppm	δ ppm		δ ppm		δ ppm	
1.049	1.047	-0.002	1.047	-0.002	1.045	-0.004
4.255 2	4.225	-0.0302	4.226	-0.0292	4.227	-0.0282
CZP	CZP- βCDNS	$\Delta \delta$ ppm	CZP-SBE βCDNS	$\Delta \delta$ ppm	CZP-HP βCDNS	$\Delta \delta$ ppm
δ ppm	δ ppm		δ ppm		δ ppm	
1.176	1.24	0.064	1.173	-0.003	1.754	-0.0006
7.193	7.245	0.052	7.1929	0.0001	7.1931	0.0001
ENP	ENP- βCDNS	$\Delta \delta$ ppm	ENP-SBE βCDNS	$\Delta \delta$ ppm	ENP-HP βCDNS	$\Delta \delta$ ppm
δ ppm	δ ppm		δ ppm		δ ppm	
1.24	1.249	0.009	1.239	-0.001	1.1838	-0.0562
7.227 7	7.2338	0.0061	7.2321	0.0044	7.189	-0.0387

In the ^1H -NMR spectra recorded for the NS complexes, the chemical shifts of protons, both positive and negative small shifts, confirm the presence of the interactions (inclusion and non-inclusion) in most of the prepared complexes. Table I presents chemical shifts for several protons in the ACE inhibitors molecules: H 2 (methyl group) and H from the carboxylic group of proline ring for CP, H 1' and H 2' (ethoxy group) for CZP and ENP. As it is obvious, the lowest shifts were recorded for the CZP-HP βCDNS and CZP-SBE βCDNS complexes suggesting weak interactions, with probably negligible effect on CZP properties. The ^1H -RMN spectra of the complexes CP-SBE βCDNS , CZP- βCDNS and ENP-HP βCDNS are presented in Figure 3.

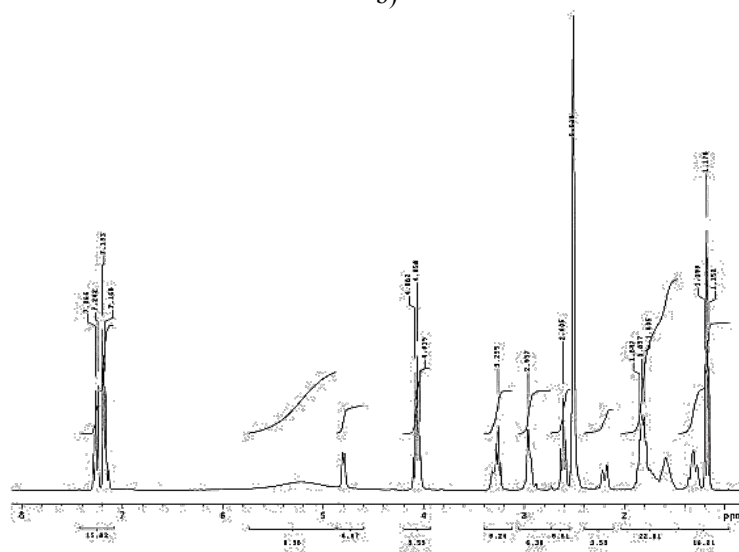
The FTIR-ATR spectra confirm the formation of the CD nanosponges: intense bands at 3,300–3,500 cm^{-1} due to the O–H stretching vibration and more intense bands for the vibration of the –CH and –CH₂– groups in the 2,800–3,000 cm^{-1} region, characteristic for the cyclodextrin IR absorption spectra, appear in the CDNS spectra, too. In the spectra recorded for the lyophilized complexes formed by captopril, cilazapril and enalapril with the three nanosponges it is obvious that an inclusion process occurs, as long as the band at 1743.30 cm^{-1} , due to the C=O vibrations is far less intense and shifted, as well as the amidic band I (1584.55 cm^{-1}) and the band at 1313.71 cm^{-1} , due to C–N vibration (Figure 4).



a)



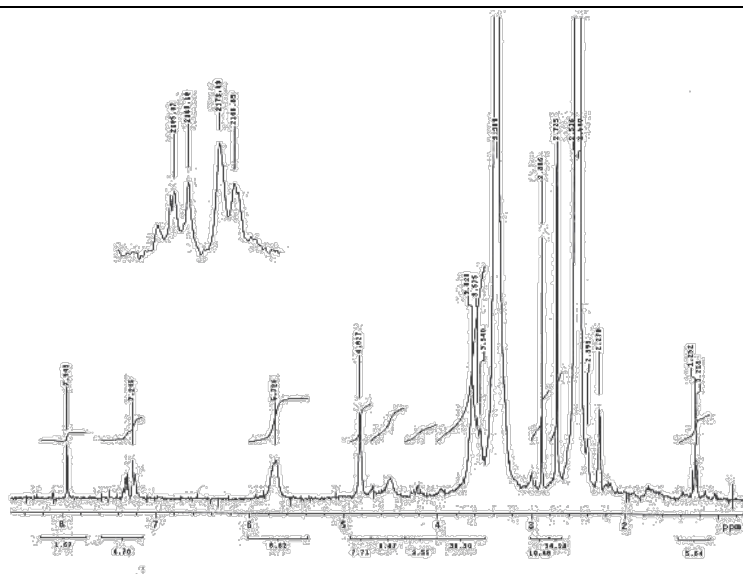
b)



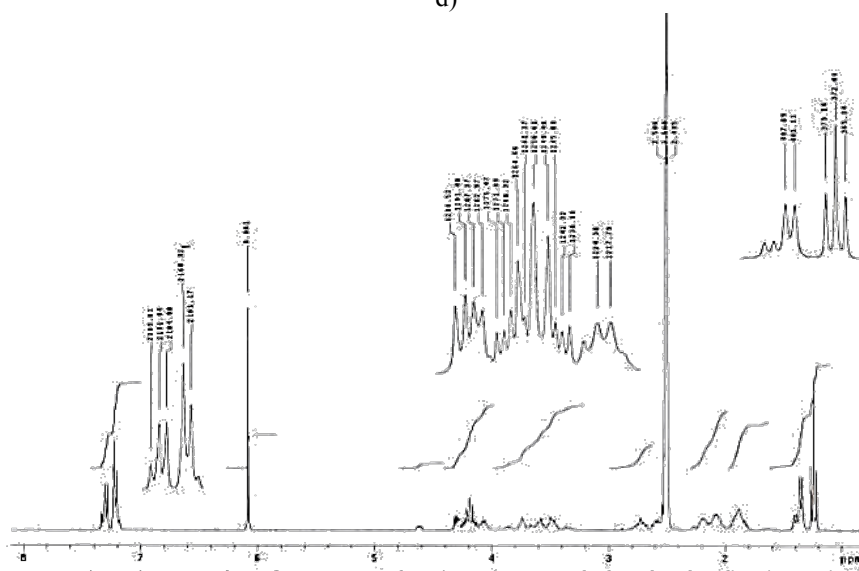
c)

Figure 3.

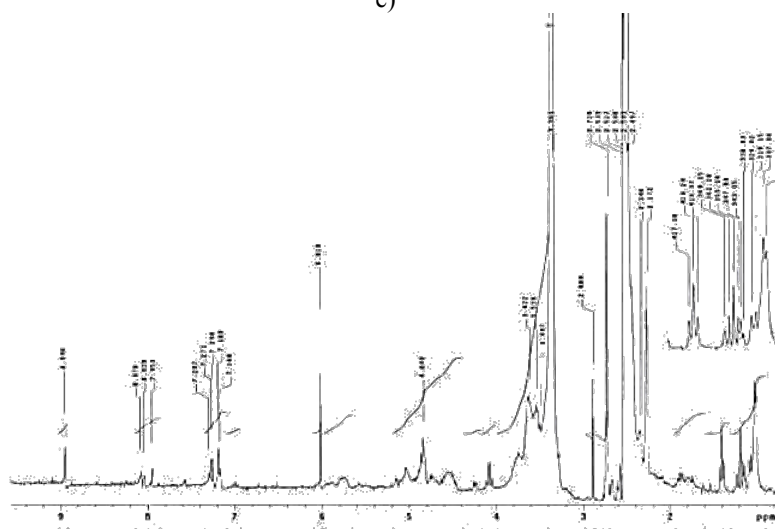
¹H-NMR spectrum of a) captopril; b) captopril:SBE β CDNS; c) cilazapril



d)



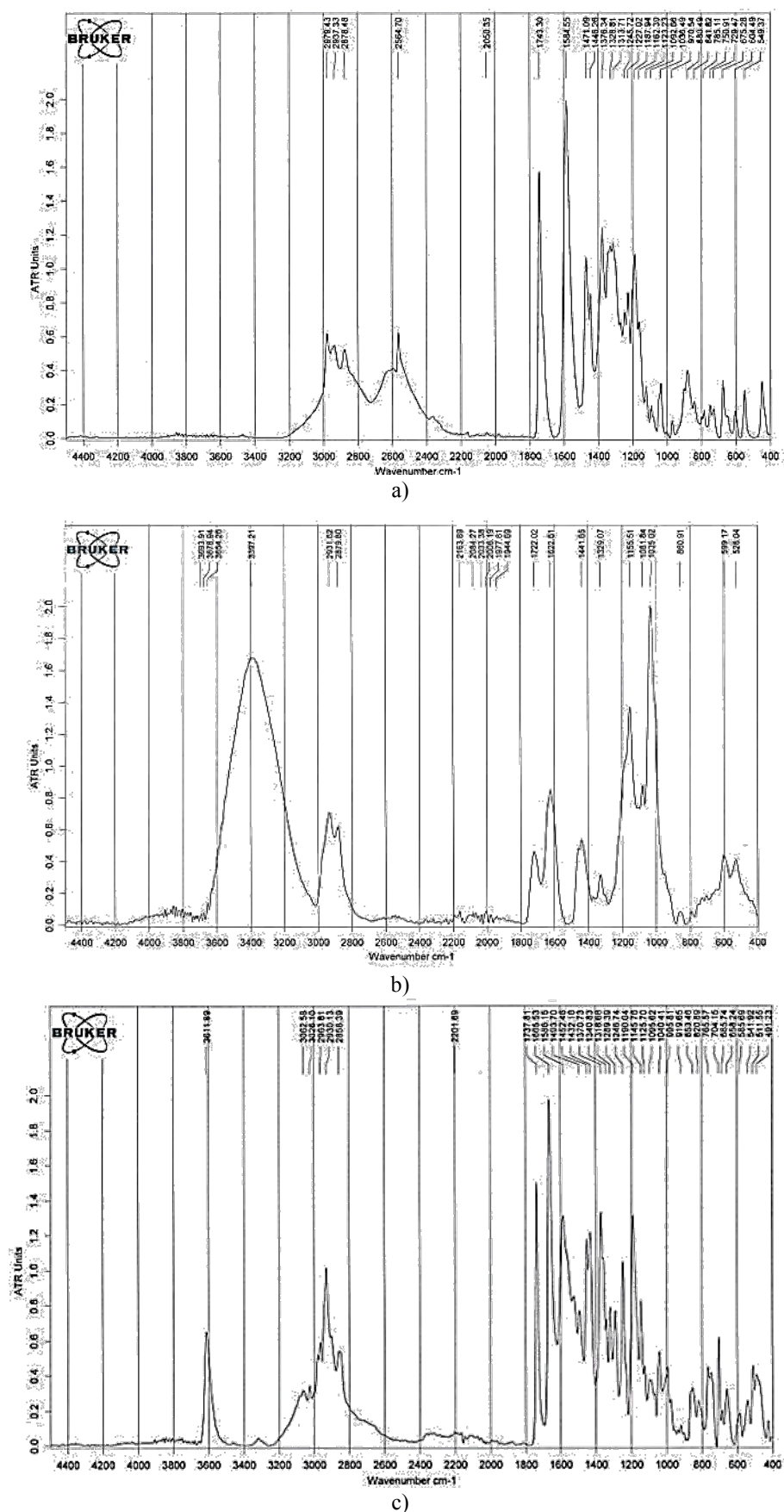
e)

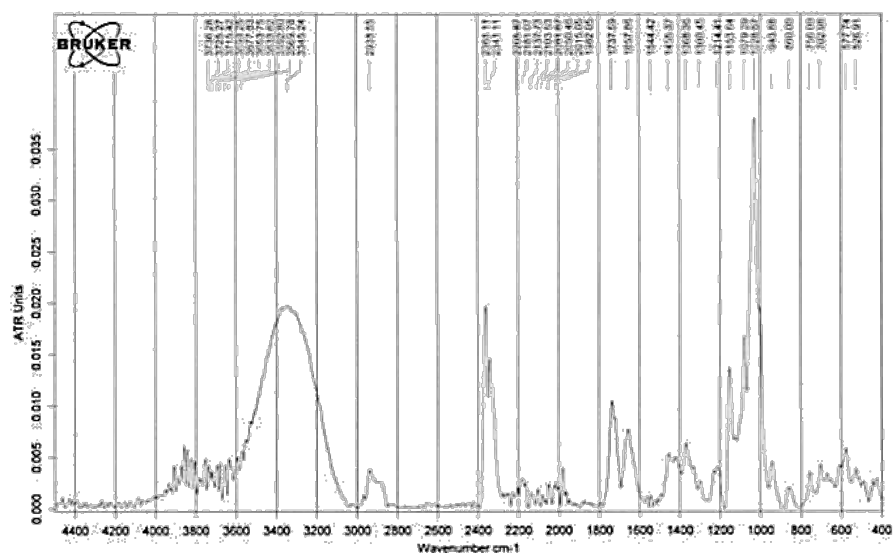


f)

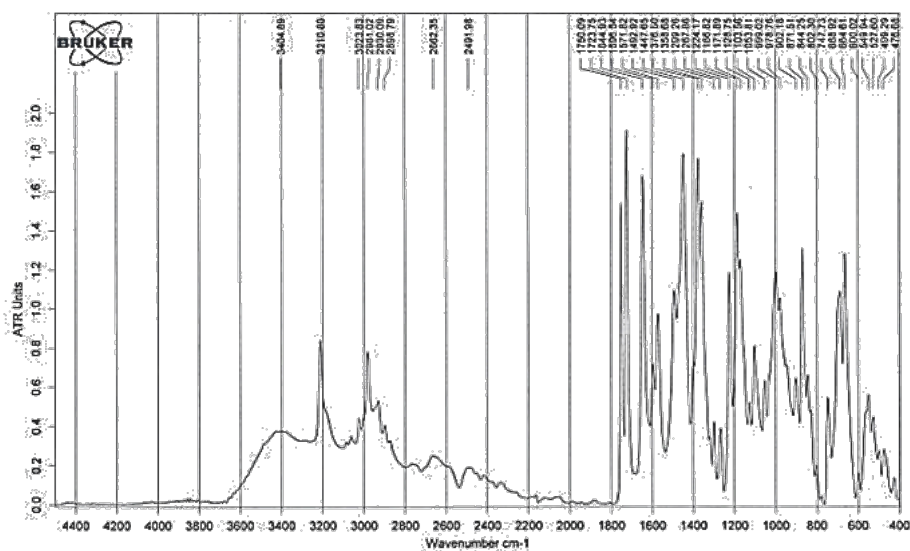
Figure 3.

¹H-NMR spectrum of d) cilazapril:βCDNS; e) enalapril; f) enalapril:HPβCDNS

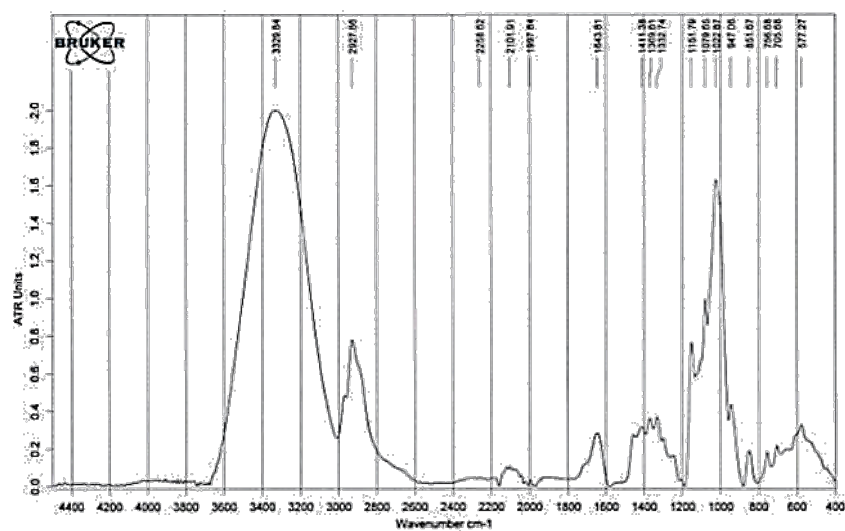
**Figure 4.**FTIR-ATR spectrum of a) captopril; b) CP-SBE β CDNS; c) cilazapril



d)



e)



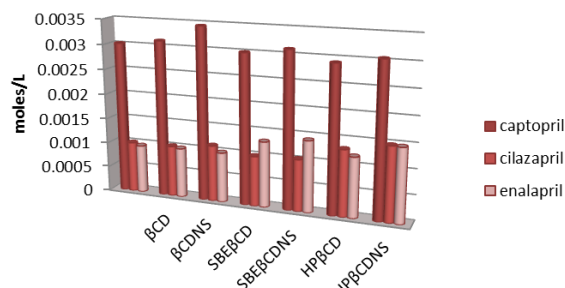
f)

Figure 4.

FTIR-ATR spectrum of d) CZP-βCDNS; e) enalapril; f) ENP-HPβCDNS

Solubilization efficiency

The effective solubility of CP, CZP and ENP provided by the β CDNS, HP β CDNS and SBE β CDNS was estimated against equivalent quantities of the CD monomers. As it can be seen in Figure 5, it is obvious that the CDNS effect is more intense for the β CDNS. This observation is consistent with the IR and NMR conclusions.

**Figure 5.**

Solubilization efficiency of cyclodextrins and cyclodextrin-based nanosponges

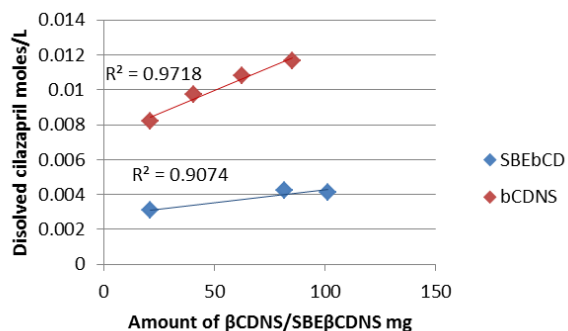
The β CDNS effect on CZP solubility is stronger and more coherent than for the other tested nanosponges; irregularities observed for the interactions between CZP and HP β CDNS and SBE β CDNS are probably due to the steric hindrance involved both in the reticulation and in the inclusion process. On the other hand, CP, as the smallest molecule is better entrapped by the β CDNS, but the resulted complexes are rather labile. For ENP, the strongest interactions are in the ENP- HP β CDNS complex because of the higher probability to form hydrogen bonds.

Phase solubility diagrams

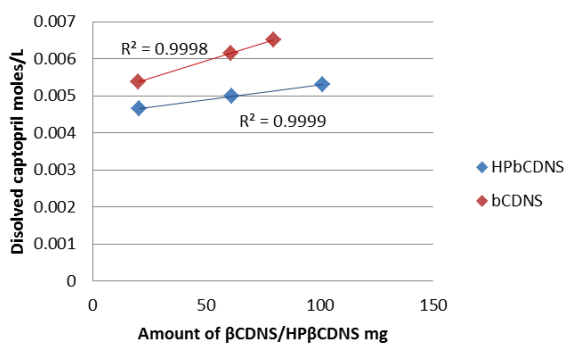
As it can be seen in Figures 6, 7, and 8, according to the Higuchi and Connors classification [16], the phase-solubility diagram for all the investigated complexes (CP- β CDNS, CP-HP β CDNS, CP-SBE β CDNS, ENP- β CDNS, ENP-HP β CDNS, ENP-SBE β CDNS, CZP- β CDNS, CZP-HP β CDNS, CZP-SBE β CDNS) are AL-type, with relative good correlations coefficients indicating the formation of the soluble complexes.

The most efficient entrapment and solubilization effect can be observed for ENP complex with HP β CDNS, when the solubility of ENP increases linearly with the amount of nanosponge in the solution, from 5.2 to 10 mg/mL for 5–15 mg added HP β CDNS.

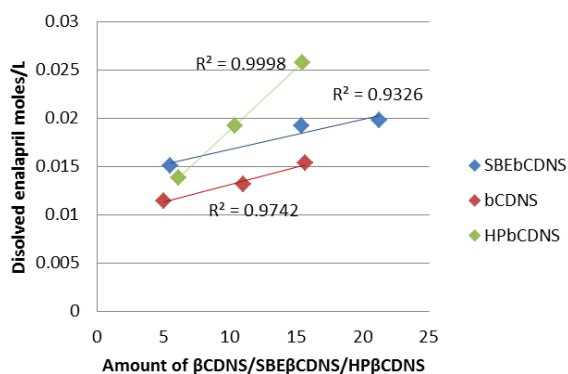
From the phase-solubility diagrams we have estimated apparent binding constants for the drug-CDNS associations. The obtained values, very small (lower than 100 M^{-1}) except for the ENP-HP β CDNS complex (770 M^{-1}) indicate that the major interaction, both with the monomeric and the studied polymeric cyclodextrins is the inclusion in the cyclodextrin hydrophobic cavity and to a much lesser extent to the entrapment in the nanosponge pores.

**Figure 6.**

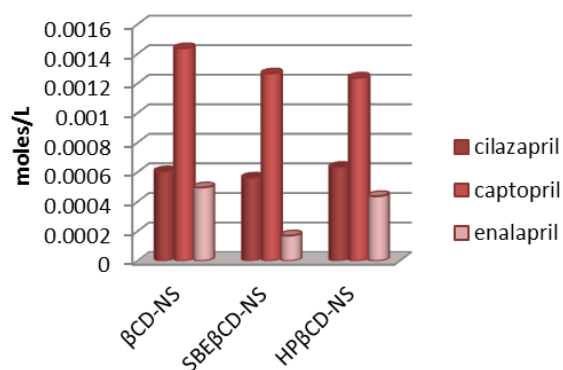
Phase solubility diagrams in water for cilazapril with SBE β CDNS and β CDNS

**Figure 7.**

Phase solubility diagrams in water for captopril with HP β CDNS and β CDNS

**Figure 8.**

Phase solubility diagrams in water for enalapril with HP β CDNS, β CDNS and SBE β CDNS

**Figure 9.**

Loading efficacy for β CD-NS, SBE β CD-NS, HP β CD-NS

Figure 9 indicates that the loading efficacy of the three types of nanosponges varies with the nature of the guest molecule. β CDNS is able to entrap larger amounts of CP and ENP, while CZP is better entrapped in the HP β CDNS.

Cytotoxicity

Inverted microscopy was used to observe the general morphological changes of HeLa cells treated with 100 μ g/mL CD complexes (Figure 10 B, C, D, E, F, and G), as compared to the control sample (Figure 10 A). The HeLa cells exhibited normal features, such as polygonal shape, homogeneous morphology in the untreated as well

as in the presence of the nanosponges and drug-NS complexes. No toxicity was detected using fluorescence-based live-dead assays in HeLa untreated and treated with 100 μ g/mL nanosponges and drug-NS complexes, for 24 h (Figure 11). On the other hand, the cellular cycle was not affected by the treatment with 100 μ g/mL nanosponges, for 24 hours (Figure 12). No appearance of apoptotic peak was observed. All of this demonstrate that the obtained nanosponge complexes are highly compatible and could be used for *in vivo* applications.

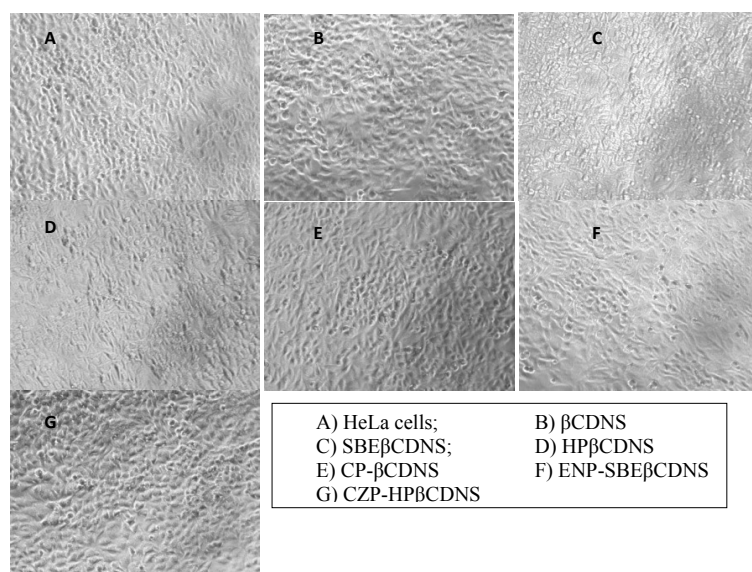


Figure 10.

Inverted microscope images of HeLa monolayers after 24 h of treatment with nanosponges and drug-NS complexes

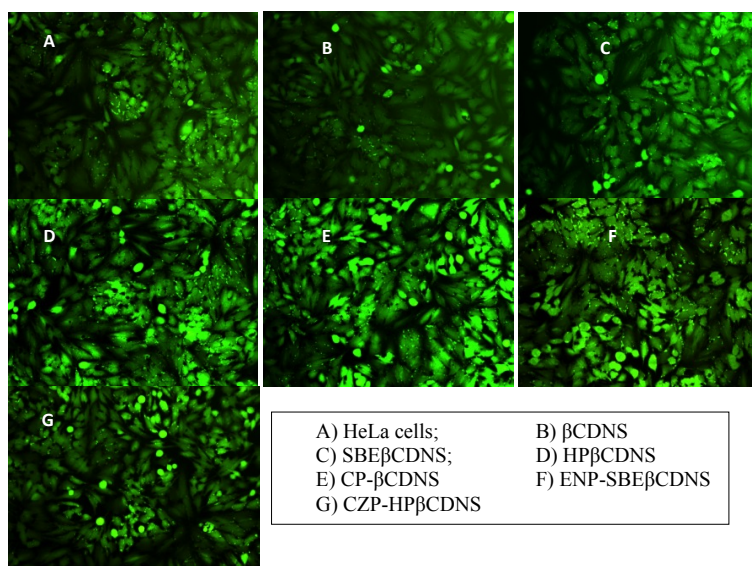


Figure 11.

Fluorescence staining of HeLa monolayers after 24 h of treatment with nanosponges and drug-NS complexes; FdA is taken up by viable cells which convert the non-fluorescent FdA into the green fluorescent metabolite fluorescein depending on esterase, while PI pass through disordered areas of dead cell membranes, and intercalates with the DNA double helix of the affected cell.

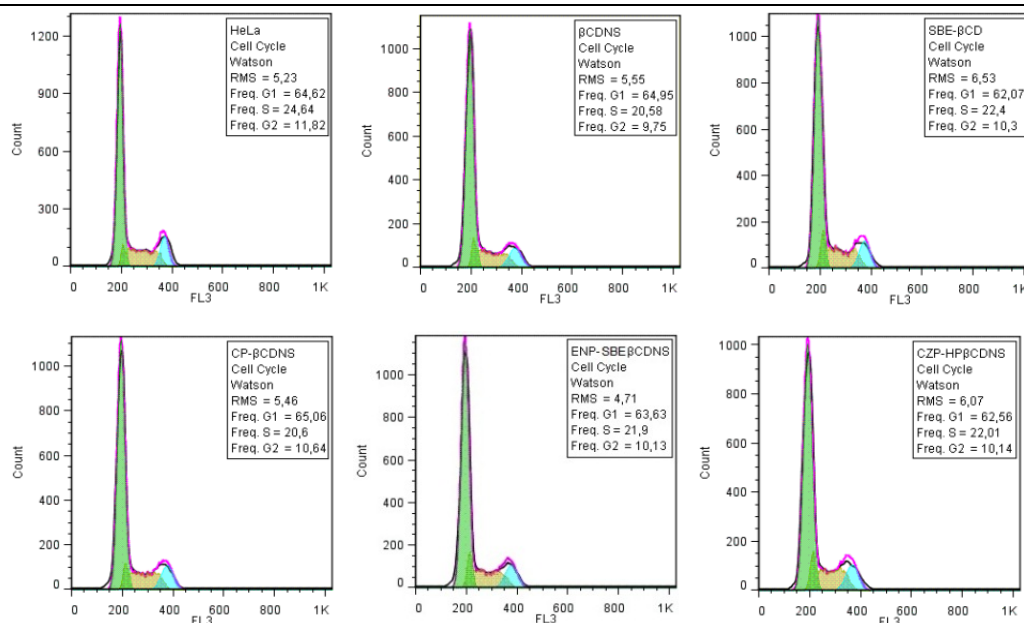


Figure 12.

Histograms of HeLa cell cycle untreated (A) or treated with nanosponges and drug-NS complexes

Conclusions

The synthesis of β CDNS, HP β CDNS and SBE β CDNS was confirmed through physico-chemical characterization techniques described in this work (^1H NMR, ATR-IR) and the interaction with CP, CZP and ENP was studied both in solid state and in solution.

The chemical shifts variations for the protons in the ACE inhibitors molecules upon complexation indicate weak interaction forces between the guest molecules (CP, CZP and ENP) and the synthesized nanosponges. The inclusion of the guest molecules was confirmed by the FTIR-ATR spectra, which show major changes in the fingerprint region ($900\text{--}1400\text{ cm}^{-1}$) of the guest molecules, due to the host-guest interactions and, also, to the entrapment in the nanosponge pores.

The solubility studies showed modest effect of the CDNS on the guest molecules solubility. However, we are entitled to assume that interactions are established, as it can be seen in the behaviour in solutions. These interactions are mostly due to the inclusion in the cyclodextrin almost hydrophobic cavities and also to other less specific interactions.

Acknowledgements

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