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Research Article

A Comparative Study of β-Cyclodextrin Nanocomposites and Their Ingredients: Characterization, Antioxidant Evaluation, and Biocompatibility Analysis

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Abstract

Background: Cyclodextrin based nanosponge (CDNS) as a novel delivery system has not been compared in terms of antioxidant and cytotoxicity potentials with its individual components. Given that oxidative stress and cytotoxicity play pivotal roles in various diseases, the authors sought to evaluate the properties of the synthesized CDNSs and their components, β-cyclodextrin (β-CD) and carbonyldiimidazole (CDI). These carriers have been widely used in pharmaceutical sciences for various purposes. Demonstrating their remarkable characteristics can guide researchers in making accurate applications and selections.

Methods: Two types of CDNSs were synthesized with different cross-linker ratios. The analysis involved dynamic light scattering (DLS), differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR), and solid-state NMR methods. The antioxidant activity was determined through DPPH scavenging activity, glutathione peroxidase assay, and detection of reactive oxygen species levels. Additionally, the cellular cytotoxicity of the materials was characterized using the MTT test.

Results: The results confirmed the synthesis of CDNSs with 1:2 and 1:4 β-CD:CDI molar ratios. The antioxidant results revealed that β-CD and CDI exhibited higher antioxidant activity compared to CDNSs with 1:2 and 1:4 ratios. Based on the MTT results, all four compounds demonstrated almost complete cytocompatibility with at least 50% cell viability.

Conclusion: Following the successful synthesis of CDNSs, it can be inferred that these materials are non-toxic. However, the process of nanosponging did not enhance the antioxidant activity of β-CD and CDI.

Introduction

Oxidative stress (OS) is a phenomenon, known as redox imbalance in favor of oxidant burden. It is considered one of the most important biological factors in cancer development and progression. In general, cancer cells signify aberrant redox homeostasis, which proposes novel therapeutic strategy by focusing on redox status regulation.[1] OS, as a physiological condition, can induce vulnerable reactions in the cancer process. This process leads to the production of a series of damaging free radicals.[2] These highly reactive molecules are generated by normal cellular processes, environmental stresses, and ultraviolet (UV) irradiation. Reactive oxygen species (ROS) may cause cellular and tissue injury by affecting cellular components such as DNA, carbohydrates, proteins, and lipids.[3] Excess production of ROS can also lead to inflammation, premature aging disorders, and several disease states, including cancer, diabetes, and atherosclerosis.[4] Organisms have developed complex antioxidant systems to protect themselves from oxidative stress; however, excess ROS can overwhelm the systems and cause severe damage.[5] As OS modulates several cancer cells malignant behaviors, antioxidant therapy can be considering as an effective strategy to stop cancer initiation and progression.

Cyclodextrin Nanosponges (CDNSs) are among the newest drug delivery carriers. They are called nanosponges due to their sponge-like structure and size.[6] Notably, cyclodextrins (CDs) used in this carrier for drug delivery include α, β, and γ-CD. By adding a series of crosslinkers, a nanosponge structure is ultimately created.[6,7] Among CDs,

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β-CD is widely used in CDNSs production because of its unique characteristics such as biocompatibility,[8] highest complexation ability, and stability with cross-linking agents.[6] CDNSs possess a series of ideal features for use in drug delivery. Among these features, we can mention the loading capacity for drugs and the enhancement of solubility and permeability of the loaded drug.[6,9] These drug delivery carriers are relatively new, and many of their properties, including antioxidant effects, have not been thoroughly investigated. Therefore, numerous studies are required for their introduction to the pharmaceutical market. It is important to note that limited studies have been conducted regarding the characterization of the CDNS' effect on the antioxidant activity of loaded agents. Some research studies about kynurenic acid,[10] vitamin E,[11] cinnamic aldehyde,[12] and caffeic acid[13] have indicated an increase in the antioxidant power of the target substances during loading into the β-CDNSs when compared to their free forms. Anandam and Selvamuthukumar[14] also indicated that the antioxidant activity of quercetin increased significantly through loading into β-CDNSs. They attributed this result to the improvement of the physicochemical properties and dissolution properties of quercetin.CDNSs have been introduced as biocompatible carriers,[9,15,16] but there has not been any comparative works about the source of this cytocompatibility. Beta-CD constitutes the main component of CDNSs in pharmaceutical applications. Its cytotoxicity depends on molecular weight, presence of charge, charge density, and also on some substitutes.[8]

Overall, CDNSs are promising candidates for drug delivery and biomedical applications due to their high biocompatibility and low cytotoxicity.[6,15] The studies conducted about CDNSs and CDs showed that these carriers can increase the antioxidant power of the loaded drugs. However, the source of this antioxidant activity remains a question that will be discussed in this article. Therefore, the authors decided to evaluate and compare the antioxidant and cytocompatibility effects of plain CDNS with its components to identify the main source of these characteristics. Here, we will also demonstrate the effect of crosslinking on CDs' antioxidant and cytocompatibility properties.

Methods

Materials

β-cyclodextrin (β-CD), 1,1′-carbonyldiimidazole (CDI), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). N Dimethylformamide (DMF), and ethanol were provided from Merck chemicals. Milli-Q water (Millipore) was prepared by the Milli-Q water purification system (ultra-pure water purification unit/laboratory max. 320 l/day, Milli-QA) and was used in all stages of this study. Glutathione Peroxidase Activity (GPX) Assay kit was purchased from ZellBio GmbH (Germany). 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2- carboxylic acid (trolox) was obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). All the chemicals used were of analytical grades.

Synthesis of β-cyclodextrin nanosponges

β-cyclodextrin nanosponges were synthesized from β-CD and CDI in 1:2 and 1:4 molar ratios. Briefly, CD was dissolved in DMF solvent, and then CDI was added with specific molar ratios. The resulting mixture was reacted at 90°C for 5 hours. After the completion of the reaction, the mixture was washed with an excess of water and ethanol to remove un-reacted CDs and the crosslinker residues.

Particle size characterization

Particle size and polydispersity of CDNSs were measured in triplicate by dynamic light scattering (DLS) (Malvern Instruments, Malvern, UK) using a 90 plus particle sizer. The analysis was performed at a fixed scattering angle of 90° and at 25°C. Zeta potential measurement was also done using an additional electrode in the same instrument.

Fourier transformed infrared study

The Fourier transform infrared (FTIR) spectra were obtained in the region from 500 to 4000 cm^{-1} with 1 cm^{-1} resolution. The spectra of β-CD, CDI, CDNSs, and their physical mixtures (PMs) were analyzed through the KBr disk method. Spectrum software by PerkinElmer located in Liantrisant, UK, was also used for spectral characterization and assessment.

Differential scanning calorimetry analysis

In current study, 3 mg of β-CD, CDI, CDNSs, and their physical mixtures were subjected to thermal analysis using a differential scanning calorimetry (DSC) instrument (TA Instruments SDT-Q600, USA). α-Alumina powder was used as the standard material. Samples were heated in the temperature range of 30–300°C at a rate of 10°C/min. The pan used in this study was made of alumina and did not have any pinholes.

Solid-state NMR analysis

Solid state NMR spectra were acquired using an Inova instrument from VARIAN, USA, operating at 500 MHz for H nuclei. A certain number of samples (5 mg for 1H) were prepared in $CdCl_3$ solvent for further analysis. The Mestrenova software was used for spectral characterization and assessment.

Antioxidant activity

DPPH scavenging activity

The antioxidant activity of β-CD, CDI, and CDNSs was determined using the DPPH (2,2-diphenyl-1picrylhydrazyl) Antioxidant Assay Kit (Zen-Bio, Inc. Cat# AOX-3). DPPH stable free radical method is a rapid and sensitive procedure to investigate the antioxidant activity which measures the reduction of the stable DPPH radical through electron transfer. Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water-

soluble vitamin E analog, served as a positive control, reducing the DPPH radical in a dose-dependent manner. Briefly, a methanol solution of DPPH and Trolox standards was prepared. Subsequently, 20 µL of samples or Trolox standards were added to wells of the assay plate. Buffer applied as a negative control. Then, 80 µL of DPPH working solution (250 µM) was added and was placed on plate shaker, incubated at 25°C for 10 minutes, kept in the dark. Finally, the optical density of each well was measured at 517 nm using a spectrophotometer plate reader (Thermo Scientific™ Multiskan™ FC Microplate Photometer). The Trolox standard curve was used to determine the Trolox equivalent antioxidant capacity (TEAC).

Glutathione peroxidase activity

In addition to our primary methodology, we employed an alternative approach to assess the antioxidant property. This involved evaluating the activity of the glutathione peroxidase enzyme, commonly referred to as GPX. The enzymatic activity of GPX was measured using the commercial chemical colorimetric assay kits (ZellBio GmbH, Ulm, Germany) based on the manufacturer's protocol. The GPX activity was expressed as units per milligram of protein, which is the amount of enzyme that catalyzed decomposition of 1 µmol of glutathione into glutathione disulfide per one minute. The decrease in absorbance was recorded with a microplate reader/ ELISA reader (Awareness Technologies Inc. Stat Fax 4200 Microplate Reader) at 412 nm. The assay sensitivity of kit was 5U/ml and the intra-assay CV and inter-assay CV for GPx concentration were 3.5% and 4.7%, respectively.

Evaluation of reactive oxygen species level

The Fluorometric Intracellular ROS Kit (Sigma) was used for ROS measurement. ROS produce as a result of the reduction of oxygen in aerobic respiration and via different enzymatic systems within the cell and can cause cell damage. ROS Detection Reagent was reconstituted with 40 µL of DMSO to generate the 500× ROS Detection Reagent stock solution. Cells were treated with 10 µL of 10× test compound solution (in a 96 well plate) in a PBS buffer. For control wells (untreated cells), the corresponding amount of buffer was added. To induce ROS, the cell plate was incubated in a 5% CO_2, 37°C incubator for 30 minutes. Then, 100 µL/well (in a 96 well plate) of the Master Reaction was mixed into the cell plate. After incubation, the fluorescence intensity was measured using a Fluorometer – QFX – DeNovix instrument (with λex = 640 nm/ λem = 675 nm).

Cell viability study

Cytotoxicity of β-CD, CDI, and CDNSs was evaluated in MCF-7 cells using MTT assay, 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide. The cells were seeded in 96-well plates at 10 $\times 10^3$ cells/well in 200 µl of RPMI-1640 with 10% FBS. After incubation time cells were treated with the different concentrations of samples, then the plates were incubated for 24 h. Next, the RPMI-1640 medium was replaced with 100µL of fresh medium and 50µL with MTT solution (5 mg/mL, Sigma-Aldrich) incubated for 4 hours at 37°C. Then 200 µL of DMSO was added to each well, and the plates were placed on a shaker until the formazan had completely dissolved. Finally, the optical absorption of each well was read using an ELISA Reader (Awareness Technologies Inc. Stat Fax 4200 Microplate Reader) at a wavelength of 570 nm. Cell viability was calculated based on Eq. 1. In this formula, OD refers to optical density.

$$Viability\ (\%) = \frac{MeanOD_{Sample}}{MeanOD_{Blank}} \times 100 \qquad \text{Eq. (1)}$$

Results and Discussion

Particle size characterization

Particle size and surface charge are among the most effective criteria affecting the stability of a suspension. The average diameter of CDNSs was determined by the light scattering method. Both nanocarriers had almost unimodal distribution with a very low polydispersity index (PDI) (Figure 1). This test was done with the aim of evaluating the nano-platform of CDNSs. The particle size of both nanosponges (CDNS 1:2 and 1:4) was found to be 107.1±24.30 and 537.6±53.57 nm (Z-average by number), with a zeta potential of −27.3±8.05 and -16.2±5.89 mV, respectively. Based on recent studies, there is no definite relation between the CD:cross-linker ratio in CDNSs and the obtained results.[9,15] The negative charge of the obtained particles allows them to be dispersed in water as a result of electric repulsion.[17] The authors attributed this negative zeta potential to the presence of free carbonyl and hydroxyl groups. Therefore, a low cross-linker ratio would result in a more negative surface charge. On the other hand, both of these nanoparticles were suggested to be cytocompatible because they possess a negative surface charge.[18,19]

FTIR study

FTIR analysis was performed on β-CD, CDI, CDNS1:2, CDNS1:4, and physical mixtures (PM) (Figure 2). The C=O peak around 1400-1500 cm^{-1} in both CDI and CDNS spectra may be evidence of the nanosponge synthesis.[9] The characteristic peak of the carbonate bond should appear around 1600-1700 cm^{-1}, but in CDI due to the presence of two aromatic rings and nitrogen atoms, it shifted to a lower wave number (around 1400_1500 cm^{-1}).[20] A similar shift was also observed in the spectrum of CDNS because of converting the carbonyl to ester.[21] In the spectrum of β-CD, a peak of unbound water is visible on 1651 cm^{-1}.[7] A broad peak observed at 3410 cm^{-1} is related to the O-H stretching of hydroxyl groups in β-CD and CDNS.[15] The FTIR of physical mixtures indicated all the peaks of β-CD and CDI. But the peaks of the imidazole group were removed in CDNSs, indicating the synthesis of CDNSs.[10,22] Physical mixtures of CDNSs were prepared at the same molar ratio of CD and CDI. The absence of characteristic peaks of

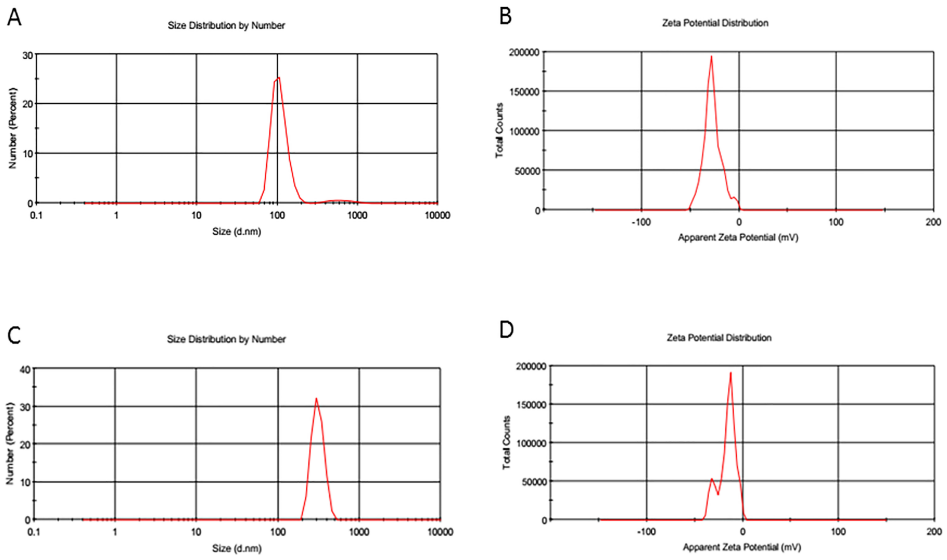

Figure 1. Particle size and zeta potential analysis of cyclodextrin nanosponge 1:2 (A, B) and 1:4 (C, D).

each ingredient may be due to some cohesive interactions between them, resulting in a non-uniform mixture.[23] On the other hand, sample weight as another important factor may influence the peak intensity of PMs, resulting in improper mixing.[24] Therefore, the authors emphasize the need to pay attention to the possible interactions of excipients in the formulations, since this may affect the mixing of solid ingredients in pharmaceutical preparations.

DSC analysis

DSC studies were performed to assess the thermal properties of the β-CD, CDI, synthesized CDNSs, and their physical mixtures (Figure 3). The thermogram of β-CD showed a broad endothermic peak around 100°C, which aligns with the loss of water molecules.[25,26] No sharp endothermic peak was observed for β-CD due to its amorphous nature.[7] In the spectrum of CDI, there should have been a sharp melting point around 120°C, but only a small peak was seen around this temperature. Here, the authors attributed the absence of the sharp peak to the low stability of CDI against heat and atmospheric moisture.[27,28] Therefore, the use of a pinhole pan is suggested for the DSC analysis of CDI to minimize possible interactions.

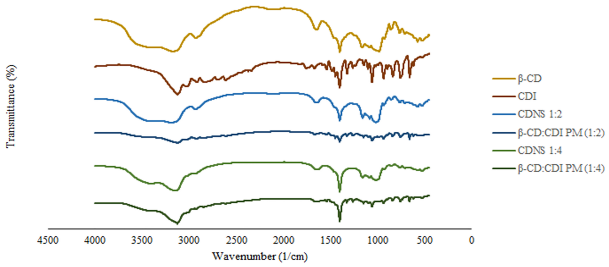

Figure 2. FTIR spectrum of β-cyclodextrin (β-CD), Carbonyldiimidazole (CDI), synthesized cyclodextrin nanosponges (CDNS 1:2, and CDNS 1:4), and physical mixtures of β-CD and CDI at 1:2 and 1:4 molar ratios (β-CD:CDI PM (1:2), and β-CD:CDI PM (1:4)).

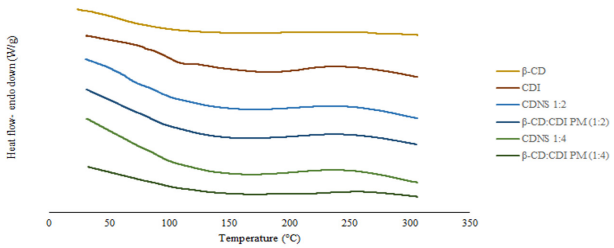

Figure 3. DSC analysis of β-cyclodextrin (β-CD), Carbonyldiimidazole (CDI), synthesized cyclodextrin nanosponges (CDNS 1:2, and CDNS 1:4), and physical mixtures of β-CD and CDI at 1:2 and 1:4 molar ratios (β-CD:CDI PM (1:2), and β-CD:CDI PM (1:4)).

An endothermic shift was also observed in the heat flow baseline of β-CD, CDNSs, and their physical mixtures, which is related to their amorphous structure. On the other hand, a broad and slight exothermic peak was also seen around 240°C for CDI, synthesized CDNSs, and β-CD:CDI PM (1:2), indicating the thermal decomposition of these materials.[29] The absence of this peak in β-CD:CDI PM (1:2) may be related to cohesive interactions that prevented proper physical mixing.

H-NMR analysis

The structural properties of β -CD, CDI, CDNSs 1:2, and 1:4, as well as the physical mixtures of β -CD and carbonyl diimidazole with ratios of 1:2 and 1:4 were studied using a ^{1}H-NMR spectrum (Supplementary data). The protons on the inside and outside of the cavities were displayed in the spectra of β-CD, CDNSs and PMs. The chemical shifts in protons of hydroxyl groups also indicated the formation of covalent bonds in CDNSs.[30] The ^{1}H-NMR spectrum of CDI showed two proton assignments at 7.03, and 7.66 ppm due to its sensitivity to moisture, atmosphere, and exposed

Table 1. Antioxidant activity results of β-cyclodextrin (β-CD), carbonyldiimidazole (CDI), and cyclodextrin nanosponges (CDNS 1:2 and 1:4).

Samples	DPPH radical-scavenging activity (µmole trolox/ mg sample)	Glutathione Peroxidase Activity (U/mg protein)	Dichlorofluorescein density (RFU/ mg sample)
Control	278.00± 37.2	51.33± 6.8	1968.67± 165.8
β-CD	165.33± 13.5	31.67± 3.5	3513.33± 265.0
CDI	194.00± 6.2	35.67± 2.5	2643.33± 56.8
CDNS 1:2	133.33± 5.5	24.00± 2.0	3900.00± 147.3
CDNS 1:4	129.67± 6.6	25.67± 3.5	4470.00± 468.0

Results are depicted as mean± SD (n=3). Here, relative fluorescence unit λem = 675 nm is abbreviated as RFU.

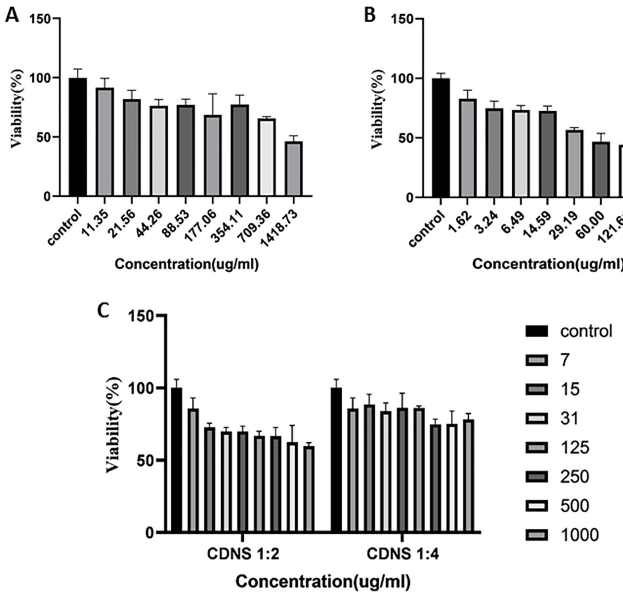

Figure 4. Cell viability in MCF-7 against β-cyclodextrin (A), carbonyldiimidazole (B), and cyclodextrin nanosponge 1:2 and 1:4 for 24 h (C).

surface area.[27] The absence of these peaks in the spectrum of CDNSs indicates their synthesis.[31] These peaks were observed in the spectrum of PMs. It is also important to note that the absence of imidazole residues in the spectrum of CDNSs may confirm the proper washing of the finally synthesized powder.[9,15]

Antioxidant activity evaluation

There are many methods to represent antioxidant activity, and they are based on different mechanisms such as radical-scavenging activity, glutathione peroxidase activity, and reactive oxygen species level. Spectrophotometry and fluorometry are the most widely used methods for antioxidant assays.[32] In current study, the antioxidant activity of β-CD, CDI, and CDNSs was measured based on the mentioned processes, and the obtained data are depicted in Table 1. The obtained results showed that the process of nanosponging does not enhance the antioxidant activity of β-CD. The authors attributed this outcome to the high content of hydroxyl groups in β-CD compared to CDNSs.[33,34] Since the number and location of hydroxyl groups play an important role in antioxidant activity.[35] CDI, a green activating reagent,[36] exhibits a more potent antioxidant effect compared to β-CD. Recent investigations showed impressive results regarding the relationship between solubility enhancement and antioxidant activity.[37] Therefore, the antioxidant potential of β-CD may be limited by the strong intermolecular hydrogen bonding and low water solubility.

Based on the results of DPPH radical-scavenging activity and dichlorofluorescein density of CDNSs, CDNS 1:2 exhibits a more potent antioxidant effect than CDNS 1:4. However, CDNS 1:4 shows more glutathione peroxidase activity than CDNS 1:2. From this, it can be concluded that CDI cannot enhance the antioxidant capacity of β-CDs. Furthermore, there is no specific relationship between CDI content in CDNSs and their antioxidant activity. It's also important to note that the type of the cross-linker may affect the solubility of synthesized CDNSs,[38] and may influence the antioxidant activity. Recent investigations showed impressive results regarding the relationship between solubility enhancement and antioxidant activity.[37]

Cellular cytotoxicity assay

Cytotoxicity of β –CD, CDI, and CDNSs against MCF-7 cell lines was investigated, and cell viability was determined using the MTT assay after 24 h. Based on the results depicted in Figure 4, cytocompatibility was observed for

β-CD (11.35-1418.73 µg/ml), CDI (1.62-243.23 µg/ml), and CDNS 1:2 and 1:4 (7-1000 µg/ml) with cell viability values higher than 50%. It is also noteworthy that a dose-dependent manner was observed for all compounds. Vukic *et al.*[30] reported that free β-CD exhibited no evident cytotoxicity toward HCT116 and MDA-MB 231 cell lines in a range of 0-100 µg/ml after 24, 48, and 72h. Moreover, other studies confirmed no substantial cytotoxicity of CDNS 1:4 at higher concentrations (1 and 2.5 mg/ml) for 24 and 48 h.[9,15] The authors attribute this cytocompatibility of CDNSs to the FDA-approved β-CD,[6] and green reagent CDI.[39,40] Based on the results depicted in Figure 4 (C), CDNS 1:4 has less toxicity than CDNS 1:2. Thus, it can be concluded that by increasing the amount of cross-linker, the toxicity of the CDNSs will decrease.

Conclusion

Overall, CDNS 1:2 and 1:4 were successfully synthetized through solvent method, and characterized with several analytical processes. Both of nanosponges were nano-sized with negative surface charge. DSC, FTIR and 1H-NMR confirmed their synthesis. Based on antioxidant tests, the process of nanosponging did not enhance the antioxidant activity of β-CD and CDI. According to cellular studies, CDNSs and their components are non-toxic and can be introduced as cytocompatible delivery systems. Furthermore, the authors suggested that more studies are necessary to fully understood the role of cross-linker ratio in antioxidant and cytotoxicity effects.

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Author Contributions

Zahra Maleki: Data Curation, Formal Analysis, Investigation, Resources, Software, Writing–Original Draft. **Elham Safarzadeh:** Formal Analysis, Funding Acquisition, Methodology, Resources, Software, Supervision, Validation, Writing - Review & Editing. **Francesco Trotta:** Conceptualization, Visualization, Writing–review & editing. **Saeideh Allahyari:** Conceptualization, Funding Acquisition, Methodology, Project Administration, Resources, Software, Supervision, Validation, Visualization, Writing - Review & Editing.

Conflict of Interest

The authors report no conflicts of interest.

Supplementary Data

Supplementary data (NMR spectra) are available at https://doi. org/10.34172/PS.024.40870

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