Improving stability of cerium oxide nanoparticles by microbial polysaccharides coating

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Abstract: Cerium oxide (CeO$_2$) nanoparticles (CONPs) are interesting biomaterials with various applications in biomedicine, cosmetics and the pharmaceutical industry, but with limited practical application because of their low stability in aqueous media. The aim of this study was to obtain CONPs with increased stability by coating the particles. Microbial exopolysaccharides (levan, pullulan) and glucose were used to prepare CONPs under different synthesis conditions. Coating was attempted by adding the carbohydrates during (direct coating) or after (subsequent coating) the synthesis of CONPs. The obtained nanoparticles were characterized by X-Ray diffraction analysis, Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The suspension stability of the uncoated and coated CONPs in aqueous media was evaluated by measuring the hydrodynamic size, zeta potential and turbidity. The FT-IR spectra revealed the differences between coated CONPs and showed the success of subsequent coating with carbohydrates. Coating with carbohydrates improved the stability the CONP suspension by decreasing the size of aggregated particles. The suspensions of levan- and glucose-coated CONPs had the best stability. In this study, CONPs were prepared using non-toxic materials, which were completely environmentally friendly. The obtained results open new horizons for CONP synthesis, improving their biological applications.

INTRODUCTION

Cerium oxide nanoparticles (CONPs) have been extensively investigated due to their excellent oxygen storage capacities based on the redox transition between Ce^{3+} and Ce^{4+} and relatively easy formation of labile oxygen vacancies on their surface.[1–7] Due to this, CONPs are powerful free radical scavengers. After reaction with free radicals, they regenerate themselves in every cycle, making them unique compared to other particles.[8,9] However, CONPs show great tendency for agglomeration due to the high number of oxygen vacancies on their surface, leading to low stability of their water suspensions, which is the main restriction for their application.[10] This problem could be reduced by coating the CONPs with polymers, thereby improving their suspension stability.

In general, for coating CONPs, polymers such as dextran,[4,6,10–15] heparin,[1,16] chitosan,[5,17] poly(ethylene glycol) (PEG),[18,19] and poly(lactic-co-glycolic acid) (PLGA)[20] are commonly used, as well as glucose,[21] citric acid and citrates,[22–24] and even drugs.[25] In selecting a polymer, attention should be paid to the type and amount of polymer coated over the nanoparticles, which would affect suspension stability.[3]

In this study, the carbohydrates levan, pullulan and glucose were used to coat CONPs. The use of pullulan as a coating agent for CONPs was conducted for the first time, according to the available literature. Glucose was used as it is a common coating material[21,26,27] and as such, suitable for comparison with novel compounds for coating CeO$_2$. Levan and pullulan (Fig. 1) are biocompatible and water soluble microbial exopolysaccharides, being the focus of various investigations.[28–30] Levan is a carbohydrate polymer that consists of *β*-D-fructofuranose attached by *β*-(2,6) linkages,[28] whereas pullulan is composed of maltotriose repeating units linked to terminal glucosyl residues by *α*-(1,6)-D-glycosidic bonds.[29,30] The aim of this study was to increase the stability of aqueous suspensions of CONP by coating the particles with the aforementioned carbohydrates. The coating was performed by two different methods, direct coating (DC) and subsequent coating (SC), at different temperatures and coating times.
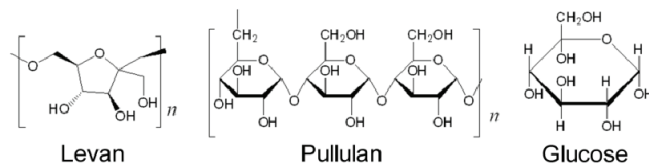

Fig. 1. Structural formulas of glucose, levan and pullulan.

<div align="center">EXPERIMENTAL</div>

Materials

Chemical reagents used in this study were $Ce(NO_3)_3 \cdot 6H_2O$ (Acros Organics, 99.5 %), NH_3 (Merck, 25 %), NaOH (Carlo Erba Reagents, 97 %) and glucose (Sigma–Aldrich, $\geq$ 99.5 %). All the chemicals were of analytical grade.

Synthesis and coating of CONPs

CONPs were synthesized by the self-propagating room temperature (SPRT) method, using $Ce(NO_3)_3 \cdot 6H_2O$ and NaOH as starting materials according to Eq. (1):

$$2[Ce(NO_3)_3 \cdot 6H_2O] + 6NaOH + 1/2O_2 \rightarrow 2CeO_2 + 6NaNO_3 + 15H_2O \qquad (1)$$
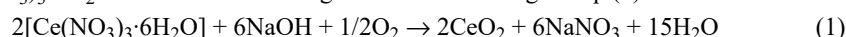
The chemicals were hand mixed in a mortar with a pestle for 5–10 min. The product obtained was rinsed three times for 10 min with deionized (DI) water and twice with ethanol in a centrifuge (Centurion 1020D) at 4200 rpm.[32] The final powders were dried overnight at 70 °C.[31]

CONPs coated with carbohydrates were obtained in two ways. According to the DC method used by Sun *et al.*,[33] levan, pullulan or glucose were individually dissolved in DI water, and $Ce(NO_3)_3 \cdot 6H_2O$ was added. The mass ratio of carbohydrates to $Ce(NO_3)_3 \cdot 6H_2O$ was 1:7. The pH of the reaction mixture was adjusted to pH 10 by adding 25 % ammonia. The mixture obtained was transferred into a sealed, Teflon™-lined stainless steel autoclave for crystallization between 100 and 200 °C in 6–48 h time interval. The obtained powder was isolated from the liquid phase by centrifugation, washed three times with DI water and dried at 60 °C overnight.

In the SC method, glucose, levan or pullulan was individually dissolved in DI water and then CONPs, synthesized by the SPRT method, were added. The mass ratio of carbohydrates to CONPs was 1:7. Further steps were the same as in the DC method.

Microbial biosynthesis of levan

Levan, a microbial polysaccharide, was prepared by cultivating *Bacillus licheniformis* NS032 in sucrose medium. The isolation, purification and physicochemical characterization of the polysaccharide are detailed in Kekez *et al.*[34]

Microbial biosynthesis of pullulan

Pullulan was produced by the *Aureobasidium pullulans*; strain CH-1 (Institute of Chemistry, Technology and Metallurgy ICTM, Collection of Microorganisms), according to the procedure used by Jakovljević *et al.*[30] and Radulović *et al.*[35]

Characterization of CONPs

X-Ray diffraction. The phase purity and crystallinity of the synthesized CONPs were examined using X-ray diffraction (Raguku Ultima IV, Japan). The X-ray beam was nickel-filtered $CuK_{\alpha 1}$ radiation ($\lambda = 0.1540$ nm) and the diffractometer was operated at 40 kV and 40 mA. The XRD data were collected from 5 to 90 ° (2θ) at a scanning rate of 5 ° min^{-1}.

Scanning electron microscopy. The CONPs were imaged on a FEI Quanta 200 (FEI Company, Czech Republic) microscope equipped with an energy dispersive spectrometer EDAX Genesis 4000. The CONPs were applied to an aluminium alloy circular grid, with sticky tape and graphite, which makes it conductive for electrons.

Transmission electron microscopy. For the TEM imaging, the CONPs were suspended in DI water and adsorbed onto a thin carbon film. The images were acquired at room temperature at a magnification of 155,000× on a TemCam F416 CMOS camera (TVIPS GmbH) in a Phil-

ips (USA) CM120 LaB6 electron microscope (Philips/FEI) which was operated at an acceleration voltage of 120 kV.

Fourier transform infrared spectroscopy. The FT-IR spectra were recorded in the wavelength range 4000–400 cm^{-1} using the KBr pellet technique at resolution of 1 cm^{-1} using a Bomem MB 100 spectrophotometer.

Dynamic light scattering and zetametry. A Zetasizer Nano ZS with a 633-nm He–Ne laser (Malvern, UK) was used to measure the hydrodynamic size and zeta (ζ) potential of CONPs in DI water (0.1 mg mL^{-1}). The hydrodynamic particle sizes and particle size distributions (radii) were determined by the dynamic light scattering (DLS) method, in which the results are given by the intensity of the scattered light. The zeta potential was calculated by the instrument software from the electrophoretic mobility of particles measured by laser Doppler velocimetry. At least three measurements were conducted and the results were averaged.

Turbidimetry. A Palintest Micro 950 Turbidity Meter was used for measuring the turbidity of aqueous suspensions of the CONPs, as a means to semi-quantitatively estimate particle concentrations, *i.e.*, stability. The suspensions of uncoated and coated CONPs were prepared in DI water at concentrations of 7.5, 15, 30 and 60 mg L^{-1}. Prior to measurements, the CONP suspensions were sonicated in ultrasound bath Ultrasons HD (J. P. Selecta s.a., Spain) for 60 min at 120 W. The turbidity is expressed in nephelometric turbidity units (NTU).

RESULTS AND DISCUSSION

Structural properties of the CONPs synthesized by two methods

The DC method was used first to coat CONPs *in situ*, *i.e.*, during the CONP synthesis, as this is a common approach for coating nanoparticles. Accordingly, a series of synthesis experiments was performed with the addition of glucose, maintaining the reaction temperature at 100 °C for different times (Fig. 2). The XRD patterns of the CONPs show that amorphous products were obtained, regardless of the synthesis duration (Fig. 2A). Thus, changing the coating time obviously had no effect on the efficiency of coating, and neither did the synthesis temperature (data not shown). Due to the impropriety of using amorphous particles in any potential biomedical application, coating of the CONPs was then attempted after their synthesis *via* the SPRT method by the SC method. Again, the coating efficiency with glucose was examined first, setting the coating process parameters as for the DC method (temperature between 100 and 200 °C; coating time between 6 and 48 h).

In contrast to the DC CONPs, the XRD patterns of all the glucose-coated CONPs obtained by the SC method showed a series of four diffraction peaks, corresponding to the reflections of the cubic fluorite structure of CeO$_2$ (Fig. 2B).[36] There was no significant change in the XRD spectra with increasing temperature (data not shown). Moreover, most importantly, it is obvious that coating with glucose did not affect the crystallinity of CONPs produced by SPRT method, as can be seen from the diffractogram of uncoated CONPs, given for comparison purposes (Fig. 2A and B). These results indicate that the SC method

is more convenient for coating CONPs with carbohydrate than the DC method, and for this reason it was used in the further investigations.

A coating time 6 h and a temperature 100 °C were chosen for further study since CONPs were not successfully coated at shorter coating times or at lower temperatures (data not shown).

The coatings of the CONPs, when they were prepared with the microbial polysaccharides, levan and pullulan,[30,35,36] were compared with that obtained when the CONPs were coated with glucose, a carbohydrate previously used for the purpose. All XRD patterns of the coated CONPs showed characteristic peaks for CeO_2, and no important differences were observed among levan-, pullulan- or glucose-coated CONPs. Therefore, it could be concluded that coating with carbohydrates did not alter the crystal structure of CONPs.
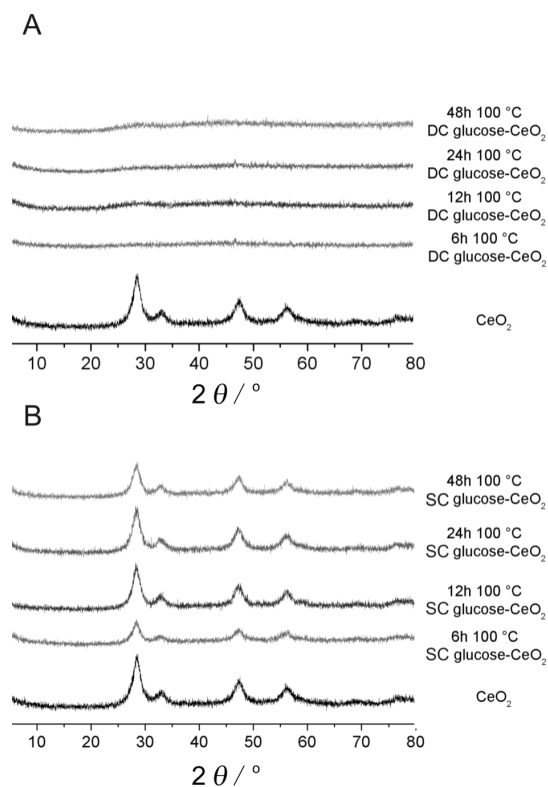

Fig. 2. XRD diffraction patterns of glucose-coated CONPs using: A) the DC and B) the SC method at 100 ºC during 6, 12, 24 and 48 h.

Surface characterization of CONPs

The carbohydrate polymer coating of CONPs was confirmed using FT-IR. In all spectra (Fig. 3) of the prepared uncoated and coated CONPs, a characteristic

broad peak with similar intensity at 3000–3500 cm^{-1}, corresponding to the O–H symmetric stretching vibration of mono- and polysaccharides, was visible. The weak bands at around 2920 and 2850 cm^{-1} correspond to the asymmetric and symmetric stretching vibrations of C–H in CH$_2$ (Fig. S-1 of the Supplementary material to this paper). The signal at about 1627 cm^{-1} suggested the presence of adsorbed water. The spectral region in the range 1200–950 cm^{-1} is considered as the fingerprint region for carbohydrates, and was dominated by ring vibrations. Additionally, a C–O–C vibration overlapped the frequencies of (C–OH) side groups,[37] while an anomeric region was located at 950–750 cm^{-1}.[38] The FT-IR spectrum of uncoated CONPs contained absorptions at similar spectral regions, but with somewhat different intensities.
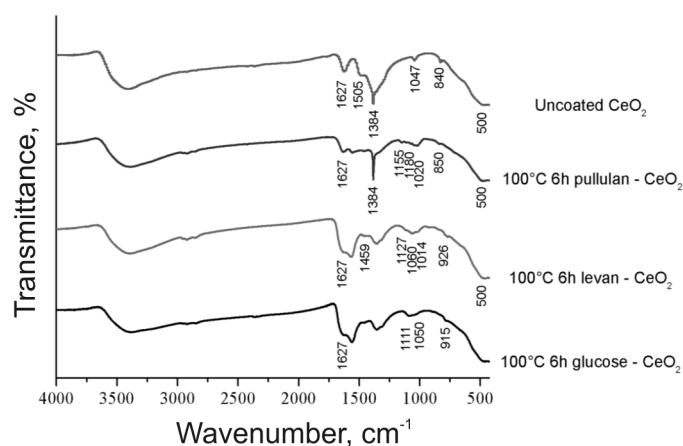

Fig. 3. FT-IR spectra of uncoated and CONPs coated by SC method with glucose, levan and pullulan at 100 °C during 6 h.

In the FT-IR spectrum of the uncoated CONPs (Fig 3), the absorption at about 1630 cm^{-1} was assigned to H–O–H bending vibration of water.[39] The sharp intensive peak at 1384 cm^{-1} corresponded to N–O antisymmetric stretching of residual nitrate groups, $\nu(NO_3^-)$, in the material.[40,41] The absorptions at about 1505 and 1047 cm^{-1} were similar to those for commercial CeO$_2$ powders and CONPs.[42,43] The disappearance of the band at 1505 cm^{-1} in polysaccharide--coated CONPs could be explained by an overlap with a strong band at 1540––1570 cm^{-1}, which may arise from the absorption of atmospheric CO$_2$. Adsorption of CO$_2$ on cerium(IV) oxide was observed and studied.[44,45] The FT-IR spectrum of uncoated CONPs contained additional absorptions at 840 and 810 cm^{-1}, which were typical peaks for Ce–O stretching vibrations, as well as a broad band at about 500 cm^{-1}, which also corresponded to Ce–O bonds.[46]

In the FT-IR spectrum of levan-coated CONPs, the absorption peak at 1060 cm^{-1} with shoulders at 1014 cm^{-1} and 1127 cm^{-1} indicated C–O–C stretching, the peak at 1459 cm^{-1} was attributed to C–H bending, while the absorption at 926 cm^{-1} is characteristic for the presence of the furanose ring of the carbohydrate monomer residues.[47]

The FT-IR spectrum of pullulan-coated CONPs contained absorption bands at 1155, 1107, 1080 and 1020 cm^{-1}, assigned to stretching vibrations of C–O and C–C, and the deformational vibrations of C–C–H and C–OH, respectively.[48] An absorption band at 850 cm^{-1} was characteristic for the α-configuration of the glucose units in pullulan. The sharp peak at 1384 cm^{-1} corresponded to N–O frequencies of residual nitrate groups.[40,41]

The FT-IR spectrum of glucose-coated CONPs showed an absorption band at 1111 cm^{-1}, which is characteristic for C–O frequencies. The band at *ca*. 1050 cm^{-1} evidenced C–O and C–C stretching and the shoulder at 915 cm^{-1} was characteristic for the C–O and C–C–H vibrations of the pyranose ring.[49] Absorption bands between 1300 cm^{-1} and 1460 cm^{-1} indicate the presence of the δ vibrations of the C–O, C–C and C–H bonds of glucose.

FT-IR analysis of the prepared nanomaterials confirmed the formation of CONPs. The spectral patterns seen were typical of carbohydrates, confirming that these carbohydrate molecules had successfully coated, and become integral parts of the CONPs.

SEM and TEM analyses

Typical SEM and TEM images of the glucose-coated CONPs synthesized by the SC method are shown in Fig. 4. The SEM image (Fig, 4A) shows dispersed CONPs agglomerates of different sizes, which could be seen individually in the TEM image (Fig. 4B). The TEM image reveals the typical agglomerate, com-
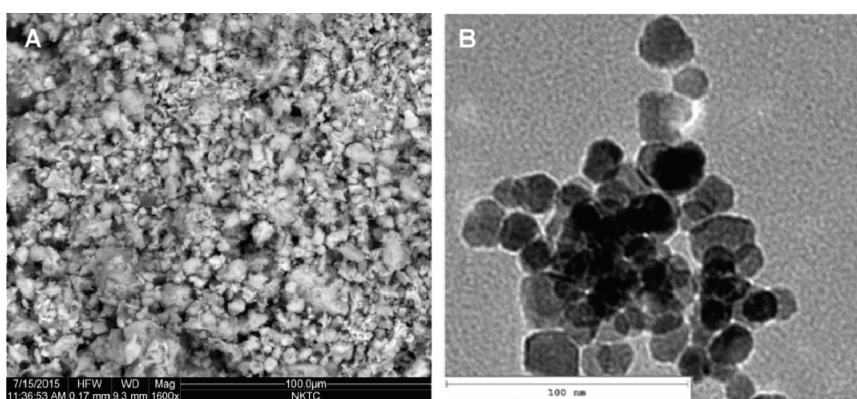

Fig. 4.A) SEM and B) TEM images of glucose-coated CONPs prepared by the SC method at 100 °C for 6 h.

posed of similarly shaped particles, sized 10–20 nm. The glucose coating of CONPs is observed as a white framework/shell surrounding the particles. SEM and TEM confirmed the glucose was intimately bonded with the CONPs, as was also evidenced by the FTIR results.

Stability of CONPs

Turbidimetry measurements. Turbidimetry was used to examine the stability of the CONPs in water. The turbidimetric data of uncoated and CONPs coated with different carbohydrates suspended in water in various concentrations are shown in Fig. 5. The slopes of linearized dependences indicate the stability of the CONPs suspensions resulting from the three carbohydrate types used as coatings. Uncoated CONPs exhibited the lowest stability (*i.e.*, had the smallest slope). Coating with pullulan increased suspension stability. The best result was achieved with levan and glucose coatings, which imparted the highest (and similar) stability (*i.e.*, the largest slope).
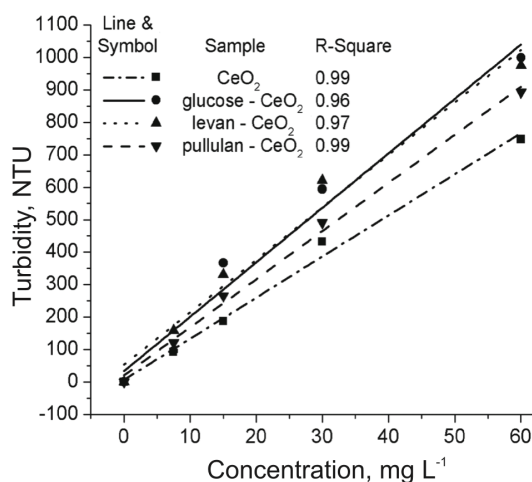

Fig 5. Linearized results of the turbidity measurements of aqueous suspensions of uncoated and CONPs coated with glucose, levan and pullulan at 100 °C during 6 h.

Particle size and zeta potential. To further understand the suspension stabilities of the investigated CONPs, the hydrodynamic size, as well as zeta potential values of the particles, were measured, and the results are given in Table I. The hydrodynamic size, determined by dynamic light scattering (DLS) by application of Stokes–Einstein equation, is that of a sphere that has the same translational diffusion coefficient as the particle being measured, assuming a hydration layer surrounding the particle.[50,51] The *Z*-average particles size (Z_{av}) or *Z*-average mean (also known as cumulant mean) is the primary parameter produced by this technique and, given certain conditions, the most commonly used DLS size parameter for quality control or comparative purposes. Another first order result from DLS is the intensity distribution of particle sizes, especially of

interest when the analyzed system is not monodisperse, but of broader distributions.[50,51] The intensity distribution is naturally weighted according to the scattering intensity of each particle fraction, and is commonly given as Intensity %. In this study, the distribution data, *i.e.*, size of fractions present with their shares to the intensity of the scattered light, are also given in order to provide a better insight into the evolution in particle size in dependence of the carbohydrates used for ceria coating.

TABLE I. Z-average particle size (Z_{av}), intensity distribution of particles size (size of fractions and intensity shares (I)), and zeta potential (ζ) of uncoated and glucose-, levan- and pullulan--coated CONPs

Sample	Z_a / nm	Size of fraction 1, nm	I / %	Size of fraction 2, nm	I / %	ζ / mV
CONPs[a]	385	345	83.8	105	15.5	32.2
Glucose–CONPs	235	235	77.6	61.0	22.4	31.3
Levan–CONPs	216	265	70.0	86.4	30.0	20.8
Pullulan–CONPs	314	306	86.3	84.3	13.7	19.8

[a]A small portion (0.7 %) of larger aggregates (2.75 μm) was also detected

Results, presented in Table I, showed good correlation with the turbidimetry data. Accordingly, the Z_{av} of uncoated CONPs was the largest, pointing to the presence of aggregates that resulted in the suspension of lowest stability. Their size was different: the most abundant (over 80 %) was a 350 nm-particle fraction, followed by a much smaller amount of a *ca.* 100 nm-sized population. A third fraction (not included in Table I) was also detected, revealing aggregates in the micrometric range. Although present in a small quantity (intensity of 0.7 %), huge particles caused an increase in the Z_{av} value, *i.e.*, with a size greater than the size of fraction 1. As an intensity-based calculated value, the Z_{av} value is sensitive to even small changes in the sample, *e.g.*, the presence of a small proportion of agglomerates, and thus will often tend towards the larger end when bigger particles are present.[50–52] A similar issue was observed for the pullulan-coated CONPs. Coating with carbohydrates reduced the size of the aggregates in all cases. A similar effect was also reported upon coating CONPs with heparine.[1]

The carbohydrates used in this study differed in terms of electrical charge and size, with both parameters playing a role in the stability of the particles in suspension. As seen in Table I, the uncoated CONPs carried a positive charge, which is at the edge of stability (a suspension is considered stable when $|\zeta| > 30$ mV). Glucose, the smallest molecule among the three used carbohydrates, is practically uncharged. Thus, the surface charge of CONPs was undisturbed when small glucose molecules penetrated its clusters, breaking them apart, which resulted in formation of smaller aggregates. In contrast, levan and pullulan both contain some negative charge, which is compensated by the positive charge on

the CONP surface, decreasing the ζ value compared to that of the uncoated CONPs. Those two molecules could impart stability to their respective CONP suspensions by steric hindrance: the levan molecule is bigger (*ca.* 150 nm) *versus* the smaller size-pullulan (60–120 nm), and thus, the stabilizing effect was more pronounced with levan, resulting in the presence of a greater quantity of smaller particles. Bondarenko *et al.*[51] also reported that the interaction of levan with other NPs led to a decrease in ζ, even to the extent of charge reversal; nevertheless the stability of the NPs was improved. The interaction was claimed to be guided by non-covalent physical adsorption.

Similar values of Z_{av} were determined for the CONPs coated with glucose and levan (Table I). The Z_{av} values showed good correlation with the turbidimetry data. The Z_{av} data, therefore, indicated the inherent stability of these suspensions, confirming the results of turbidimetry.

CONCLUSIONS

In this study, it was demonstrated that DC method should be modified in order to avoid obtaining amorphous CONPs samples at all the aforementioned synthesis conditions. For this reason, the SC method was proposed, providing a successful coating of CONPs with carbohydrates at 100 °C during 6–48 h. The XRD results confirmed that the SC method was suitable for the coating of CONPs and no undesirable effect on the CONP structural properties was observed. However, the FTIR spectra showed surface differences among the CONPs coated with different carbohydrates. Based on turbidity and zeta potential measurements, the carbohydrate-coated CONPs were more stable in water compared to the uncoated CONPs; the CONPs coated with levan and glucose were more effective than those coated with pullulan. The Z_{av} results showed that coating with carbohydrates reduced the size of the CONPs aggregates in all cases.

All results indicated that 100 °C and 6 h were the most suitable conditions for coating CONPs with carbohydrates by SC method in terms of all the tested parameters, due to the cost/time effectiveness and a minimal possibility to change their physicochemical properties. This is essential for their biological activity and further application. However, since there is an increasing quantity of CONPs in the environment due to their more frequent application, there is a need to estimate the risk of CONP exposure to the environment and to human health. Future research will be focused on these problems.

SUPPLEMENTARY MATERIAL

ИЗВОД

ПОБОЉШАЊЕ СТАБИЛНОСТИ НАНОЧЕСТИЦА ОКСИДА ЦЕРИЈУМА ОБЛАГАЊЕМ МИКРОБНИМ ПОЛИСАХАРИДИМА

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Церијум-оксид (CeO_2) наночестице (CONPs) су интересантан биоматеријал чија је разноврсна примена у биомедицини, козметици и фармацеутској индустрији ограничена због нестабилности у воденој средини. Циљ овог истраживања је добијање CONPs веће стабилности облагањем. Микробни егзополисахариди (леван, пулулан) и глукоза су коришћени за синтезу CONPs при различитим условима. Испитано је облагање додатком угљених хидрата током (директно облагање) или након (накнадно облагање) синтезе CONPs. Добијене наночестице су окарактерисане рендгенском дифракцијом (XRD), инфрацрвеном спектроскопијом са Фуријеовом трансформацијом (FT-IR), скенирајућом електронском микроскопијом (SEM) и трансмисионом електронском микроскопијом (TEM). Стабилност суспензије необложених и обложених CONPs у воденој средини је испитана мерењем хидродинамичке величине, цета потенцијала и турбидитета. FT-IR спектри су открили разлике међу обложеним CONPs и показали да је накнадно облагање угљеним хидратима успешно. Облагање је побољшало стабилност суспензије CONPs смањењем величине агрегираних честица. Суспензије CONPs обложених леваном и глукозом су имале највећу стабилност. CONPs у овом раду су припремљене коришћењем нетоксичних материјала, који су потпуно безопасни по животну средину. Добијени резултати указују на нове могућности за синтезу CONPs омогућавајући њихову ширу биолошку примену.

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