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Enhancement of hydrosolubility and *in vitro* antiproliferative properties of chalcones following encapsulation into β -cyclodextrin/cellulose-nanocrystal complexes.

Benjamin Rioux^a, Christelle Pouget^{a,*}, Gautier M.A. Ndong-Ntoutoume^b, Robert Granet^b, Aurélie Gamond^a, Aurélie Laurent^a, Aline Pinon^a, Yves Champavier^{a,c}, Bertrand Liagre^a, Catherine Fagnère^a and Vincent Sol^b.

^a Laboratoire PEIRENE EA 7500, Université de Limoges, Faculté de Pharmacie, rue du Dr Marcland, 87025 Limoges Cedex, France.

^b Laboratoire PEIRENE EA 7500, Université de Limoges, Faculté des Sciences et Techniques, 123 avenue A. Thomas, 87060 Limoges Cedex, France.

^c BISCEM NMR platform, GEIST, Université de Limoges, 2 rue du Dr Marcland, 87025 Limoges Cedex, France.

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ABSTRACT

This paper describes the preparation of two chalcone/ β -cyclodextrin/cellulose-nanocrystals complexes and the study of their antiproliferative activities against two colorectal and two prostatic cancer cell lines. The aim of this work was to enhance hydrosolubility of chalcones thanks to the hydrophilic character of cellulose nanocrystals. These latter were linked, through ionic interactions, to a cationic derivative of β -cyclodextrins whose lipophilic cavity allowed the encapsulation of hydrophobic chalcones: 3-hydroxy-3',4,4',5'-tetramethoxychalcone (**1**) and 3',4,4',5'-tetramethoxychalcone (**2**). First, we showed that encapsulation allowed hydrosolubilization of chalcones. Then, chalcone/ β -cyclodextrin/cellulose-nanocrystals complexes demonstrated enhanced *in vitro* antiproliferative activities, compared to the corresponding free-chalcones.

Cancer, which is characterized by its high incidence and mortality, is a major health hindrance; an estimated 18.1 million new cancer cases and 9.6 million cancer deaths occurred in 2018 worldwide.¹ Despite the availability of various chemotherapeutic protocols, associated acute side effects of the clinically used anticancer compounds (including the most recently introduced drugs) are still significant obstacles to effective cancer chemotherapy.^{2,3,4} Consequently, discovery and development of new effective anticancer agents and/or therapies remain the major goal of medicinal chemists.

Most anti-cancer agents suffer from a lack of specificity, since they also attack healthy cells which rapidly divide (cells from oral mucosa, hair follicles, bone marrow, etc.), and then generate adverse side effects. Moreover, anticancer agents are also characterized by poor water solubility and low bioavailability. To overcome these problems, many research groups have designed nano-sized drug delivery systems. Polymeric nanocarriers are the most widely investigated nanotechnology platform for cancer therapy, due to their easy chemical modification. According to our projects on drugs vectorization for anticancer applications, we have developed cellulose nanocrystals (CNCs) which can specifically target tumors, through the Enhanced Permeation and Retention (EPR) effect, and destroy them by action of the

vectorized drug.⁵ In this way, Ndong- Ntoutoume *et al.* have demonstrated the efficiency of curcumin I included into β -cyclodextrins/CNCs complexes, on colorectal and prostatic cancer cells.⁶

CNCs are obtained after sulfuric acid-hydrolysis of cotton fibers that results in the removal of the amorphous regions of cellulose. CNCs are rod-like nanoparticles, 100-200 nm long, 10-20 nm wide and 5-10 nm thick. They gather several interesting characteristics: uniform nano-rod shape, good mechanical strength, high specific surface area, biocompatibility and biodegradability. Another great advantage of these cellulose nanocrystals is the negative charges (O-sulfate ester functions present on their surface) which enhanced their hydrophilic character and, above all, allow the formation of ionic complexes with cationic compounds, in our case, a cationic derivative of a β -cyclodextrin (β -CD). β -CD are cyclic oligosaccharides made of seven D-glucopyranose units linked by α -(1,4) glycosidic bonds. Cyclodextrins are well known to form inclusion complexes with guest hydrophobic molecules caged into their lipophilic inner cavity while their surface is sufficiently hydrophilic to endow cyclodextrins with water solubility.⁷

Chalcones belonging to the flavonoid family, received significant attention due to their wide range of anticancer

* Corresponding author.

E-mail address: christelle.pouget@unilim.fr

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activities, including antimetabolic properties, *via* disruption of the tubulin-microtubule system,⁸⁻¹¹ inhibition of NF- κ B activation¹² or induction of apoptosis.¹³

Nevertheless, in some cases, the interesting properties of some chalcones are hindered by a poor solubility and a low bioavailability owing to their hydrophobicity. Our encouraging results relative to the encapsulation of curcumin into β -CD/CNCs complexes prompted us to vectorize two chalcones (chalcones **1** and **2**, Figure 1) which, according to literature data and our own results, already have demonstrated potential anticancer effects. These two chalcones possess a trimethoxyphenyl ring which has been found to be of great interest for anticancer activity.¹⁴⁻¹⁶ Chalcone **1** (3-hydroxy-3',4,4',5'-tetramethoxychalcone) was shown to possess a strong antiproliferative activity against K562, A549 and MCF-7 cell lines^{15, 17} as well as against colon (HCT-116 and HT-29) and prostatic (PC-3 and DU145) cancer cells.^{18, 19} Chalcone **2** (3',4,4',5'-tetramethoxychalcone) was found to inhibit the growth of ovarian cancer cells²⁰ and we also demonstrated its antiproliferative activity against colorectal cancer cells (HT-29 and HCT-116) and prostatic cancer cells (PC-3 and DU-145).¹⁸

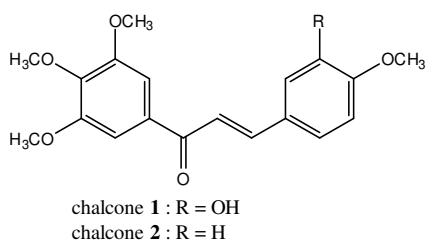
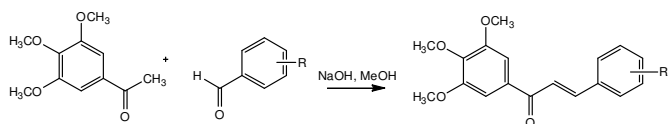


Figure 1. Chemical structures of chalcones **1** and **2**.

In the present paper, we describe the encapsulation of chalcones **1** and **2** into β -CD/CNCs complexes and we compare the antiproliferative properties of these complexes with those of the corresponding free chalcones; biological tests were performed on colon (HT29 and HCT-116) and prostate (PC3 and DU145) cancer cell lines.

The overall synthetic pathway is described in Schemes 1-3. Firstly, chalcones **1** and **2** were prepared using the Claisen-Schmidt condensation (Scheme 1) from building-blocks, namely 3,4,5-trimethoxyacetophenone and the appropriate benzaldehydes. ¹H NMR spectra showed that the (*E*)-stereoisomers were specifically generated since the coupling constant between the two ethylenic protons was about 15-16 Hz. Chalcone **2** was obtained from 4-methoxybenzaldehyde in 55% yield while chalcone **1** was prepared in a good yield (69%) from 3-hydroxy-4-methoxybenzaldehyde.

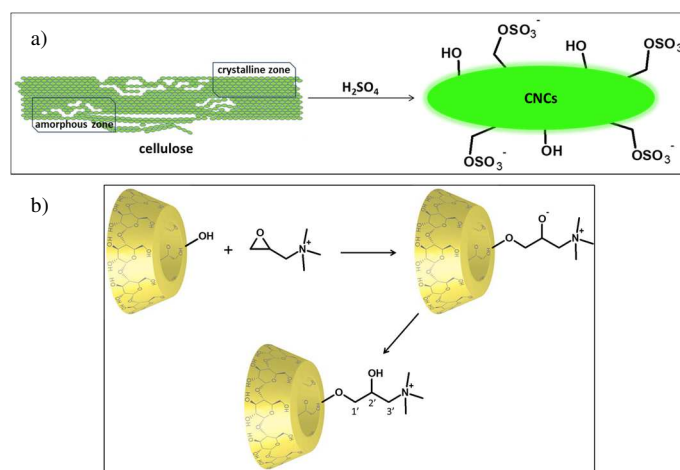


Scheme 1. Synthesis of chalcones **1** and **2** *via* Claisen-Schmidt condensation.

CNCs preparation was achieved by classical sulfuric acid-hydrolysis (Scheme 2a) and characterization data match the results obtained by Ndong-Ntoutoume *et al.*⁶ The zeta potential of CNCs, -51 mV, confirmed the presence of O-sulfate ester functions (Figure S1). A transmission electronic microscopy (TEM) image of cellulose nanocrystals is shown in Figure 2; nanoparticles (100–200 x 10–20 nm) were observed after uranyl acetate staining.

In parallel, cationic β -CD were obtained through reaction of glycidyltrimethyl ammonium chloride (GTMAC) with β -CD in alkaline aqueous medium (Scheme 2b) according to the

Chisholm and Wenzel procedure.²¹ Purification of cationic β -CD was performed by dialysis against deionized water (MWCO: 600 Da). Chisholm and Wenzel demonstrated that the best inclusion capacity of cationic β -CD was reached for a degree of substitution (DS) slightly superior to 1. In the present study, DS is defined as the number of GTMAC units *per* anhydroglucose unit of cyclodextrin. An average DS of 1.7 was calculated from NMR data as the following ratio: integral of the H1 (anomeric proton) of the glucose unit / integral of H2' proton of the grafted chain (Scheme 2b and Figure S2a). The presence of quaternary ammonium functions on cationic β -CD was revealed by FTIR spectroscopy, through observation of stretching vibration bands at 1478 cm^{-1} and 988 cm^{-1} (Figure S2b).



Scheme 2. a) Synthesis of CNCs; b) Synthesis of cationic β -CD.

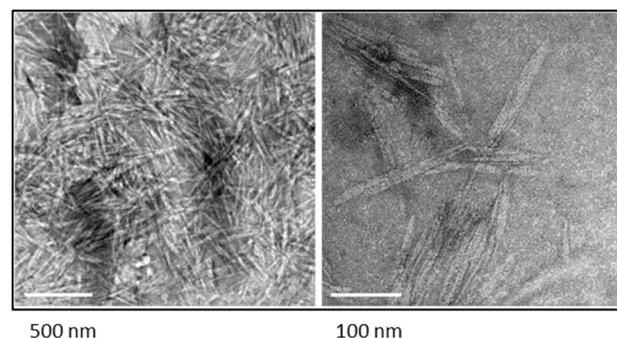


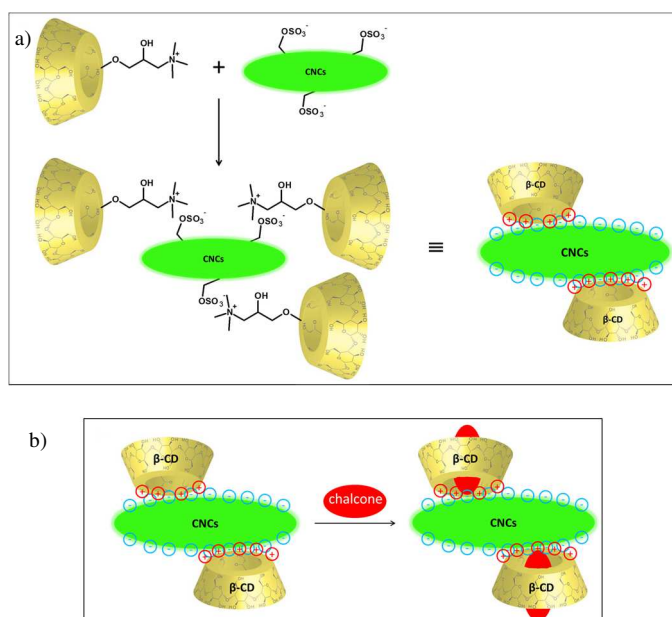
Figure 2. TEM image of cellulose nanocrystals

Then, cationic β -CD were brought into contact with cellulose nanocrystals in aqueous medium (Scheme 3a), under ultrasonic stirring. β -CD/CNCs complexes, resulting from an electrostatic coupling, were centrifuged at 13,000 rpm for 10 min and thus separated from free cationic β -CD. FTIR (KBr disc) spectra of β -CD/CNCs and CNCs differ by the presence of a new signal at 1478 cm^{-1} corresponding to the $-\text{N}^+(\text{CH}_3)_3$ group of cationic β -CD (Figure S3). A significant increase of zeta potential was also observed due to the positive charge of cationic β -CD: -41 mV for β -CD/CNCs vs -51 mV for CNCs (Table 1).

Table 1. Size (DLS), polydispersity index (PDI) and zeta potential (ζ) characteristics of CNCs intermediates and final β -CD/CNCs/drug complexes in deionized water.

CNCs Type	DLS (nm)	PDI	ζ -potential (mV)
CNCs	141.5 \pm 0.2	0.239 \pm 0.015	-51.0 \pm 0.7
β -CD/CNCs	198.1 \pm 2.1	0.122 \pm 0.018	-41.0 \pm 1.1
β -CD/CNCs/1	206.8 \pm 1.6	0.151 \pm 0.013	-29.2 \pm 1.8
β -CD/CNCs/2	205.8 \pm 0.9	0.141 \pm 0.021	-30.1 \pm 1.5

These β -CD/CNCs complexes, obtained as an opalescent aqueous suspension, were very stable, due to the repulsion between the nanocrystal particles which bear the negatively charged O-sulfate ester functions (Schemes 2 and 3). Therefore, they were ready for the inclusion of non-polar drugs, i.e. chalcones **1-2**, into the β -CD hydrophobic cavity. Acetone solutions of chalcones were mixed with β -CD/CNCs suspensions (Scheme 3b), and β -CD/CNCs/chalcone complexes were purified by centrifugation. These complexes were obviously much more soluble in water than free chalcones as shown in Figure 3. Finally, the loading of β -CD/CNCs with chalcones **1** and **2** increased, at the same time, the size of the complexes and ζ -potential (Table 1).²²



Scheme 3. a) Formation of β -CD/CNCs complexes via electrostatic coupling between CNCs and cationic β -CD. b) Inclusion of chalcones **1** and **2** into β -CD/CNCs complexes.

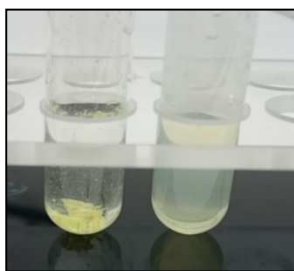


Figure 3. Chalcone **1** on the left and β -CD/CNCs/chalcone **1** complex on the right, both in aqueous medium.

Loading ratios of drug into β -CD/CNCs were evaluated by UV-Visible titration, for each one of the two chalcones (Table 2): a small volume of β -CD/CNCs/chalcone was taken and the drug was extracted by addition of chloroform. Then, concentration of the chalcone in the organic phase was determined by a UV-Visible titration.

Table 2. Loading ratios of chalcones into β -CD/CNCs complexes.

Complexes	Loading ratio (w/w)	Chalcone-loaded β -CD/CNCs (mol.L ⁻¹)
β -CD/CNCs/1	13.5%	$7.84 \cdot 10^{-4}$
β -CD/CNCs/2	23.3%	$1.41 \cdot 10^{-3}$

Cell viability of cancer cell lines was evaluated in presence of this nanoformulation. We first checked that β -CD/CNCs nanocarriers alone only slightly reduced cell proliferation (Figure 4). Then, cell lines were incubated 48 h in presence of chalcones, either complexed or free, in the 0.05-50 μ M concentration range. IC₅₀ values are presented in Table 3. We evaluated the cytotoxic properties of the complexed chalcones and compared the results with those obtained for equivalent concentrations of free chalcones to show the interest of the encapsulation (Figure 5).

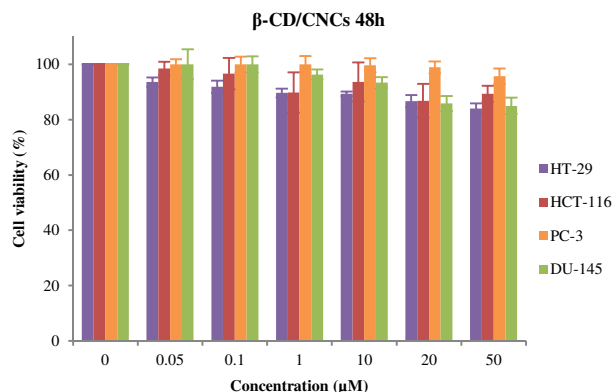


Figure 4. Effect of β -CD/CNCs complexes on cancer cells proliferation.

Table 3. IC₅₀* values (μ M) from MTT assay for β -CD/CNCs/chalcones complexes and free chalcones **1** and **2**.

Drugs	HT-29	HCT-116	PC-3	DU-145
β -CD/CNCs	ND**	ND**	ND**	ND**
β -CD/CNCs/1	13.98 ± 0.74	0.83 ± 0.01	0.77 ± 0.05	0.72 ± 0.02
1	27.8 ± 1.7	0.8 ± 0.1	0.10 ± 0.01	6.3 ± 1.2
β -CD/CNCs/2	4.5 ± 0.5	8.9 ± 1.0	6.3 ± 1.5	7.9 ± 0.4
2	7.4 ± 0.6	10.7 ± 0.6	7.8 ± 0.5	13.8 ± 1.7

* IC₅₀ values are the average of three determinations at least.

** IC₅₀ undetermined, cell viability is superior to 84% at 50 μ M.

Samples of β -CD/CNCs/chalcone complexes were studied in water while free chalcone solutions had to be made in DMSO. This confirmed hydrosolubilization of chalcones through encapsulation into β -CD/CNCs complexes.

The β -CD/CNCs/1 complex presents the lowest IC₅₀ in this series. When tested against HT-29 cells, the IC₅₀ value is almost divided by 2 ($13.98 \pm 0.74 \mu$ M for β -CD/CNCs/1 vs $27.8 \pm 1.7 \mu$ M for **1**). Against DU-145 cells, the IC₅₀ is approximately 9-fold lower than for **1** ($0.72 \pm 0.02 \mu$ M vs $6.3 \pm 1.2 \mu$ M). Concerning HCT-116 cells, IC₅₀ values for the complex and the free chalcone are identical (0.83μ M). IC₅₀ against PC-3 cells only is not improved, but nevertheless remains less than 1μ M ($0.77 \pm 0.05 \mu$ M vs $0.10 \pm 0.01 \mu$ M). Therefore, encapsulation of chalcone **1** into β -CD/CNCs proves to be a relevant choice since it makes it possible to significantly improve its antiproliferative potential on HT-29 and DU-145 cells, while preserving the antiproliferative activity on HCT-116 and PC-3 cells.

Concerning the β -CD/CNCs/2 complex, the IC₅₀ values are slightly lower than those of chalcone **2** against the four cell lines studied. As for chalcone **1**, we can say that encapsulation of this chalcone by the β -CD/CNCs nanoformulation is a relevant strategy to improve the antiproliferative effect.

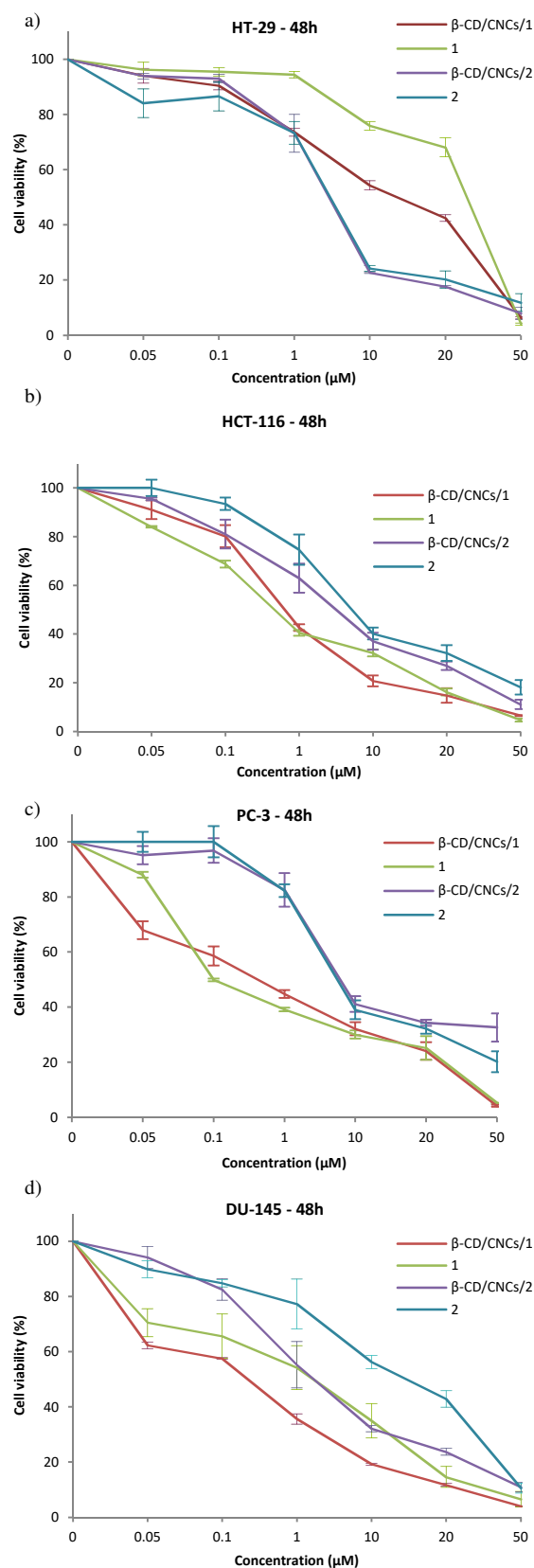


Figure 5. *In vitro* antiproliferative effect of free chalcones and β -CD/CNCs/chalcones complexes against HT-29 (a), HCT-116 (b), PC-3 (c) and DU-145 (d) cancer cell lines after 48 h treatment.

In conclusion, we described the preparation of β -cyclodextrin/cellulose nanocrystals complexes, stabilized by ionic interaction and able to encapsulate hydrophobic chalcones which were selected for their antiproliferative potency. The first aim of the chalcone hydrosolubilization was achieved through

these β -CD/CNCs/chalcone complexes, which allowed biological evaluation in aqueous medium. Then, β -CD/CNCs were found to be suitable for efficient drug loading and release, as described in our previous studies.^{5, 6} Loading ratio was around 20% for the two complexes. Then, β -CD/CNCs/chalcone complexes demonstrated *in vitro* antiproliferative effect against colorectal and prostatic cancer cell lines. As β -CD/CNCs alone were shown to have no effect on cell proliferation, the antiproliferative activity was due to the release of drugs from the encapsulated form. Considering chalcones **1** and **2**, the use of nanocarriers enhanced the antiproliferative effect observed with corresponding free chalcones. As demonstrated by Ndong-Ntoutoume *et al.*, encapsulation may increase internalization of hydrophobic chalcones into cancer cells.⁶ *In vivo* studies have to be performed to evidence the role of these nanocarriers in the targeting of tumor cells *via* the Enhanced Permeation and Retention (EPR) effect. The use of these complexes (β -CD/CNCs/chalcone) may therefore be a relevant strategy in cancer therapies.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/xxxxxxxxxxx>.

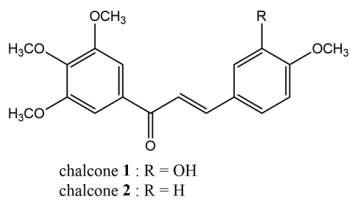
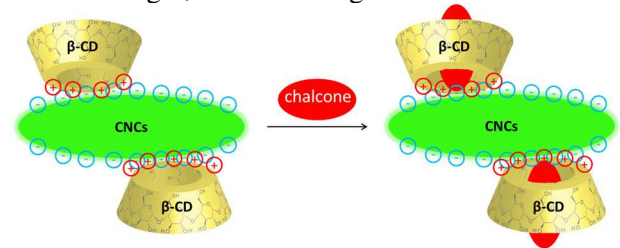
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