Surface modification of nanocellulose through carbamate link for a selective release of chemotherapeutics

Surface Modification of Nanocellulose through Carbamate Link for a Selective Release of Chemotherapeutics

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Abstract

Herein we report the synthesis of cellulose nanocrystals covalently bound to a model chemotherapeutic drug (DOXO) via a novel spacer arm, which acts both as linker and as selective releasing agent. The carbamate linkage present in the linker, shows stability in aqueous environments for a wide range of conditions and can only be hydrolyzed in the presence of cells, freeing the active drug, with unmodified chemotherapeutics properties.

Keywords

Cellulose nanocrystals, carbamate linker, doxorubicin, selective release.

Introduction

Drug delivery is an important challenge in therapeutics. Setting up new formulations, technologies, and systems for properly achieving the delivery of pharmaceutical compounds is highly required

(Locatelli et al. 2014; Grünwald et al. 2016). In the past years, this field of research has increased in terms of studies and publications, mostly focused on polymer-based nanocarriers (Parhi et al. 2012; Sun et al. 2014).

Nanomaterials modification for the introduction of active agents onto their surface is the first requisite for creating an effective drug delivery system (Locatelli et al. 2015). Surface modification of nanomaterials with links and spacers has been the key for the introduction of active agents, such as proteins for targeted drug delivery, but it is also a powerful strategy to link on the drug' surface that should be selectively delivered: this is, nowadays, a topic in continuous development (Cellante et al. 2018).

Cellulose is the most abundant biopolymer in the world and, in its nanometric form, has wide potential as a component in bio nanocomposites. The use of nanocellulose in drug delivery systems is quite recent: until 2014 nanocellulose was mostly employed for reinforcing the structure of biomaterials (Dufresne 2013; Fatah et al. 2014; Gazzotti et al. 2019; Peres et al. 2019). After 2014, advancements on its chemical processing have strongly pushed the research in biomedical engineering, also due to the low cost of the starting material and the tunable properties of the final nanosystems (Habibi 2014; Khine et al. 2020).

One potential and partially explored application of nanocellulose may be in cancer therapy, as nanocarrier for drug transport and release, since its sugar-based backbone allows for easy chemical modification and for a total biocompatibility, absence of cyto- and systemic toxicity, and easy excretion via cell digestion (Lin and Dufresne 2014). Anyways, there are still many issues to be addressed: indeed, the main challenge remains the development of strategies for a controlled surface modification in order to link or incorporate a drug in a stable and reproducible way and, most important, to have a long and controlled release over time.

Dash et al. in 2012 worked on cellulose nanocrystals (CNCs) for a new 'alternative' drug delivery system: with the help of aromatic linkers and spacer molecules, they tried the attachment of active amine-containing drugs to the CNCs (Dash and Ragauskas 2012). However, only the introduction of a spacer arm was completed, but neither the creation of a cleavable linker nor the incorporation of the drug was attempted.

Carbamates, the esters of carbamic acid, are well known in organic chemistry, especially for their presence in amine-protecting group such as tert-butyloxycarbonyl (Boc), fluorenylmethoxycarbonyl (Fmoc) and carboxybenzyl (Cbz). Due to their hydrolytic stability, carbamates have been used in the design of prodrugs in order to achieve prolonged systemic circulation (Ghosh and Brindisi 2015). Conversion of carbamate prodrugs in body requires the presence of specific enzymes (e.g. esterase) for the release of the parent drug. Upon hydrolysis, carbamate esters release the corresponding alcohol and carbamic acid, which, due to its chemical instability, immediately breaks down to the corresponding amine, thus releasing the free drug and carbon dioxide.

Achieving the release of payloads at the target sites in a spatial- and/or time-controlled manner has been reviewed as the real peculiarity of advanced controlled drug delivery systems (DDSs), which can effectively reduce the dosage frequency, while maintaining the drug concentration in targeted organs/tissues for a longer period of time. This became of the utmost importance when applying therapeutics with high toxicological side effects, such as chemotherapeutics. The local, prolonged and smart release of drugs is the only possibility in future for personalized sustained and sustainable therapies, and the research of novel carriers, such the one proposed in this paper, is of tremendous demanding (Liu et al. 2016).

Inspired by these medical needs and by the above mentioned data, in this paper we report the linkage of an amino-containing model drug (Doxorubicin, DOXO) onto CNCs via the surface modification of the nanosystem and the creation of a linker that can be easily shortcut by cellular enzymes, finally releasing the active molecules unaltered. Several release conditions were investigated finding an extreme stability of the linkers. Indeed, the release can only happen by an enzyme-cleaved initiation step to separate the linker from the drug and, through decomposition of the linkers, to deliver the unmodified drug (Casey Laizure et al. 2013). With our strategy the drug appears fully active after the release and its efficacy has been proved *in vitro* by cells death assessing.

Results and Discussion

Synthesis and Chemical Modification

The nanocarrier was synthetized using pure cellulose filter paper as starting material. This has been digested in acidic environment to produce cellulose nanocrystals, which have been chemically modified on their surface to stably bind the active drug via a series of 5 consecutive reactions (**Fig. 1**).

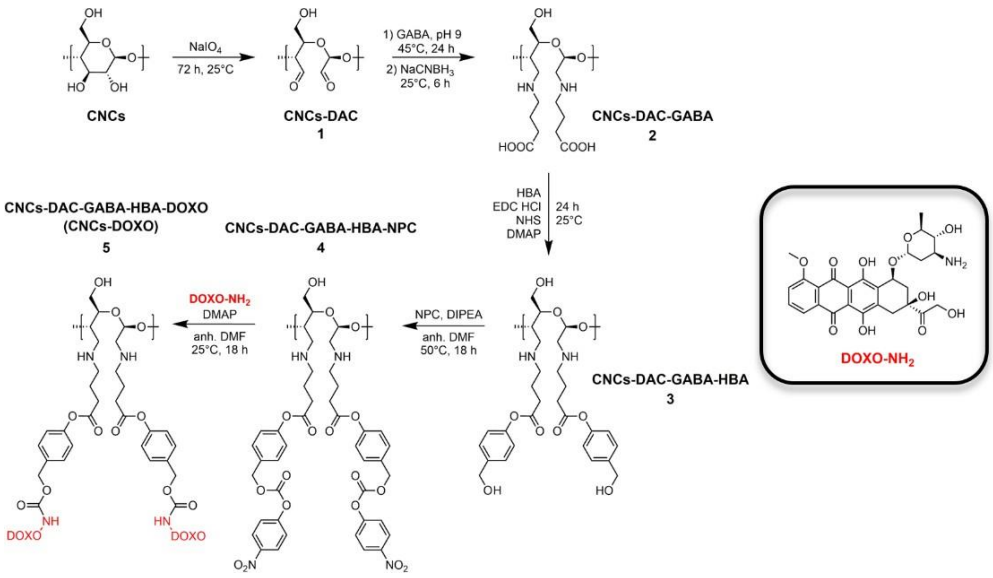


Fig. 1. Schematic representation of the synthetic step for the preparation of Doxorubicin-loaded CNCs. Abbreviations: CNCs = cellulose nanocrystals, DAC = cellulose dialdehyde, GABA = γ-amino butyric acid, HBA = 4-hydroxy benzyl alcohol, EDC HCl = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, NHS = N-hydroxy succinimide, DMAP = 4-dimethylamino pyridine, NPC = 4-nitrophenyl chloroformate, DIPEA = N,N-diisopropyl-N-ethylamine, anh. DMF = anhydrous dimethylformamide, DOXO-NH_2 = doxorubicin

Cellulose nanocrystals (CNCs) were prepared according to a reported procedure, with slight modifications (Jiang et al. 2010). The DLS (Dynamic Light Scattering) analysis of hydrolyzed CNCs revealed a ζ-potential value of -11.0 mV as expected for fully hydroxylated sugars-based structures (Mahouche-Chergui et al. 2014). The IR spectrum is reported in **Fig. 2a**.

The so-obtained CNCs have been then treated with the strong oxidizing agent $NaIO_4$, so as to lead to the opening of the sugar ring with selective rupture of the σ C_2-C_3 bond and to the introduction of two aldehydic residues. The resulting product, dialdehyde cellulose (CNCs-DAC, **1**), showed an unmodified ζ-potential value of -11.2 mV, in accordance with the introduction of non-charged functional groups. The IR analysis (**Fig. 2b**) revealed the presence of a signal (even if with poor intensity) at 1734 cm^{-1} that can be attributed to the C=O of the aldehydes. The low intensity of the carbonyl stretching absorption can be related to the equilibrium that exist in water between aldehydes and geminal diols, catalyzed by both acidic and alkaline conditions, and by the occurrence of intramolecular hemiacetals (Buschmann et al. 1982). Moreover, the aldehyde content of periodate-oxidized CNCs was determined to be as high as 0.15 mmol of aldehydes per gram of CNCs-DAC, revealing extensive oxidation of CNCs surface.

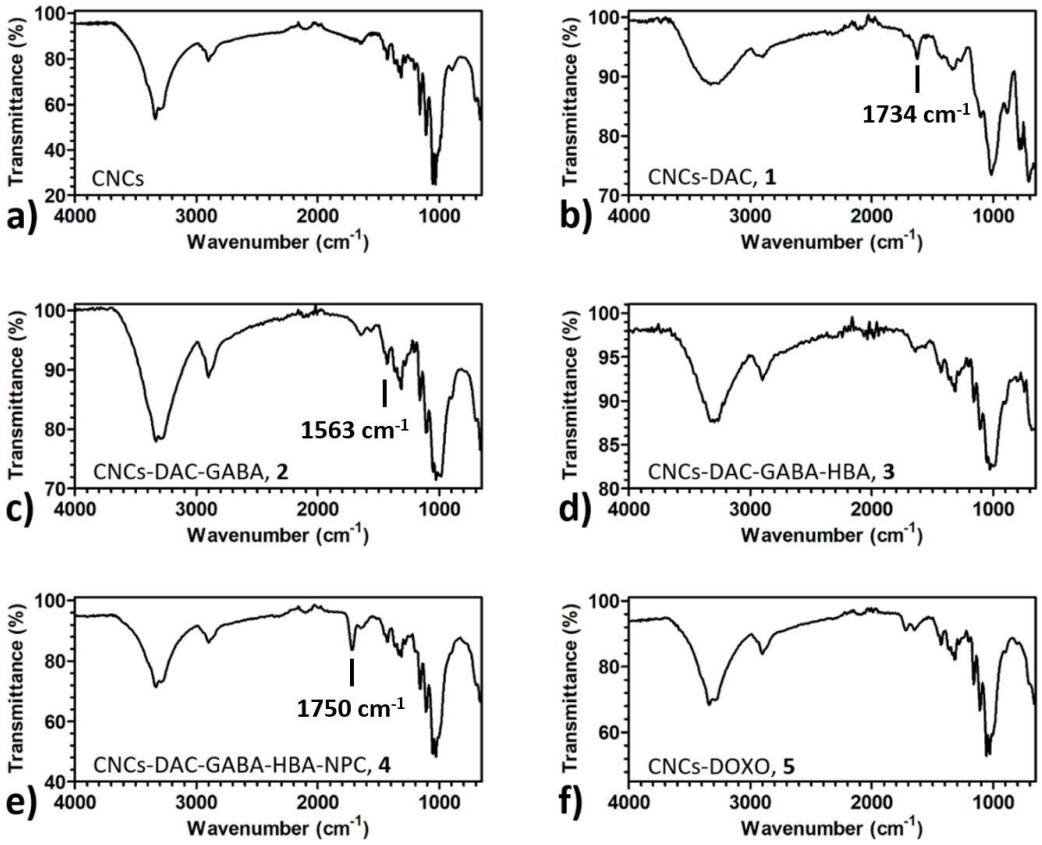
CNCs-DAC has been then coupled with γ-aminobutyric acid (GABA) through a reductive amination in presence of the reducing agent sodium cyanoborohydride ($NaCNBH_3$): in this reaction, each of the CNCs aldehyde groups react with the amino group presents in the GABA, thus forming an imine

(Schiff base), which is then reduced in the second step, linking the GABA residues to the cellulose backbone through stable amino groups. The obtained product (CNCs-DAC-GABA, **2**) is then purified and characterized: the ζ-potential value has been found equal to - 31.6 mV, which agrees with the introduction of carboxylic groups that are generally deprotonated at neutral pH and able to confer a high negative surface charge. The FTIR analysis (**Fig. 2c**) showed the disappearance of the signal attributed to the aldehyde groups at 1734 cm^{-1} and the appearance of a new signal at 1563 cm^{-1} related to the stretching of N-H bonds.

At this point, the nucleophilic substitution with the aromatic linker 4-hydroxybenzyl alcohol (HBA) has been carried out in presence of the coupling agent 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDC·HCl) that allows the esterification reaction to proceed in water. The obtained CNCs-DAC-GABA-HBA, **3** presents a ζ-potential value of -15.2 mV, in agreement with the disappearance of -COOH groups in favor of less charged hydroxyl groups. The FTIR analysis (**Fig. 2d**) showed little or no variation in comparison to the previous one.

Dash and Ragauskas described the synthesis until this step, but nothing more was attempted for the actual linkage of a drug to CNCs (Dash and Ragauskas 2012). In order to proceed with the functionalization of CNCs and the creation of a cleavable linker, an acylation reaction with 4-nitrophenylchloroformiate was performed in anhydrous DMF. The product (CNCs-DAC-GABA-HBA-NPC, **4**) presents now a carbonate group, suitable for nucleophilic substitution with amino groups. Since this functional group tends to hydrolyze in aqueous environments, the HBA-linked CNCs has been preventively freeze-dried so as to remove most of the water before dissolution in DMF. ζ-potential analysis was not performed because of this inconvenience. Anyways, the FTIR analysis (**Fig. 2e**) showed an intense signal appearing at 1750 cm^{-1} due to the carbonate group, thus confirming the reaction's success.

Finally, an amino containing drug could be loaded to CNCs by nucleophilic substitution on the carbonate group on the linker (**Fig. 2f**). As a model drug, Doxorubicin was selected since it contains a single amino group able to react with the linker, and it produces a strong fluorescence that allows for its easy determination and quantification. The selected solvent for the coupling reaction remains anhydrous DMF, but the final product (CNCs-DAC-GABA-HBA-DOXO or CNCs-DOXO, **5**) can be re-dispersed in water after purification, due to the creation of water-stable carbamide bonds. A strong red coloration easily indicates that the reaction proceeds in the desired way and that Doxorubicin has been linked to CNCs. Fluorometric analysis revealed a Doxorubicin loading equal to 0.25% w/w, which is a satisfactory result since only the surface of CNCs can be exploited for functionalization, while the entire core of the nanosystem remains unaffected by the reactions.



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152 **Fig. 2**. ATR-FTIR analysis of the modified cellulose products. CNCs (a); CNCs-DAC, **1** (b); CNCs-
153 DAC-GABA, **2** (c); CNCs-DAC-GABA, **3** (d); CNCs-DAC-GABA-NPC, **4** (e) and CNCs-DOXO,
154 **5** (f)

155

156 The mechanism of drug release by this carbamate linker was also discussed by Dash et al., who
157 theoryzed the possible drug release via 1-6 elimination of the 4-hydroxy benzyl alcohol. This would
158 lead to the release of DOXO-NH-CO-O-CH2PhOH rather than DOXO-NH_2, with unknown effects
159 on the drug efficacy. In order to demonstrate the efficient release of the pristine drug, we
160 analyzed by ^{1}H-NMR the chemical nature of the released species at different pH
161 (**Supplementary Fig. 2**). At pH 12, DOXO-NH2 was released without the presence of any sign
162 of 1-6 elimination of the 4-hydroxy benzyl alcohol, therefore demonstrating the integrity of the
163 released drug by cleavage of the carbamate bond.

164 The crystallinity of CNCs was confirmed to be unchanged during the conjugation steps by applying
165 XRD on freeze-dried CNCs-DOXO compared to pristine CNCs (**Fig. 3** and **Supplementary Table**
166 **2**). This analysis revealed no variation in the crystallinity of CNCs after the formation of the DOXO
167 linkage.

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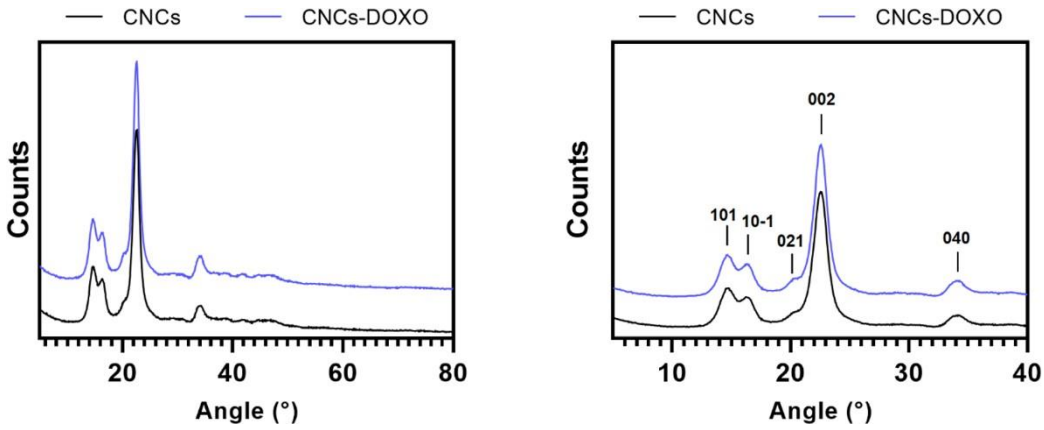
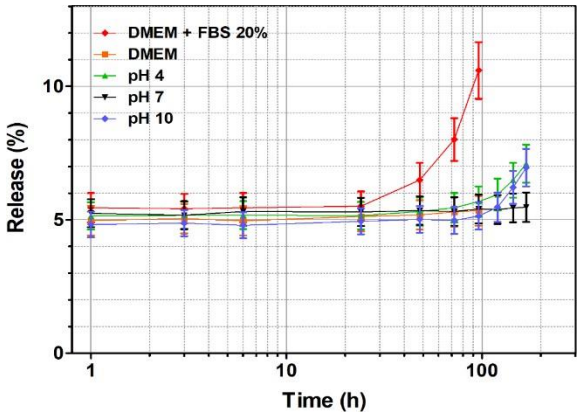


Fig. 3. XRD analysis (Cu Kα) on freeze-died CNCs compared to CNCs-DOXO. Full spectrum (left) and its expansion with the *hkl* assignment of the peaks (right).

Release studies

In order to investigate the stability of the carbamate linker and the conditions required to achieve drug release in vitro, several tests have been performed. In particular, CNCs-DOXO was incubated at 37°C in D.I. water (pH 6.5), in alkaline (pH 10) and acid (pH 4.5) environments, in DMEM and finally in DMEM containing 20% of fetal bovine serum (FBS). Drug release was followed over 96 h or 1 week when possible and compared to the total amount of doxorubicin loaded onto CNCs (**Fig. 4**). A burst release of drug (around 5.5% of the total) was observed within the first hour independently on the release medium: this could be related to the release of physisorbed Doxorubicin from CNCs, not related to the cleavage of any covalent linking. Interestingly, little drug release was observed only in the presence of FBS after at least 24 h, revealing the great stability of the proposed linker in aqueous environment. We believe enzymes and/or proteins in FBS are able to partially trigger the release of DOXO, with an empirical kinetics by which the amount of released drug is proportional to squared time (**Supplementary Fig. 3 and 4**). As expected, after 96 h DMEM medium starts to degrade: this is a peculiar property of this agent which helps biologists to monitor cell growth and maintenance, but does not allow us to prolong the corresponding release study longer.



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193 **Fig. 4**. Release kinetics of Doxorubicin from CNCs-DOXO in different solutions at 37°C,
194 determined by fluorometric analysis. Release is observed in DMEM + FBS 20%.

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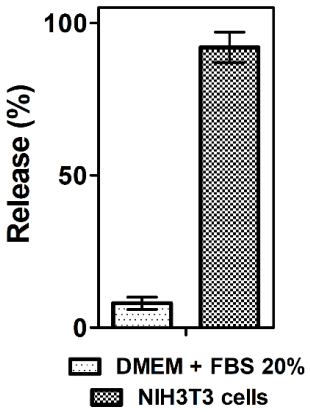
196 Since the drug binding to the nanocarrier demonstrated its stability in normal conditions, we tested
197 whether the release of DOXO could be triggered by cells themselves, which contain a much greater
198 variety of enzymes (such as carboxylesterases) that could cleave the carbamate linker. In order to
199 explore that, CNCs-DOXO has been dispersed in cell culture media containing NIH3T3 cells. After
200 96 h of incubation at 37°C, a portion of the medium is withdrawn and centrifugated to remove
201 nanostructures and cells. Fluorescence analysis on the obtained supernatant revealed that around
202 94% of the CNCs-loaded DOXO was released as free drug, revealing that the complete release of
203 DOXO can only be achieved by the direct action of cells, while the drug remains stably connected
204 to cellulose in non-biological systems (**Fig. 5**).



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206 **Fig. 5**: Comparison between the released amount of Doxorubicin after 72 h of incubation at 37°C
207 either in DMEM + FBS 20% or in NIH3T3 cell culture.

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In vitro studies – Cell Viability Assay

The cytotoxicity of CNCs-DOXO was evaluated in NIH3T3 cells by a MTS cell viability assay. Specifically, NIH3T3 cells were treated with increasing concentrations of CNCs-DOXO and compared to equivalent amounts of bare CNCs and free DOXO (**Fig. 6**). Interestingly, the obtained IC_{50} values showed almost identical effects of free doxorubicin (IC_{50} = 1.139 μM) and CNCs-DOXO (IC_{50} = 1.148 μM), confirming that cells are able to cut the carbamate linker between CNCs and DOXO, thereby releasing the drug for its cytotoxic action. Importantly, bare CNCs showed very little toxicity at concentrations required for carrying sufficient concentrations of active DOXO (IC_{50} = 9.505 μM).

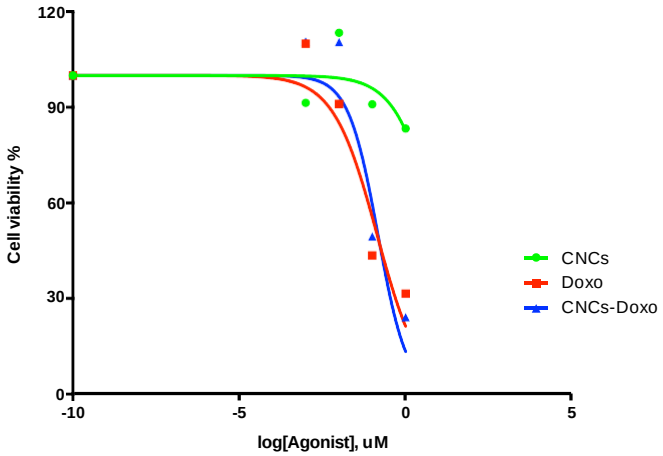

Fig. 6. Cell viability assay in NIH3T3 cells. MTS assay was performed after 72 h treatment of NIH3T3 cells with different concentrations of CNCs, DOXO and CNCs-DOXO, to establish relative IC50 values. The results shown are averages of triplicate samples from a typical experiment.

Conclusions

In conclusion, we have reported for the first time an effective linker based on carbamate cleavable group for the attachment of model drug (Doxorubicin) to nanocellulose crystal backbone. The protocol for linking the drug is reproducible and easy to handle. The linker, consisting of a spacer arm and the carbamate prodrug, has been proved to be stable under harsh conditions, such as basic or acidic pH. On the other hand, the release of the active and unaltered drug is achieved in almost quantitative way in presence of cells owning proper enzymes for carbamate cleavage.

To the best of our knowledge this surface modification of CNCs represents the first successful obtainment of a cleavable linker for incorporation of drugs, thus allowing for the very first time the

usage of nanocellulose as proper smart drug delivery system. This modern approach represents the first step towards the use of completely bio-based nanomaterials able to ensure more stimulus-sensitive and sustained nanomedicine for clinical applications.

Experimental section

All chemicals have been purchased from Sigma-Aldrich Co. (St Louis, MO, USA) and used as received. Amount of DOXO linked to the nanocarrier was determined by fluorescence analysis with an Edimburg FLSP920 spectrofluorimeter equipped with a 450 W Xenon arc lamp. All aqueous solutions were prepared with deionized water obtained using an ultrafiltration system (Milli-Q; EMD Millipore, Billerica, MA, USA) with a measured resistivity above 18 MΩ/cm. XRD analysis has been performed with a vertical goniometric diffractometer (Bragg – Brentano geometry) Philips PW 1050/81 with a PW 1710 chain counting employing Cu Kα radiation. ^{1}H-NMR spectra were obtained on a Varian Inova (14.09 T, 600 MHz) NMR spectrometer. In all recorded spectra, chemical shifts have been reported in ppm of frequency relative to the residual solvent signals for both nuclei (^{1}H: 4.79 ppm for D_2O). Solvent-suppressed ^{1}H-NMR spectra have been recorded with pre-saturation pulse sequences.

Synthesis of CNCs

1.5 g of Whatman® cellulose filter paper is finely cut and placed in a 250 mL round-bottomed flask. At this point, 25 mL of ice-cold 4 M aqueous HCl solution are added under stirring. After all the filter paper was soaked in the acid solution, the flask is moved to an oil bath set at 80°C under continuous stirring for 4 h. Hydrolysis is stopped by addition of 70 mL of cold water. Then, the milky suspension is summarily purified by centrifugation (6000 rpm, 15 min) and redispersion in water, and finally by dialysis. Reaction yield (68%) was determined by gravimetric analysis of the final aqueous dispersion, and CNCs was stored in the fridge at a concentration of 2%.

Synthesis of CNCs-DAC, 1:

In a 100 mL flask, 15 mL of the 2% CNCs dispersion are added to 155 mg of NaIO4. The flask is then wrapped in aluminium foil to protect it from the light, and the reaction is allowed to take place for 72 h by stirring at room temperature. Then, the synthetized CNCs-DAC is separated by centrifugation, dispersed in water and dialyzed. The final concentration of DAC was again 2%. The total aldehyde content was determined exploiting the fluorimetric method reported by Nonsuwan in

2009, in which aldehydes are reacted with acetoacetanilide and ammonium acetate exploiting Hantzsch's reaction to quantitatively produce a fluorophore. (Nonsuwan et al. 2019).

Synthesis of CNCs-DAC-GABA, 2:

10 mL of 2% CNCs-DAC are added to 10 mL of 0.2 M sodium acetate solution (pH 9) in a 50 mL round-bottomed flask, and stirred for 15 min. Then, 1.143 g of γ-aminobutyric acid (GABA) are added. Reaction is carried out by stirring at 45°C for 24 h. After cooling, 175 mg of $NaCNBH_3$ are added, and the mixture is further stirred for 6 h. Reaction mixture is purified by dialysis and centrifugation. Gravimetric analysis on the purified product reveals a 32% yield and a concentration of the final solution equal to 0.63% w/w.

Synthesis of CNCs-DAC-GABA-HBA, 3:

From the previous step, 10 mL of a 0.63% dispersion of 2 are transferred to a 50 mL flask, to which 576 mg of EDC HCl, 33 mg of NHS, 384 mg of DMAP and 337 mg of HBA are sequentially added. The mixture is magnetically stirred for 24 h at room temperature, then purified by dialysis and freeze-dried. Final yield was 79%.

Synthesis of CNCs-DAC-GABA-HBA-NPC, 4:

In a dry 50 mL flask and under N2 atmosphere, 78.2 mg of 3 are dispersed in 20 mL of anhydrous DMF. Then, 715 mg of 4-nitrophenyl chloroformate (NPC) and 50 μL of DIPEA are added. The mixture is placed in an oil bath set at 50°C and stirred for 18 h, after which the mixture was washed by addition of 5 mL of water and successive centrifugation. Then, in order to remove most of the water, the precipitated pellet is dispersed in anhydrous DMF and centrifugated several times, each time discarding the hydrate DMF supernatant. Finally, the pellet was dispersed in 5 mL of anhydrous DMF to proceed with the conjugation of the drug.

Linkage of Doxorubicin: synthesis of CNCs-DAC-GABA-HBA-DOXO, 5:

The entire amount of product obtained by the previous step is added to 121 mg of DMAP dissolved in 5 mL of anhydrous DMF, in a round-bottomed flask under N2 flow. Then, 1 mL of 1 mg mL^{-1}

doxorubicin solution is added. Coupling reaction is allowed to happen by stirring at room temperature for 18 h, after which the unbound excess of doxorubicin was eliminated by repeated centrifugation and washes with water, until clear supernatant and red pellet were obtained. The suspension is then freeze-dried and stored at room temperature under vacuum.

Quantification of Doxorubicin

The amount of doxorubicin (DOXO) in all solutions was determined by fluorometric analysis. Four standard CNCs aqueous solutions at 0.1, 0.2, 0.4 and 0.8 μg mL^{-1} are prepared and their fluorescence emission intensity was detected at 594 nm while exciting at 288 nm. Linear correlation was observed between the counts at the detector and the concentrations, allowing to use fluorescence intensity to measure DOXO concentration, maintaining constant all other experimental parameters (**Supplementary Fig. 1**).

NMR analysis of the released species

In order to establish the chemical nature of the released species at different pH, 15 mg of freeze-dried CNCs-DOXO have been disperded in 1.5 mL of D_2O and divided in three 500 μl portions that are added to 500 μL of 2 M, 0.02 M and 0.0002 M NaOH in H_2O, respectively, to achieve pH values of 10, 12 and 14. At pH 12 the solution turned purple to the deprotonation of DOXO and blue at pH 14 due to DOXO decomposition. After overnight incubation at room temperature, samples have been acidified with aqueous HCl and the dispersions have been microfiltered using 440 nm pore-size syringe filters and underwent ^{1}H-NMR analysis with solvent suppression pulse sequence.

Release Studies

Release studies were performed by placing 800 μL of a 25 mg mL^{-1} dispersion of CNCs-DOXO inside a Slide-A-Lyzed MINI dialysis device equipped with a membrane with MWCO of 2 kDa and placed into 10 mL of the release medium at 37°C. At predetermined time steps, 2 mL of the release medium are withdrawn to undergo free DOXO quantification by fluorescence analysis and replaced with 2 mL of pure water at 37°C. Buffer at pH 7 was prepared using NaH_2PO4/Na_2HPO_4 0.1 M, buffer at pH 4 using CH_3COOH/CH_3COONa 0.1 M and at pH 10 using $NaHCO_3/Na_2CO_3$ 0.1 M.

In vitro studies – Cell Viability Assay

The antiproliferative effect of CNCs, DOXO and CNCs-DOXO in NIH3T3 cells was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA), following the manufacturer's protocol. Briefly, approximately 1×10^3 cells were seeded into each well of a flat-bottom 96-well cell culture plate in 100 µL of recommended culture medium and were allowed to grow for 24 h at 37°C with 5% CO_2. After 24 h of incubation, culture medium was replaced by fresh medium containing the different test agents. The concentrations of CNCs, DOXO and CNCs-DOXO were gradually increased from 0 to 10 µM (0.000, 0.001, 0.010, 0.100, 1.000, 10.000 µM). Seeded 96-well plates were then incubated for 72 h at 37°C with 5% CO_2. 20 µL of CellTiter 96 AQueous One Solution Cell Proliferation Assay reagent were then added for 2 h at 37 °C. The signal was detected using VersaMax MicroPlate Reader (Molecular Device, San Jose, CA USA). The relative growth (compared with the cell viability at 0 h) of the cells was then calculated using the equation: $[A]_T/[A]_{T0}$, where $[A]_T$ is the absorbance at time point T and $[A]_{T0}$ is the absorbance at 0 h. The assay was performed in triplicate.

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