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Conjugation of folic acid with TEMPO-oxidized cellulose hydrogel for doxorubicin administration

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ABSTRACT

The development of new drug delivery systems for cancer therapy is critical in order to further increase efficacy and reduce peripheral toxicity of current antitumor agents, and so far various targeting strategies have been investigated in this regard. Here we report on the synthesis and characterization of a new drug carrier system where folic acid is grafted at the surface of the oxidized cellulose nanofibers using the Diels Alder strategy. The oxidized cellulose nanofibers grafted with folic acid formed a colloidal system that was loaded with doxorubicin as a model antitumor agent. The system was tested in-vitro using both tumor and normal human cells, and results indicated an increased ability to kill human cells when compared to free doxorubicin. No significant effect of the CNF-bound folic acid causing an increased specificity towards tumor cells versus human fibroblast cells was observed, possibly as a result of a reduced binding potential of folic acid after conjugation to CNF. However, the system demonstrated an increased cellular uptake of doxorubicin by human cells, suggesting the potential to improve the therapeutic efficacy of pharmaceutical actives.

Introduction

Chemotherapy remains a major approach in treating many cancers, albeit still facing drawbacks such as low cellular uptake efficiency, drug resistance, and side effects that limit clinical applications and treatment efficacy (Liang, Chen, Zhao, & Wang, 2010). The use of appropriate drug carriers that can selectively target tumor cells leaving the normal ones unaffected represents one of the strategies commonly employed in an attempt to reduce the side effects of many anticancer drugs (Michael C. Perry, Donald C. Doll, 2012) (Urruticoechea et al., 2010) Frei (1985). The antitumoral agent can be conjugated with specific ligands that recognize molecular targets at the surface of cancer cells, increasing the internalization of the drug (Richter & Zhang, 2005). These molecular targets are normally found on the surface of tumor cells in larger amount compared to normal cells, and their chemical nature is variable; they can be glycoproteins and proteoglycans, for instance, lactobionic acid, lactose and hyaluronic acid; examples of antibody targets are the antigens of haematopoietic differentiation (CD20, CD30, CD33, among others), glycoproteins expressed by solid tumours (EpCAM, CEA, mucins, among others), the growth factors and differentiation signalling molecules (carcinoembryonic antigen CEA), epidermal growth factor receptor (EGFR) and the insulin-like growth factor 1 receptor (IGF1R). The fast proliferation of tumor cells demands an excess of certain vitamins such as folate, biotin, retinoic acid, and dehydroascorbic acid to sustain their rapid growth, therefore their receptors are also upregulated (Li et al., 2016) (Scott, Wolchok, & Old, 2012) hence these molecules can be used as targets for specific chemotherapeutics. The folate receptor is a glycosyl-phosphatidylinositol-anchored cell surface protein forming a sophisticated transport receptor for the internalization of folic acid (Kamen & Smith, 2004), which is required for proper cell growth Kelemen (2006) ChemicalBook (2016). Folate receptors are widely studied and used in antitumor therapies because they are overexpressed on the surface of many tumor cells found in lung, breast or ovary, among other organs, while only scarcely expressed at the surface of normal cells Kelemen (2006) (Nunez et al., 2012) (Kalli et al., 2008). The use of folic acid as a targeting ligand in drug delivery aims to minimize non-specific attack of normal cells and to increase cellular drug uptake *via* receptor mediated endocytosis in tumor cells, thereby improving therapeutic efficacy (Dong, Cho, Lee, & Roman, 2014). Its use is well justified by a high receptor affinity, non-immunogenicity, relatively low molecular weight, good stability in various conditions, and ease of chemical conjugation. Most of the delivery systems designed to target the FA receptor rely on drug conjugation to folic acid and its transport being facilitated by the affinity of the folic acid moiety for the FA receptor Leamon & Low (1991).

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The drug carriers investigated for the delivery of antitumorals using surface cell targets include liposomes, dendrimers, micelles or nanospheres Agudelo, Bérubé, & Tajmir-Riahi (2016). The systems based on natural polymers have been receiving an ever increasing attention because of their intrinsic properties such as biocompatibility and degradability. In addition to lack of toxicity and good biocompatibility, natural polymers have chemical groups easily amenable to chemical modifications and that facilitate the design of alternative structures for a variety of applications. Cellulose deserves special attention due to its remarkable biocompatibility, water absorption and chemical functionalities that make it an excellent material for applications in the biomedical field, including drug release (Klemm, Heublein, Fink, & Bohn, 2005) Jorfi & Foster (2015) (Salimi et al, 2019). When oxidized, cellulose displays surface carboxylic groups that impart to the material distinct features such as high water affinity and increased biodegradability thereby overcoming the limitations of pristine cellulose (Brodin, Gregersen & Syverud, 2018). TEMPO oxidized cellulose nanofibers (ToCNF) represent an interesting alternative for the development of drug delivery systems because of the increased water compatibility and the presence of carboxylic functionalities that are easily amenable to further chemical modifications (Singh, Ray, & Vasudevan, 1982) (Weishaupt et al., 2015). Recent reports also point to other advantageous features for drug release applications such as pH-responsiveness (Hujaya et al, 2018).

Here we propose a strategy for grafting folic acid to oxidised cellulose nanofibres using a Diels Alder coupling reaction aimed at harnessing the versatility of ToCNF towards potential applications that involve drug loading/release and tumor cell recognition. Doxorubicin (DOX) was selected as model drug because its wide application in antitumor therapies (Agudelo, Bourassa, Bérubé, & Tajmir-Riahi, 2016). We rationalised our approach in terms of a preferential affinity of the system ToCNF-FA/doxorubicin (ToCNF-FA/DOX) for tumor cells, a clear advantage in the search for new delivery systems aiming to decrease the amount of antitumoral drugs needed for treatment.

Materials and methods

Materials

2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO catalyst), 1-ethyl-3- (3-dimethylaminopropyl) - carbodiimide (EDC), and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (Saint Louis, US). Sodium hypochlorite (NaClO, 2–2.5 % available chlorine) was purchased from Anidrol (Brazil), and folic acid (pharmaceutical grade) was sourced from a local pharmacy (Araraquara, Brazil). The sugar cane bagasse was supplied by a local farm (Sao Carlos, Brazil). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum were purchased from Nutricell, Campinas, Brazil); doxorubicin was purchased from Carbosynth (Berkshire, UK), penicilin and streptomicin 10 U/mL were sourced from Sigma Aldrich (US); 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium salt (MTT) from Sigma Aldrich (Saint Louis, USA). Isopropyl alcohol, dimethyl sulfoxide, sodium hydroxide, sodium hypochlorite (NaClO, Synth Brazil) and phosphate buffer saline (PBS) purchased from Synth (Sao Paulo, Brazil), trypsin and deuterated solvents dimethyl sulfoxide (DMSO) and chloroform were purchased from Sigma-Aldrich (Saint Louis, US). All chemicals were used as received, without further purification.

Methods

Preparation of TEMPO-modified nanofibrillated cellulose (ToCNF)

Bleached sugar cane bagasse pulp was oxidized with 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) following the method previously described by Saito et al. Briefly, sodium bromide (1.2 mmol/g cellulose) and TEMPO reagent (at a ratio of 0.08 mmol / g of cellulose) were added to the suspension of pulp in water (3.3%w; 5 g), followed by the slow addition of sodium hypochlorite solution (13 mL/g cellulose); the suspension was maintained at pH 10 (with NaOH 0.5 M) under continuous stirring (4 h) until stable. The modified cellulose was filtered and washed on the filter with deionized water to neutral pH. The oxidized fibers were homogenized using a kitchen blender for 5 min, and then were sonicated in an ice bath using an ultrasonicator (Hielscher UP400S, 400 W, 2.4 KHz), until the formation of a transparent gel (3 min sonication + 5 min break, to avoid heating, repeated to 1 h total time). The content of carboxylic groups was determined by conductometric titration according to a known procedure (Isogai, Saito, & Fukuzumi, 2011) (Tsuguyuki Saito, Satoshi Kimura, Yoshiharu Nishiyama, & Akira Isogai*, 2007).

Coupling of maleimide moiety to ToCNF

This process was carried out in two steps, following a previously reported method (Gandini, 2013) (Trovatti, Cunha, Carvalho, & Gandini, 2017):

Synthesis of furan-protected amino maleimide (FPAM). Furan (0.5 mol) was added to a solution of maleic anhydride (0.25 mol) in diethyl ether (200 mL) and the reaction mixture was kept stirring at room temperature for 24 h; after precipitation, the Diels Alder adduct (DA) was filtered and dried at room temperature. The DA adduct (3.32 g; 0.02 mol) was then dissolved in anhydrous methanol (30 mL) and hexamethylenediamine (2.32 g; 0.02 mol) was added under stirring, followed by refluxing the reaction mixture for 8 h, followed by cooling overnight. FPAM was obtained as a white precipitate that was washed with methanol before drying (95% yield).

Coupling of FPAM to ToCNF. An aqueous suspension of ToCNF (40 g; 0.16% wt), EDC (58 mg; 0.37 mmol), NHS (33 mg; 0.28 mmol) and FPAM (66 mg; 0.25 mmol) was ajusted with HCl to pH 6.5, and then stirred further for 3 h at 40 °C. The suspension was then filtered using a 0.6 μm pore membrane and washed with distilled water. The deprotection of the maleimide group (with the removal of furan) was carried out by heating the Diels Alder adduct of the modified ToCNF for 2 h in an oil bath (100 °C), yielding N-hexamethylamino maleimide functionalized ToCNF (ToCNF-AM).

Coupling of furan moiety to folic acid

Furfurylamine (Fu, 100 μL; d = 1.1 g/mL; 0.271 mmol) was added dropwise to a fine dispersion of folic acid (FA; 100 mg; 0.226 mmol) in water (10 mL), under continuous stirring at room temperature. Aqueous solutions (in 2 mL water each) of N- (3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC; 52.6 mg; 0.339 mmol) and N-hydroxysuccinimide (NHS; 31.2 mg; 0.271 mmol) were added to the flask, and the reaction was stirred at room temperature for 4 h, after which the orange precipitate was separated by centrifugation. The product (FA-Fu) was recovered by vacuum filtration (0.45 μm membrane), washed with deionized water until neutral pH, and dried in the oven at 60 °C.

Coupling of ToCNF-AM to FA-Fu

An excess of FA-Fu (20 mg) was added to the aqueous suspension of AM-ToCNF (20 g; 0.2% wt) and the mixture was stirred for 3 h at 50 °C (water bath). The product (ToCNF-FA) was filtered and further washed with deionized water in excess to remove all the unreacted reagents.

Spectroscopic characterization

Fourier-transform infrared (FTIR) spectra were recorded using a PerkinElmer Spectrum 100 instrument equipped with an ATR module with diamond crystal (parameters: 4 cm^{-1} resolution; 16 scans; range 650–4000 cm^{-1}). ^1H NMR spectra were acquired using an Agilent Technologies 400/54 Premium Shielded NMR spectrometer at 400 MHz; samples were dissolved at 1 % concentration (w/v) in deuterated solvent (DMSO, chloroform).

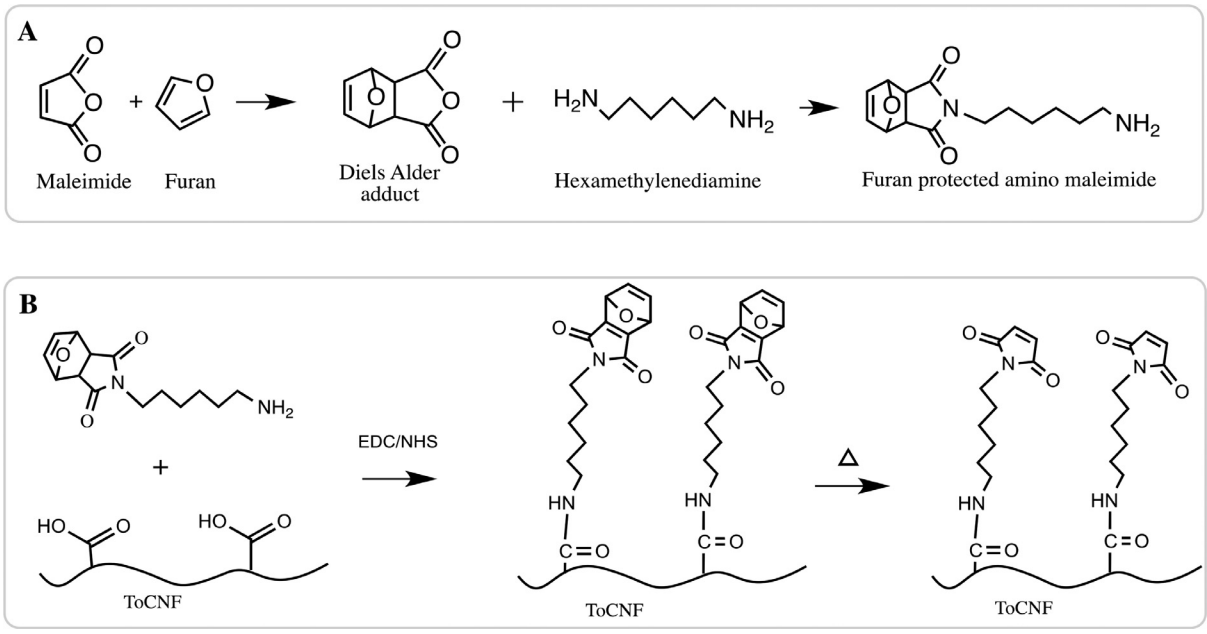

Fig. 1. The synthetic routes for the preparation of: (A) furan-protected amino maleimide (FPAM), and (B) FPAM coupling to ToCNF followed by deprotection, affording maleimide-functionalised ToCNF.

Cytotoxicity studies

The effect of ToCNF-FA/DOX on the viability of normal vs. tumour human cells was investigated on fibroblasts (GM07492) and breast tumor cells (MCF7) using a standard MTT assay. The cells were grown in DMEM supplemented with 10% of fetal calf serum and antibiotics (penicillin 100 U/mL; streptomycin 0.1mg/mL). The cultures were maintained at 37 ± 2 °C in 5% CO_2 atmosphere and trypsinized when 80–90% confluent. In each case, the cell suspension was centrifuged for 3 min at 1200 g, and 0.1 mL/well of the cell suspension (1×10^4 cells) was seeded into a 96-well plate. The plates were incubated for 24 h to complete cell adhesion, and then were treated with 100 μL of either: a positive control (10% v/v of dimethylsulfoxide); a negative control (PBS); ToCNF-FA (DMEM + 20 % v/v ToCNF-FA suspension); DOX (DMEM + 20 % v/v Doxorubicin solution); or ToCNF-FA/DOX (DMEM + 20 % v/v ToCNF-FA/DOX suspension). The concentration of Doxorubicin above was 6.5 μM for both the doxorubicin solution and the ToCNF-FA/DOX suspension; this corresponds to the IC_{50} value previously determined for the same culture conditions (supplementary material). ToCNF-FA/DOX was prepared by mixing 20 mg of ToCNF-FA in 100 μL of doxorubicin solution (6.5 μM). At predetermined time points (10; 24; 48 h), the treatment was removed and the cells were washed with phosphate buffer saline (PBS). MTT solution (50 μL; 0.5 mg/mL in PBS) was added to each well and the microplates were incubated at 37 ± 2 °C for 4 h, protected from light, to allow the formation of formazan violet crystals, which were then solubilized with isopropyl alcohol (100 µL). The absorbance was read at 570 nm in a microplate reader (Polaris-Celer). The cytotoxicity assays were performed in triplicate, and the percentage of viable cells was calculated relative to the negative control. The results are reported as mean values ± SD. The data were analyzed using repeated measures ANOVA and Tukey's post-hoc test was used for multiple comparisons; values of $p < 0.05$ were considered to be significant.

Results and discussion

Preparation of ToCNF-FA-DOX

Oxidised cellulose nanofibers (ToCNF) were prepared by oxidation of cellulose obtained from bleached sugar cane bagasse pulp *via* a controlled TEMPO-mediated reaction, using a known method (Saito 2007).

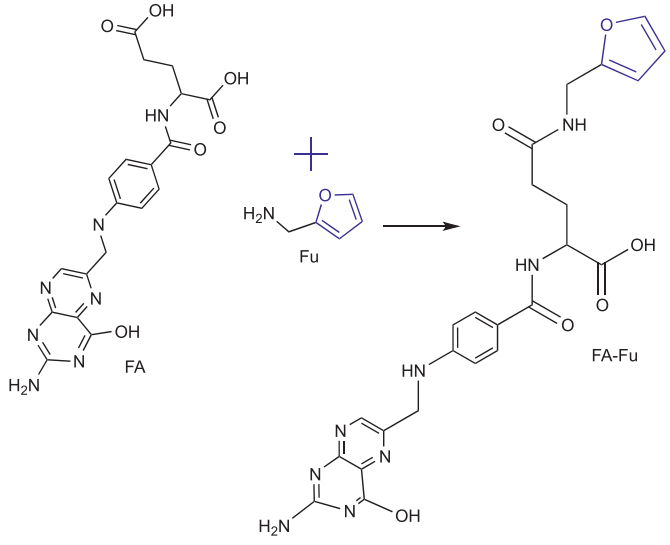

Fig. 2. Schematic representation of the coupling reaction of folic acid (FA) with furan (Fu) generating the furan modified folic acid (FA-Fu).

(Tsuguyuki Saito et al., 2007) Successful oxidation was confirmed by the appearance in the FTIR-ATR spectra of the product (Supplementary material) of a new peak at 1600 cm^{-1}, indicative of the presence of carboxylic groups; the COO^- content (2.3 mmol g^{-1}) was determined by conductometric titration. Optical and SEM images of the transparent aqueous ToCNF suspension and of a freeze-dried sample, demonstrating the nanometric scale of the fibers, are also presented in the Supplementary material. It is expected that the presence of carboxylic groups on the surface of the cellulose nanofibers will facilitate further chemical modification and support at the same time drug loading via electrostatic interactions with positively charged actives such as doxorubicin.

In preparation for its functionalisation with folic acid, ToCNF was further modified with amino maleimide (Fig. 1). The overall reaction scheme for the preparation of amino maleimide-modified ToCNF (Fig. 1B) involved ToCNF conjugation - mediated by carbodiimide in

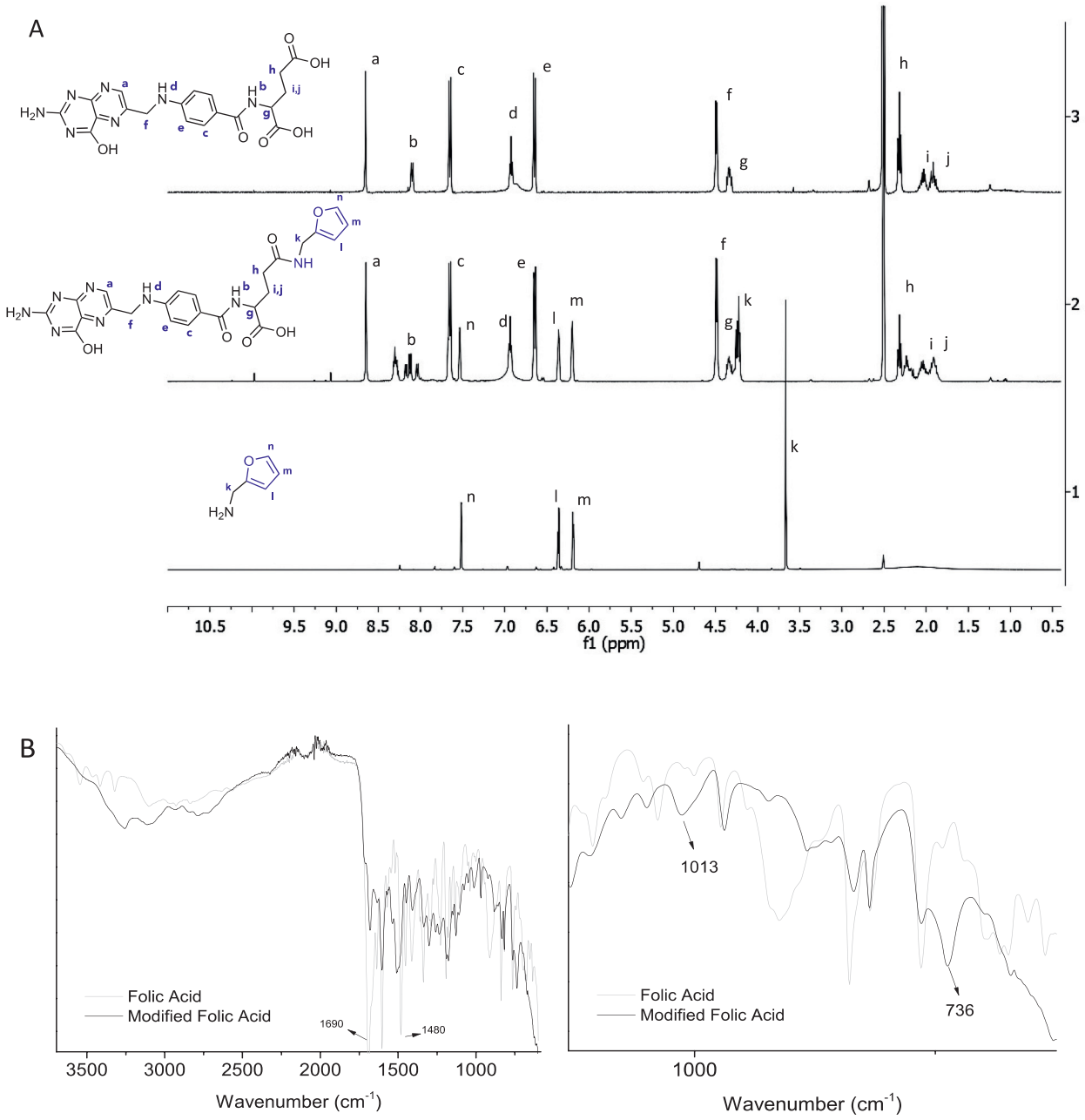

Fig. 3. A) ^{1}H-NMR spectra of FA, FA-Fu and Fu (DMSO-d_6). B) FTIR spectra of FA and FA-Fu (left - full range; right – magnification in the area of interest).

the presence of NHS - with furan-protected amino maleimide (FPAM), followed by deprotection *via* a thermally controlled retro Diels Alder reaction Gandini (2013) with elimination of furan. FTIR and ^{1}H NMR spectroscopic data are included in the Supplementary material; The synthesis of FPAM is presented in Fig. 1A.

In preparation for its conjugation to maleimide-ToCNF, folic acid was modified with 2- (aminomethyl)furan *via* a carbodiimide-mediated reaction (Fig. 2). Though a regioisomer mix is expected (due to the presence of two COOH groups in the molecule of folic acid), γ-conjugates are usually obtained as the major product with high selectivity when using carbodiimide chemistry (Trindade et al., 2014), as the γ-carboxyl in folic acid has been shown to be more reactive than the α group Lee (2006). ^{1}H-NMR spectra of the obtained product (Fig. 3A) showed a ratio of 1:1 FA:Fu, confirming that there is no di-substitution (that could ultimately prevent the FA moiety from binding to the FR receptor); detailed assignments are presented in Table 2 (supplementary information S5). In the FTIR spectra of the product, the main absorption bands that are typical of pure FA (appearing between 1700 and 1000 cm^{-1}) are replaced by main bands at 736 and 1013 cm^{-1}, which correspond to the mono-substituted furan ring and the furan ring breathing, respectively (Fig. 3B). The decrease in intensity of the peak at 1690 cm^{-1} (corresponding to the C=O vibration of the carboxyl group) with the appearance of a new peak at 1480 cm^{-1} (attributed to the stretching vibration of C-N from the amide bond) also confirms the successful FA-FU conjugation.

In the final synthetic step, FA-Fu was conjugated to maleimide-ToCNF (Fig. 4A), using again the diene/dienophile Diels Alder reaction between the furan from FaFu and the maleimide from ToCNF-AM (Fig. 4A). The FTIR spectrum of the final ToCNF-FA product (Fig. 4B) shows characteristic absorption bands at 1650 cm^{-1} (shifted from 1700 cm^{-1} in maleimide-ToCNF) and 1315 cm^{-1}, both indicative of the presence of maleimide fragment (in red), together with a shoulder at

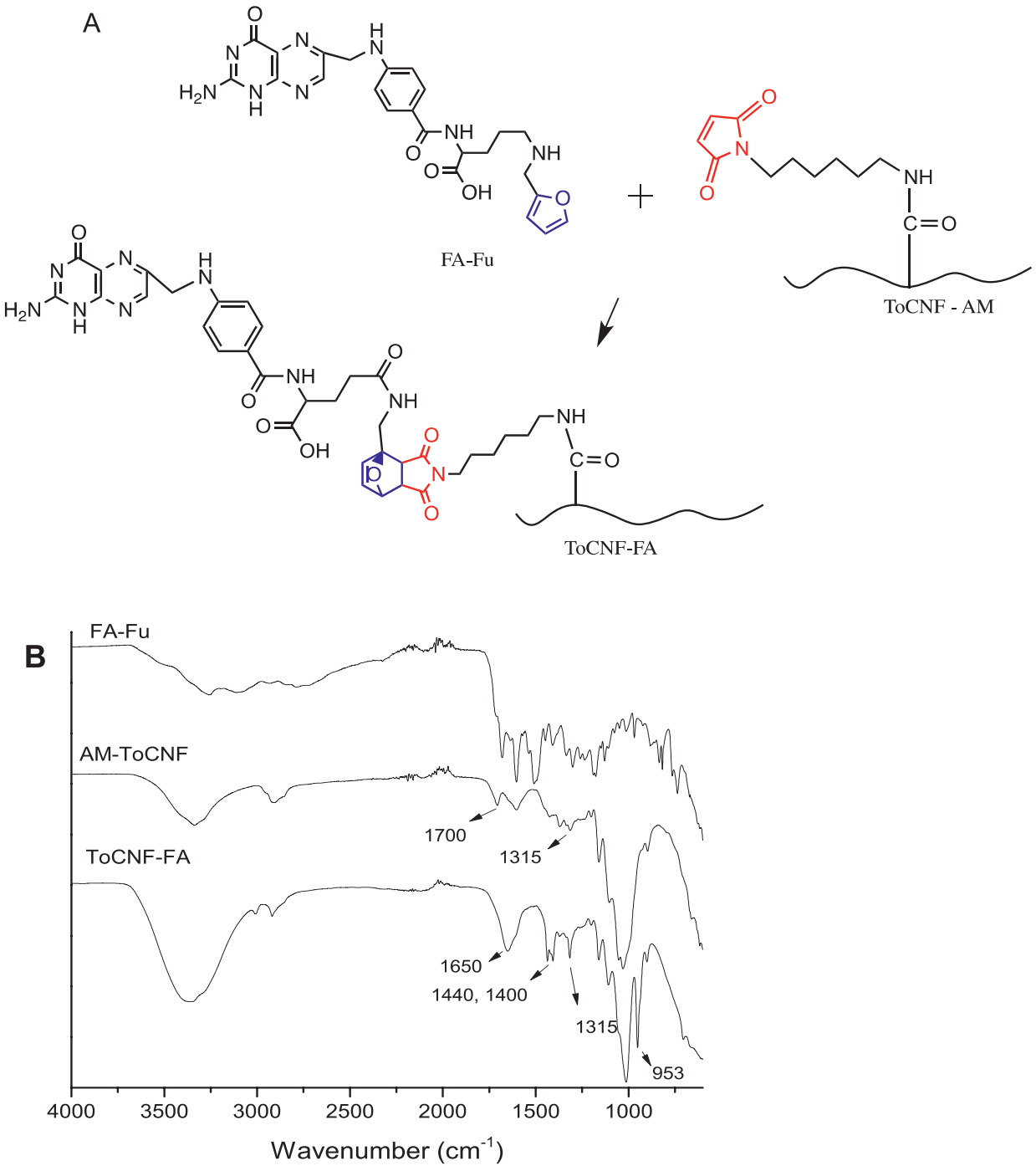

Fig. 4. (A) Schematic representation of the reaction of FA-Fu with ToCNF-AM, leading to the formation of ToCNF-FA via Diels Alder reaction between the furan from folic acid and the maleimide from ToCNF; (B) FTIR spectra of reactants and product (ToCNF-FA).

about 1600 cm^{-1} (N-H in FA) and a strong absorption band at 953 cm^{-1} attributable to the C=C bond in furan (fragment in blue), Figure 5.

The potential of different modifications of cellulose to deliver doxorubicin to cancer cells has been the subject of a number of recent investigations. Folate-decorated carboxymethyl cellulose has been shown to be a promising drug carrier for doxorubicin Movagharnezhad & Moghadam (2016), and disulfide-functionalized carboxymethyl cellulose nanogels were found to exhibit a superior antitumoral effect in vivo compared to the free drug (Qian et al., 2014).

FTIR of DOX, ToCNF-FA and ToCNF-FA/DOX was carried out in order to study if DOX, could interact with ToCNF-FA, once their chemical nature suggest the electrostatic interactions between the positive charges of DOX amine groups and the carboxylic negative charges present in the ToCNF matrix. Such interactions were evidenced by the changes exhibited in the 1500–1650 cm-1 region of the FTIR spectra: the ToCNF-FA carboxylate band (1650 cm-1) and the protonated amino groups band from DOX (1580 cm-1) were found to merge in the ToCNF-FA/DOX drug carrier into a new absorption band at 1600 cm-1, supporting the formation of a strong donor–acceptor ionic complex typical of the interaction of carboxylated cellulose with amines (Rosca, Popa, Lisa, & Chitanu, 2005) (Grande, Trovatti, Carvalho, & Gandini, 2017).

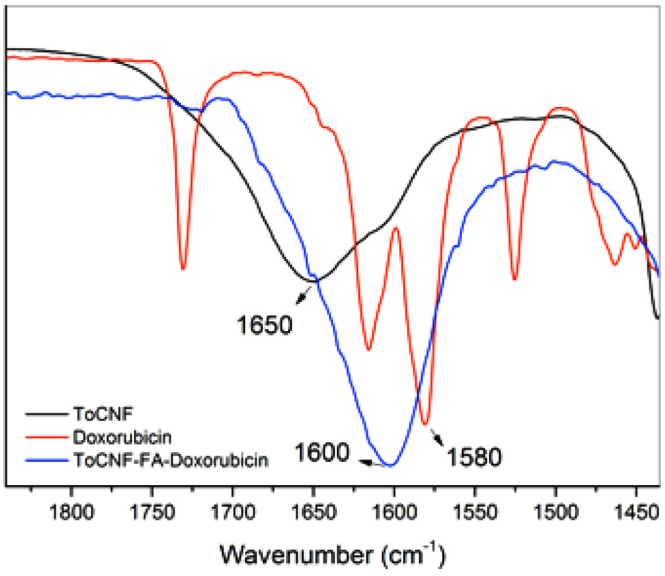

Figure 5. FTIR spectra of DOX, ToCNF-FA and ToCNF-FA/DOX.

Application of ToCNF-FA/DOX as an anticancer drug delivery system

The capability of the proposed system to impart cancer cell targeting selectivity was tested on normal fibroblast GM07492 and tumor breast MCF7 cell lines, using a standard MTT viability assay. According to ISO 10993-5, the substance is cytotoxic when the viability is lower than 70%, as used in the interpretation of these results. The results obtained with negative control (NC), positive control (PC), ToCNF-FA, ToCNF-FA-DOX and pure DOX on GM07492 and MCF7 viability are summarized in Fig. 6.

The negative control was used as 100 % viability reference. ToCNF-FA was found to be non-toxic against both cell lines in all the tested time points (10, 24 and 48 h). The toxic effect of DOX and ToCNF-FA/DOX was low at 10 h of incubation, and increased at 24 h and 48 h.

At 10 h incubation, no significant difference was detected in the viability of the cells treated with com DOX or ToCNF-FA/DOX ($p = 0.1545$). However, the viability of MCF-7 treated with DOX or ToCNF-FA/DOX showed a significant difference ($p = 0.005$) in its viability, with higher cytotoxicity for ToCNF-FA/DOX system. Similar behavior was found to GMO7284 ($p = 0.025$).

At 24 h incubation, the results indicated a significant difference in the viability of the cells treated with ToCNF-FA/DOX ($p = 0.001$), however, MCF-7 showed higher viability, indicating no selectivity effect for them, the tumor cells. Again, when comparing the viability of MCF-7 cells treated with DOX or ToCNF-FA/DOX a significant difference in the viability was found ($p = 0.0283$), with higher cytotoxicity for ToCNF-FA/DOX. Similar behavior was found to GMO7284 ($p = 0.0001$).

The values obtained after 48 h incubation with DOX and ToCNF-FA/DOX showed for both a similar effect to the positive control, possibly due to the age of the cells.

The results indicated the system formed by doxorubicin in combination with ToCNF-FA (ToCNF-FA/DOX) displayed increased cytotoxic effect (at 10 and 24 h time points) when compared to the model drug doxorubicin, suggesting its potential to increase doxorubicin efficiency in antitumor treatments. The results did not reflect an expected increase in selectivity for tumor cells, however the viability of both types of cells have been significantly affected following treatment with ToCNF-FA/DOX (compared to DOX solution), indicating the delivery system based on ToCNF-FA/DOX was significantly more efficient compared to DOX on its own.

Several systems based on cellulose or modified cellulose have been investigated recently for the incorporation and release of doxorubicin. Notable examples are the pH-responsive release studies involving cellulose nanofibrils polyion complexes (Hujaya et al, 2018), the pH responsive release of doxorubicin from oxidized-cellulose nanoparticles (Kumari et al, 2018), and the prodrug composed of carboxymethylcellulose conjugated with doxorubicin hydrogels (Capanema et al, 2018). All these systems have been shown to have the ability to tailor in vitro the drug release kinetics and the cytotoxicity against cancer cells however the cytotoxicity studies were limited to using tumor cells. In contrast, the results reported here were obtained using a novel drug carrier based on oxidized cellulose bound to folic acid and show for the first time, to the best of our knowledge, a comparison of the cytotoxicity performance of cellulose-based systems loaded with doxorubicin between normal and tumor cells.

Conclusions

Grafting folic acid to oxidized cellulose nanofibers *via* a Diels Alder synthetic approach with the aim to develop a novel doxorubicin carrier system is reported here for the first time. The grafting reaction was confirmed by FTIR and ^{1}H-NMR analysis, and the resulting hydrogel drug carrier system was investigated in vitro using both normal and tumor cells. Though no statistically significant decrease in the viability of the tumor cells relative to human fibroblast cells was observed, cytotoxicity measurements demonstrated that the viability of both types of cells

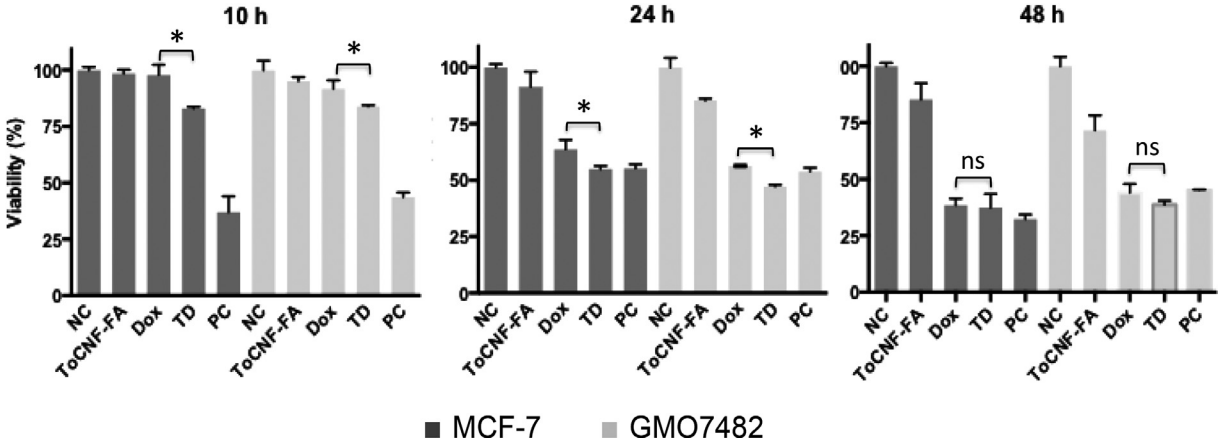

Fig. 6. Cell viability after treatment with doxorubicin (DOX), ToCNF-FA and ToCNF-FA/DOX. NC= negative control, PC= positive control. * indicates significant difference ($p < 0.05$) between Dox and TD samples for each time point; '**ns**' indicate differences statistically not significant.

has been significantly affected following treatment with the proposed formulation (ToCNF-FA/DOX) when compared to free doxorubicin solution used as control. Overall, these results advocate the potential of ToCNF-FA/DOX to improve the therapeutic efficacy of antitumorals and possibly of other pharmaceutical actives. The system developed here may lead to future scientific and technological advances once new applications are proposed based on these findings and on the versatility of TEMPO-oxidized cellulose hydrogels.

Credit author statement

Renata A Carvalho: Data curation, Investigation, Antonio J. F Carvalho and André C. Amaral: Methodology, Visualization, Investigation, Eugen Barbu and Eliane Trovatti: Conceptualization, Writing- Original draft preparation, Reviewing and Editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.carpta.2020.100019.

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